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Biological activity of endophytic fungi associated with palm trees

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Endophytic fungi were isolated from leaves, petioles and roots of palm trees in King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand, and tested for biological activity test. A total of 60 isolates were obtained and morphological identification. *Phoma exigua.*, *Fusarium chamydosporum*, *Phialophola* spp. and *Nigrospora* spp. were examined for bi-culture antagonisict test against *Colletotrichum coffeanum* causing coffee Anthracnose. The result showed that all of these 4 species of endophytics exptressed the antifungal activity inhibited colony growth and spore production of *colletotrichum coffeanum*, which were 6.68cm, 5.89cm, 6.25cm, 5.66cm, in colony diadmeter and 0.875×10^6, 0.375×10^6, 1.125×10^6, 1.0625×10^6 in spore numbers. And, the inhibition percentage of colony growth were 25.75%, 35.28%, 30.55%, 37.02%, respectively, the inhibition percentage of spore production were 76.66%, 89.99%, 69.99%, 71.66% respectively. *Nigrospora spp.* showed the best inhibition percentage of colony growth, and, *Fusarium chamydosporum* gived the best inhibition percentage of spore production.

Key words: endophytic fungi, palm trees, *Colletotrichum coffeanum*, bi-culture antagonistic test

Introduction

Endophytes are contained within the plant without disease and plant tissues remain entire and functional. A wide range of plants have now been examined for endophytes, and endophytes have been found in nearly all of them. 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

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distributed with the host, suggesting a long standing, close and mutually beneficial interaction.

Endophytic fungi have attracted great attention in the past few decades 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).

Palm trees are a botanical family of perennial lianas, shrubs, and trees. They are in the family Arecaceae (Due to historical usage, the family is alternativel called Palmae or Palmaceae). They are flowering plants, the only family in the monocot order Arecales. Roughly 202 genera with around 2600 species are currently known, most of them restricted totropical, subtropical, and warm temperate climates. Most palms are distinguished by their large, compound, evergreen leaves arranged at the top of an unbranched stem. Palms are one of the best known and most widely planted tree families. They have held an important role for humans throughout much of history. Many common products and foods come from palms. They are often used in parks and gardens that are in areas that do not have heavy frosts.

Nowadays, many endophytic fungi associated with palms had reported, including temperature palms and tropic palms (Rodrigues and Samuels, 1990; Fröhlich and Hyde, 2000; Hyde *et al.*, 2000). Frohlich *et al*. (2000) reported endophytic fungi from three unidentified *Licuala* sp. palms in Brunei Darussalam and from three *L. ramsayi* palms in Australia and got 75 fertile species in 48 genera and 60 sterile morphospecies including 10 *Xylaria* anamorphs, *Phomopsis* sp., *Phoma* sp., *Trichoderma* sp., *Colletotrichum* sp., *Pestalotiopsis palmarum*., *Lasiodiplodia* sp. *Hyphomycete* sp., *Nodulisporium* sp., *Dictyochaeta* sp., *Phyllosticta* sp., *Distocercospora* sp., *Verticillium* sp., *Coelomycete* sp., *Aspergillus niger*, *Beltraniella* spp., *Botrytis allii.* The endophyte communities of both palms were composed of a single, dominant xylariaceous species.

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.

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.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 purpose of this reseach focused on isolation of endophytic fungi from palm trees and their bioactivity against *Colletotrichum coffeanum* causing anthracnose of Arabica coffee using bi-culture antagonist test.

Materials and methods

Collection of plant samples

Plant samples were randomly collected from 10 species healthy palms:- Ptychosperma macarthuri (MacArthur Palm); Elaeis guineenis (African Oil Palm); Rhapis humilis (Slender Lady Palm); Wodyetia bifurcata (Foxtail Palm); Chrysalidocarpus lotescens; Cocos nucifera (Coconut); *Rhapis Laosensis* (Thailand Lady Palm); *Licula grandis* (Fan Palm); *Livistona chinensis* (Chinese Fan Palm) and *Mascarena Lagencuulis* (Bottle Palm) at King Mongkut's Institute of Technology Ladkrabang (KMITL), Ladkrabang, Bangkok 10520, Thailand. All the samples were randomly selected plants of healthy leaves, petioles and roots then cut off and taken to laboratory and processed within 24 h.

Isolation and identification of endophytic fungi

Plant specimens were thoroughly washed in running tap water for 5 minutes to remove dust and debris and then air dried. The cleaned leaves, petioles and roots were surface sterilized with 75% ethanol 1 min and 10% sodium hypochlorite 3-5min, and then, cut into small pieces(3×3×3mm) under sterile conditions (petioles and roots were removed outer epidermal tissues and cuticle before cut into small pieces). Briefly, fragments were cleaned in sterilized water and sterilized with 75% ethanol 30s and then cleaned in sterilized water again and placed on water agar(WA) medium, incubated at room temperature. The endophytic fungi growing out from the plant tissue were transferred into potato dextrose agar (PDA) plates and incubated for two to six days for observation. Continuous plates were subculture until get pure culture.

All the isolates were identified by the morphology of the fungal culture, the mechanism of spore production and characteristics of the spores . The sterile isolates were grown on PDA with decoction of host leaf medium to observe sporulation. For tentative identification, microscopic slides of each endophytic fungi were prepared and examined under binocular compound microscope for morphological identification.

Isolation of pathogen and pathogenicity test

The plant pathogen *Colletotrichum coffeanum* causing coffee anthracnose in Arabica variety were isolated by tissue transplanting technique from the leaf with obvious symptoms. The disease leaves were cleaned with running tap water and after air-dry cut the advance margin of symptom between healthy tissue and diseased tissue to small pieces and then sterilized with sterilized water, 75% alcohol and sterilized water again. Then, transferred onto WA medium and followed by potato dextrose agar (PDA) to obtain pure culture. *Colletotrichum coffeanum* were identified by morphological characteristic under binocular compound microscope.

The pathogen was tested for pathogenicity using detached leaf method in the laboratory. Select healthy leaves of coffee and washing in the running water and air-dried. A sterilized filter paper was placed in 9cm diameter sterilized petri dish and two sterilized microslide were also put on the filter paper, and the filter paper were moistened by sterilized distilled water. Coffee leaves were wounded by sterilized needle and then placed on the microslide in the petri dish then the spore surspention of *Colletotrichum coffeanum* was prepared at concentration is 1×10^6 spores/ml. Spore surspention were sprayed on the surface of coffee leaves including the wounded areas, incubated for two weeks at room temperature. At the same time, sterilized water was also sprayed to coffee leaves as controls and incubated. Lesions on inocultated areas were obsereved on the coffee leaves, then re-isolated pathogen from lesion invaded with inoculated pathogen according to the above mentioned method and identified the re-isolates under microscope and get pure culture.

Bi-culture antagonistic tests

Some isolates of endophytics were tested for antagonistic bi-culture. These isolates were tested to determine their bioactivity against *Colletotrichum coffeanum.* The experiment was conducted using a Completely Randomized Design (CRD) with 4 replications by the methods of Soytong (1992), Sibounnavong *et al*. (2009) and Charoenporn *et al.* (2010). The antagonistic fungi and pathogen were separately cultured on PDA at room temperature (30-

32 ºC) for 7 days. And 0.5 cm diameter sterilized cork borer was used to remove agar plugs from the actively growing edge of cultures of the antagonistic fungi and pathogen and then transferred onto the same sterilized 9 cm-diameter PDA plates, an agar plug of the pathogen was placed on one side of the plate which opposited an agar plug of an antagonistic fungus. The single plug of antagonistic fungi and pathogen were transferred into two separate PDA plates as the controls. And then, all the plates were incubated at room temperature (30-32 ºC) for 30 days. Data were collected regarding to diameter of colony (cm) and the number of conidia produced by the pathogen in the bi-culture plates and control plates. A haemcytometer was used to count the number of conidia of pathogen.

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 on the control plates and B is the diameter of colony or number of conidia produced by the pathogen in the bi-culture plate.

Analysis of variance was statistically computed and treatment means were compared using Duncan Multiple's Range Test (DMRT) at P = 0.05 and 0.01.

Results and discussion

Isolation and identification of endophytic fungi

Ten species of endophytic fungi were found including *Cladosporium* spp., *Phialophora* spp., *Pestalotiopsis* spp., *Phoma* spp., *Phomopsis* spp., *Nigrospora* spp., *Xylaria* spp., *Fusarium* spp., *Colletotrichum* spp. and *Rhizoctonia* spp., and other 20 isolates belonging to mycelia sterilia fungus .(Table 1)

A total of 65 isolates, 41.5% isolates isolated from leaves, 21.5% isolates isolated from petioles and 37.0% isolates isolated from roots. The leaf of palm can habor more endophytic fungi than petiole and root. And in this study showed higher frequency (30.7%) of sterilia fungus were isolated from palm trees. Four species of endophytic fungi were selected and tested antagonists by bi-culture method as follows: *Phoma exigua*, *Fusarium chamydosporum*, *Phialophora* spp. and *Nigrospora* spp. (Fig.1-4).

Table 1 . Isolates of endophytic fungi from different parts of 10 palm trees

Endophytic fungi	Isolates			Total
	Leaf	*Petiole*	*Root*	
Cladosporium spp.	-	2	1	3 (4.6%)
Phialophora spp.	1	-	3	4 (6.2%)
Pestalotiopsis spp.	1	-	1	2 (3.1%)
Phoma spp.	2	1	2	5 (7.7%)
Phomopsis spp.	3	1	1	5 (7.7%)
Nigrospora spp.	3	-	2	5 (7.7%)
Xylaria spp.	3	1	-	4 (6.2%)
Fusarium spp.	2	1	4	7(10.8%)
Colletotrichum spp.	1	-	2	3 (4.6%)
Rhizoctonia spp.	2	3	2	7(10.8%)
Sterilia fungus	9	5	6	20(30.7%)
Total	27 (41.5%)	14 (21.5%)	24 (37.0%)	

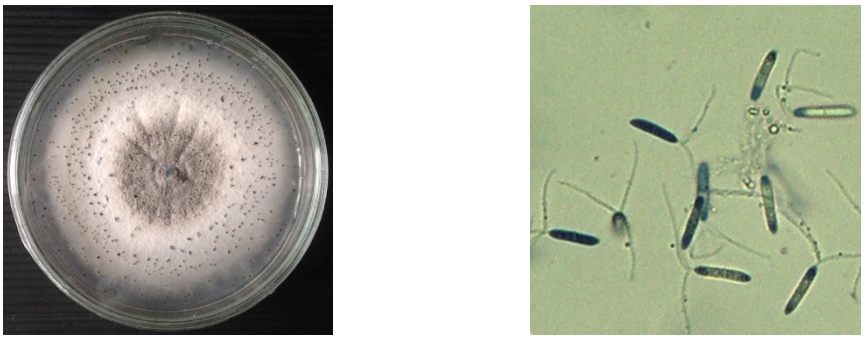

Fig. 1. *Phoma exigua* pure culture and spore

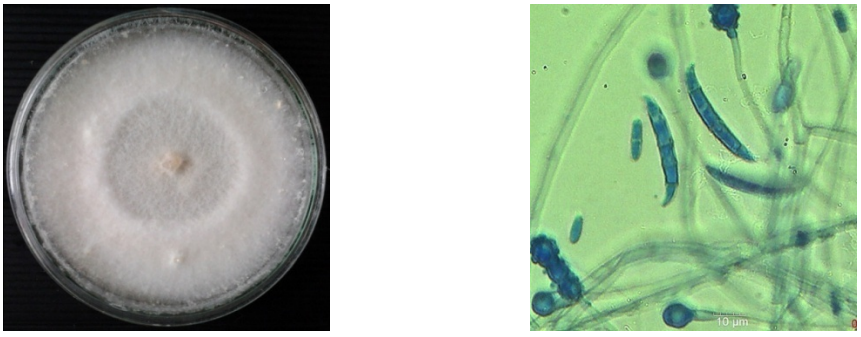

Fig. 2. *Fusarium chamydosporum* pure culture and spore

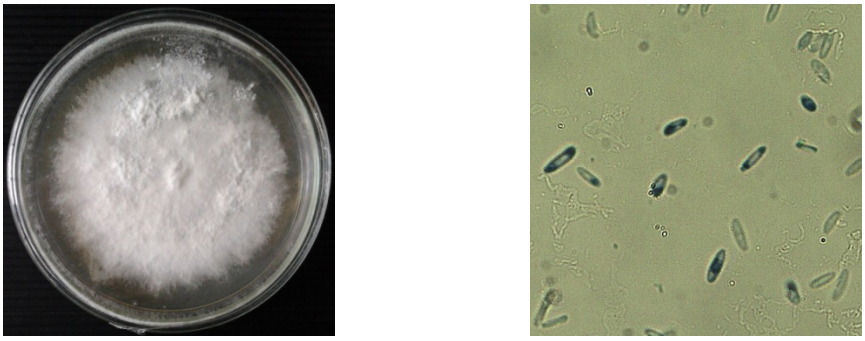

Fig. 3.*Phialophora spp*. pure culture and conidia

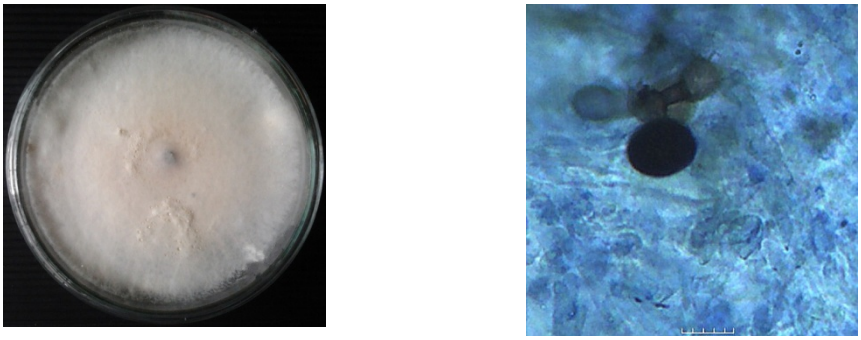

Fig.4. *Nigrospora spp.* pure culture and sporangiospore

Isolation of pathogen and pathogenicity test

Colletotrichum coffeanum were isolated from anthracnose of Arabica coffee leaves and identified from coffee leaves with obvious symptom of coffee anthracnose to pure culture (Fig.5). The isolate was confirmed pathogenic isolate from patyhogenecity test (Fig. 6). The result showed that pathogenic isolate could be infected in the coffee leaf and caused symtomp with the same symptom caused by *Colletotrichum coffeanum* causing leaf anthracnose on coffee leaves.

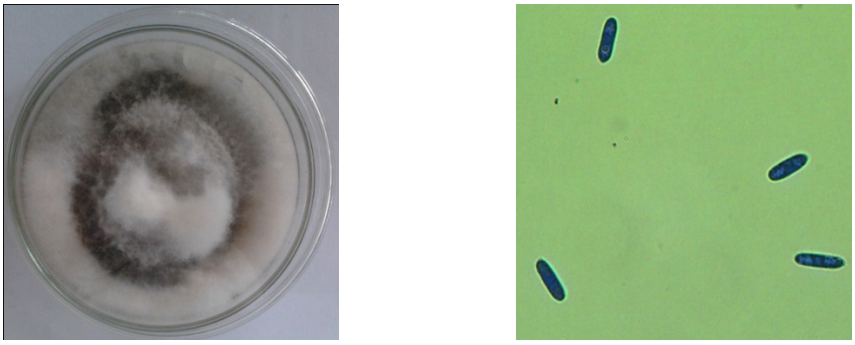

Fig.5 *Colletotrichum coffeanum* pure culture and spore

Treatment **Control**

Fig. 6. Pathogenicity test on coffee leaves

Bi-culture antagonistic tests

Phoma exigua, *Fusarium chamydosporum*, *Phialophora* spp. and *Nigrospora* spp. were proved their abilities to inhibit the growth of *Colletotrichum coffeanum* by using bi-culture tests. (Fig.7).

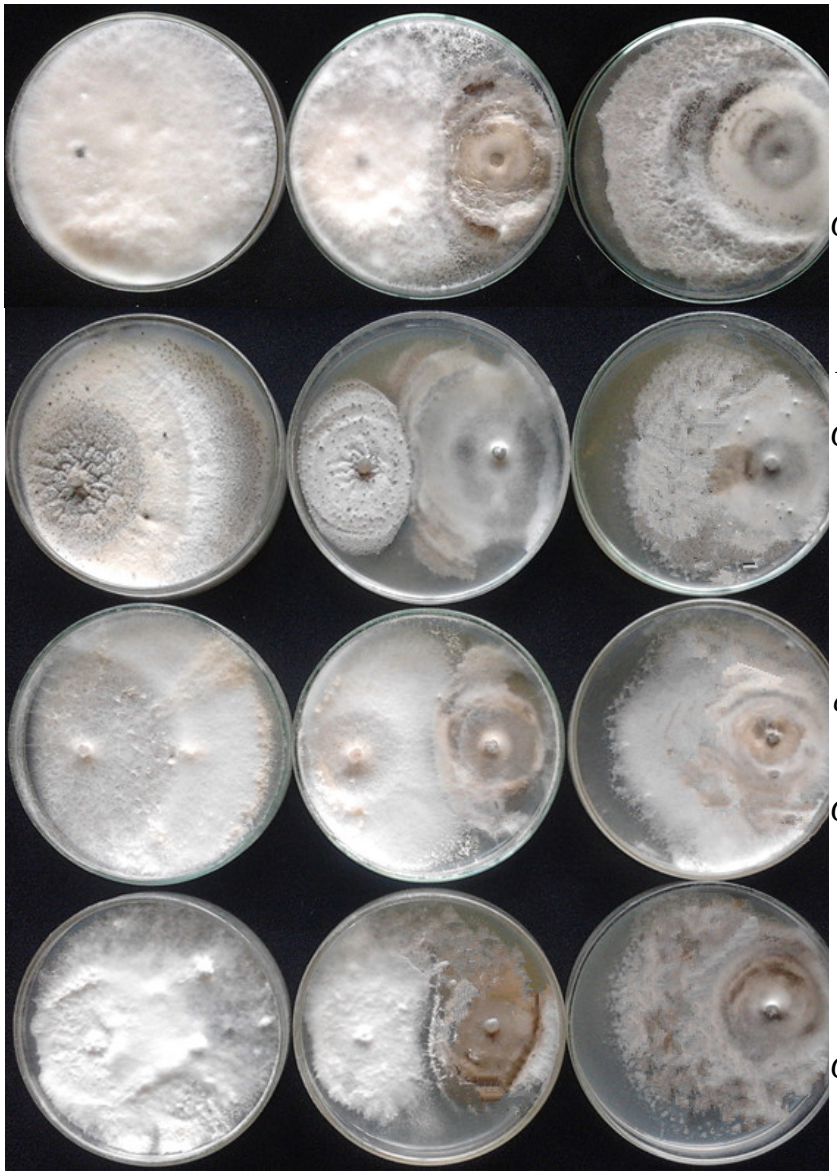

Fig. 7. Bi-culture antagonistic tests

The results showed that all the endophytics *Phoma exigua*, *Fusarium chamydosporum*, *Phialophora* spp. and *Nigrospora* spp. gave significantly inhibition of *Colletotrichum coffeanum* which were 6.68cm, 5.89cm, 6.25cm, 5.66cm in colony diameter, respectively when compared to the control plate (Table 2). *Nigrospora* spp. showed higher inhibition percentage of colony

diaemeter which was 37.02 % than *Fusarium chamydosporum*, *Phialophora* spp. which were 35.28% and 30.55%, respectively. But, *Phoma exigua* gave lowest inhibition percentage of colony diameter which was 25.75% (Fig. 8). This result was similar to the study from Luiz H. Rosa *et al.* (2012) who reported that the extracts of endophytic fungi, *Fusarium* spp. was able to inhibit the phytopathogens, *Colletotrichum acutatum, C. fraga- riae, and C. gloeosporioides* and endophytic *Nigrospora* spp also can inhibited some plant pathogen, *Colletotrichum* spp.

Table. 2. Colony growth on antagonistic bi-culture tests

Antagonist fungi	***Colletotrichum coffeanum***	
	Colony(cm)	% inhibition of colony
Control	9.00[a1/]	-
Nigrospora spp.	5.66[d]	37.02[a]
Fusarium chamydosporum	5.82[d]	35.28[a]
Phoma exigua	6.68[b]	25.75[c]
Phialophora spp.	6.25[c]	30.55[b]
CV%	1.77	4.57

1/: Average of four replications. Means followed by the same letter in a column were not significantly different by DMRT at P = 0.01

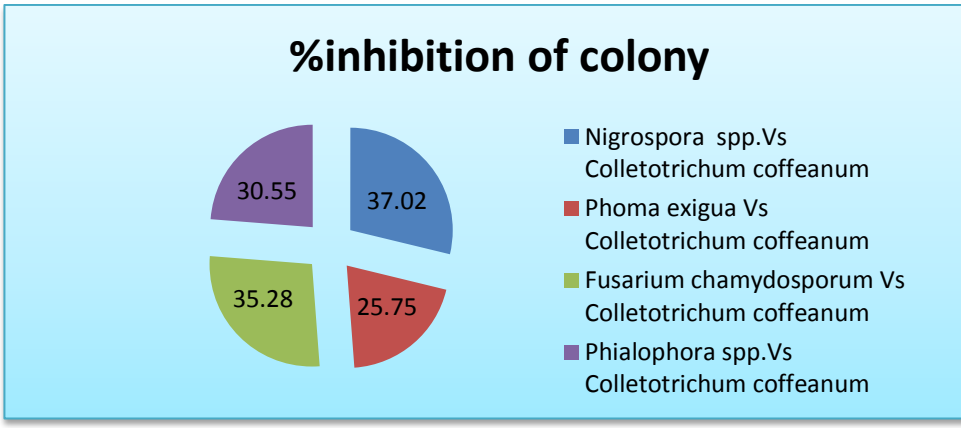

Fig. 8 Percentage inhibihion of colony diameter

The number of spores that producing by the pathogen was counted by using Hemacytometer. The results showed that *Phoma exigua*, *Fusarium chamydosporum*, *Phialophora* spp. and *Nigrospora* spp. significantly inhibited

number of pathogen spores which were 0.87×10^6, 0.37×10^6, 1.12×10^6, 1.06×10^6 spores, respectively when compared to the control plate (Table 3). The pathogen spore production was inhibited 76.66 %, 89.99%, 69.99% and 71.66% respectively (Fig.9). It is illustrated that all the tested endophytic fungi showed inhibition of spore production of *Colletotrichum coffeanum* and *Fusarium chamydosporum* gave the best inhibition of spore production. 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. 3. Number of pathogen spores on antagonistic bi-culture tests

Antagonist fungi	***Colletotrichum coffeanum***	
	Spores($\times10^6$)	% inhibition of number spores
Control	$3.75^{a2/}$	-
Nigrospora spp.	1.0625^{b}	71.66^{ab}
Fusarium chamydosporum	0.375^{c}	89.99^{a}
Phoma exigua	0.875^{bc}	76.66^{ab}
Phialophora spp.	1.125^{b}	69.99^{b}
CV%	19.95	11.09

2/ Average of four replications. Means followed by the same letter in a column were not significantly different by DMRT at P = 0.01

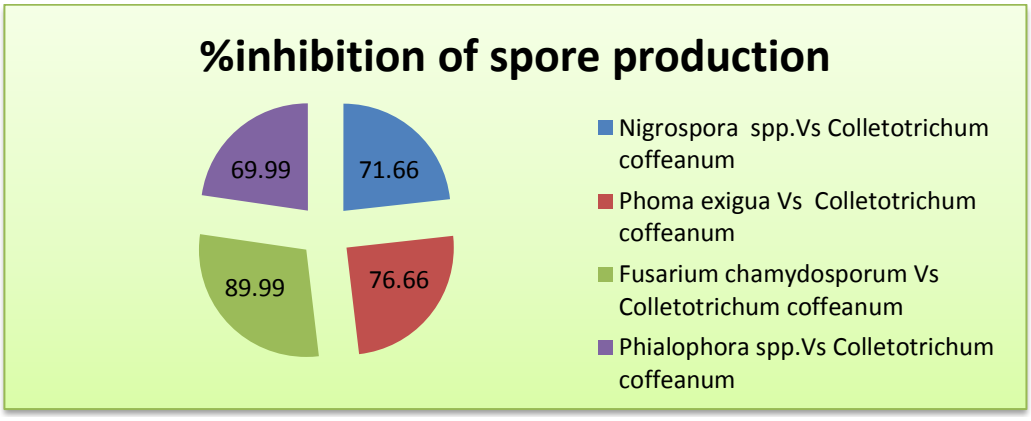

Fig. 9 Percentage inhibition of pathogen spore production

Conclusion

In this study revealed that endophytic fungi isolaed from palms can grow in any parts of palm trees. The tested endophytic fungi (Phoma *exigua*, *Fusarium chamydosporum*, *Phialophora* spp. and *Nigrospora* spp.) were proved biological activity against *Colletotrichum coffeanum* causing coffee anthracnose. The results illustrated that *Nigrospora* spp. showed the best inhibition of colony growth, *Fusarium chamydosporum* gave the best inhibition of spore production of *Colletotrichum coffeanum*.

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