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Biological activity of endophytic fungi from palm trees against chili anthracnose caused by *Colletotrichum capsici*

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Endophytic fungi are those living inside the host plant without causing any apparent negative effect on host plant. Endophytic fungi from palm trees in King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand, were isolated and identified by morphology. Crude hexane, ethyl acetate and methanol. of endophytic *Fusarium* spp. isolated from *Mascarena Lagencuulis* and *Nigrospora* spp. isolated from *Chrysalidocarpus lotescens* were yielded and examined for bioactivity test against *Colletotrichum capsici* causing chili anthracnose. The results showed that crude methanol from *Nigrospora* spp., and *Fusarium* spp. gave the highest inhibition of colony growth of *C. capsici*, which were 48.75% and 34.50%, respectively at concertration of 1000 ppm. Crude methanol from *Nigrospora* spp. gave significantly highest inhibition of spore production of *C. capsici* as 87.26% at concentration of 500 ppm and crude methanol from *Fusarium* spp. showed highest inhibition of spore production as 74.52% at concentration of 1000 ppm. The methanol crude extract from *Nigrospora* spp. expressed the ED_{50} value of 41.51 ppm and ethyl acetate crude extract from *Fusarium* spp. expressed the ED_{50} of 42.40 ppm to inhibit *C. capsici*. The research findings are reported that the metabolites from *Nigrospora* spp. and *Fusarium* spp. inhibited *C. capsici.*

Key words: Endophytic fungi; palm trees; bioactivity test; chili; *Colletotrichum capsici*

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 due to its ability to produce novel secondary metabolites for medical,

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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.

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).

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). Jane 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.

Chili (*Capsicum* spp.) is the fourth important vegetable in the world and the first in Asia, with world production in 2006 at approximately 25.9 million t for fresh chili and 2.8 million t for dry chili (FAOSTAT, 2008).Anthracnose, caused by *Colletotrichum* spp., is a serious problem for chili production in the tropics and subtropics worldwide. The pathogenic fungi *C. capsici* were reported as chili anthracnose in Thailand (Sangchote *et al*., 1998; Sangchote, 1999). This fungus is one of the major diseases for chili and causes severe damage on chili fruits in both pre and post harvest stages. And, these infections together account for more 50% of the crop losses (Pakdeevaraporn *et al*., 2005).The fungus is both internally and externally seed-borne (Ramachandran *et al*., 2007). The black wound found on infected fruits will expand very quickly under high moisture condition, especially in tropical countries. Fruiting bodies and spores of *C. capsici* will be abundantly produced on those black lesions.

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 associated with palm trees, *Mascarena Lagencuulis* and *Chrysalidocarpus lotescens* to inhibit *C. capsici* causing anthracnose of chili.

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. Plant specimens were randomly selected from healthy leaves, petioles and roots of palm trees and taken to laboratory and processed within 24 h. All the samples 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 isolates were identified by the morphology of the fungal culture, the mechanism of spore production and characteristics of the spore. The isolates were grown on PDA with decoction of host leaf medium to observe sporulation. For tentative identification, microscopic slides of each endophytic fungus was prepared and examined under binocular compound microscope for morphological identification.

Isolation of pathogen and pathogenicity test

The plant pathogen *C. capsici* causing chili anthracnose were isolated by tissue transplanting technique from chili fruit with obvious symptoms and performed pathogenicity test followed Koch's Postulate. The disease chili 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. *C. capsici* were identified by morphological characteristic under binocular compound microscope.

Then, the isolates were tested for pathogenicity using detached fruit method in the laboratory. Select healthy fruit of chili and washing in the running water and air-dried. A sterilized filter paper was placed in 9cm diameter sterilized petri dish and two sterilized glass slide were also put on the filter paper, and the filter paper were moistened by sterilized distilled water. Chili fruits were wounded by sterilized needle and then placed on the glass slide in the petri dish then the spore suspension of *C. capsici* was prepared at concentration was 5×10^6 spores/ml. Spore suspension were sprayed on the surface of chili fruits including the wounded areas, incubated for two weeks at room temperature. At the same time, sterilized water was also sprayed to chili fruits as controls and incubated. Lesions on inoculated areas were observed 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.

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). Endophytic fungi were cultured in potato dextrose broth (PDB) at room temperature (28-30℃) for 45 days. The fungal biomass of endophytics were removed from PDB, filtered through cheesecloth and air-dried overnight. The fungal biomass were grounded with electrical blender， and placed in triangular flask. And then dissolved

with equal volume hexane 5 days at room temperature, the biomass were separated by filtration through whatman filter paper. The solvent was evaporated in *vacuum* to yield crude hexane. The marc was further extracted with ethyl acetate (EtOAc) and methanol (MeOH) respectively using the same procedure as hexane. Each crude extract was weighted, and then kept in refrigerator at 4 C until to use.

Bioactivity against C. capsici

The crude extracts were tested for inhibition of *C. capsici*. 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 tested for bioactivity against *C. capsici*. They were *Nigrospora* spp. (Fig. 3) from *Chrysalidocarpus lotescens* (Fig. 1) and *Fusarium* spp. (Fig. 4) from *Mascarena Lagencuulis* (Bottle palm) (Fig. 2).

Fig. 1 *Chrysalidocarpus lotescens*

Fig. 2 *Mascarena Lagencuulis* (Bottle palm)

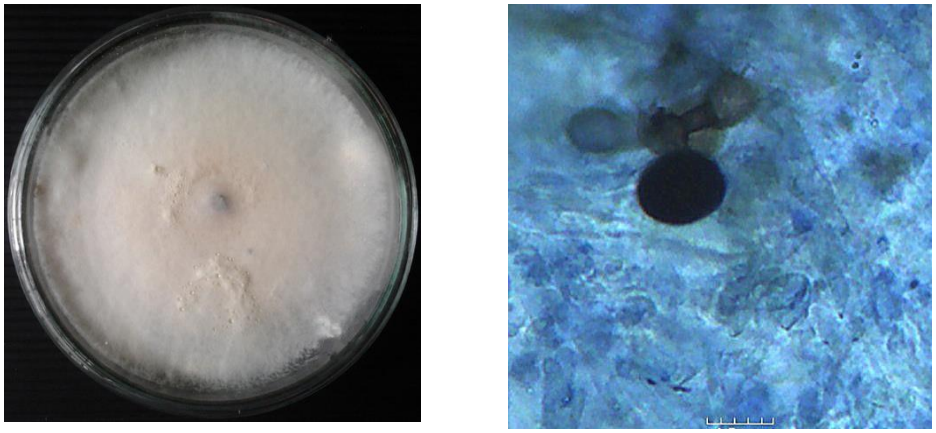

Fig. 3 *Nigrospora* spp. pure culture and spore

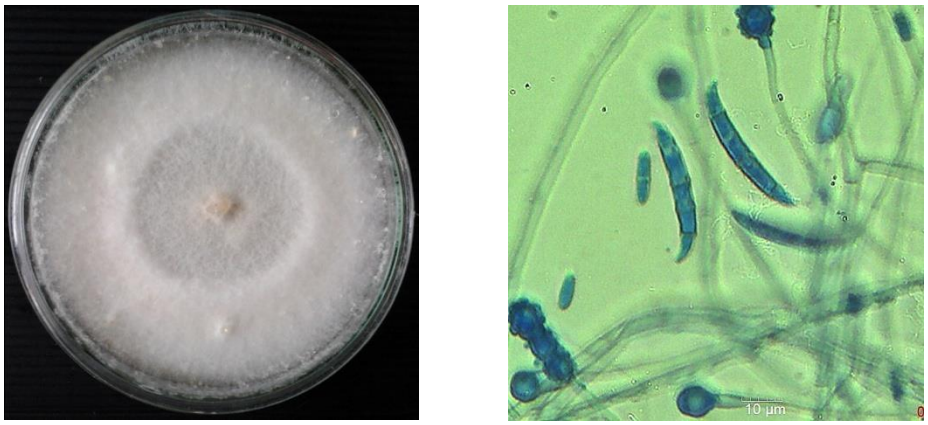

Fig. 4 *Fusarium* spp. pure culture and spores

Isolation of pathogen and pathogenicity test

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

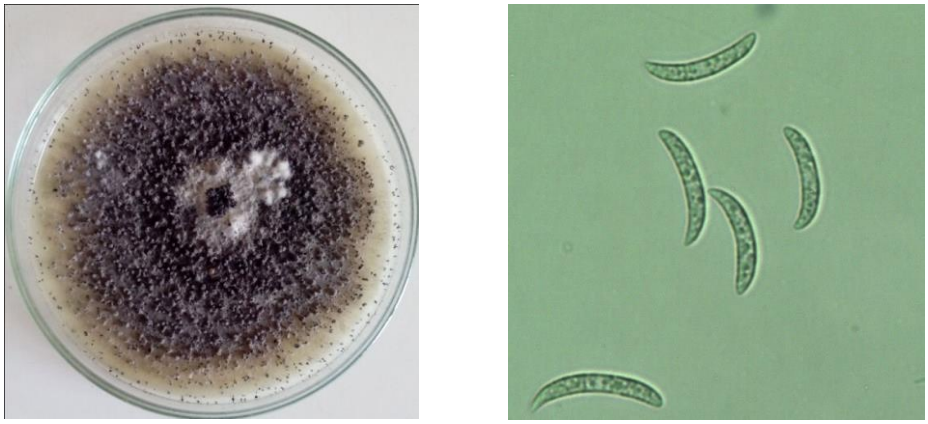

Fig.5 *Colletotrichum capsici* pure culture and spores

Non-inoculated control Inoculated one

Fig. 6. Pathogenicity test on chili fruits

Crude Extraction of Bioactivity Substances

The pure cultures of *Nigrospora* spp. and *Fusarium* spp. were cultured in PDB for 45days. Each fungal biomass was separately extracted to get crude hexane, crude ethyl acetate and crude methanol. With this, the crude

hexane, crude ethyl acetate and crude methanol from *Nigrospora* spp. yielded 0.37, 0.67 and 2%, respectively. The crude hexane, crude ethyl acetate and crude methanol from *Fusarium* spp. yielded 1.74, 0.83 and 3.85 %, respectively (Table. 1)

Table 1. Extraction of biological active substances from fungal biomass

endophytes	Fresh weight (g)	Dry weight (g)	Yield[1]			
			Crude Hexane(g)	Crude EtOAc(g)	Crude MeOH(g)	Total
Nigrospora sp.	2750	90	0.34 (0.37%)	0.60 (0.67%)	1.80 (2%)	2.74 (3.04%)
Fusarium sp.	3670	250	4.36 (1.74%)	2.08 (0.83%)	9.62 (3.85%)	16.06 (6.42%)

Bioactivity against C. capsici

Endophytic fungi *Nigrospora* spp. and *Fusarium* spp. were selected to yield metabolite as crude extracts and examined for bioactivity test against anthracnose of chili caused by *C. capsici*. The results showed that crude methanol from *Nigrospora* spp. gave significantly highest inhibition of 48.75% for the colony growth of *C. capsici* at concentration of 1,000 ppm when compared to the control, followed by crude hexane which inhibited 43.25% at concentration of 100 ppm (Table 2). Methanol crude extract from *Nigrospora* spp. gave significantly highest inhibition for the spore production of *C. capsici* as 87.26% at concentration of 500 ppm and the effective dose (ED_{50}) inhibited spore production of *C. capsici* at concentration of 41.51 ppm. Followed by, crude ethyl acetate gave 80.93% inhibition at concentration of 1,000 ppm, and ED_{50} at concentration of 130.90 ppm. Crude hexane showed 78.36% inhibition at concentration of 1,000 ppm, and ED_{50} at concentration of 259.56 ppm. (Table 3) 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.

The crude methanol from *Fusarium* spp. expressed highest inhibition percentage of *C. capsici* colony growth which was 34.50% at the concentration of 1,000 ppm. (Table 4). Methanol crude extract from *Fusarium* spp. showed significantly highest inhibition for the spore production of *C. capsici* as 74.52% at the concentration of 1,000 ppm, and the ED_{50} inhibited *C.capsici* spore production at concentration 47.39 ppm. Crude hexane, crude ethyl acetate showed inhibition as 65.18, 68.03% respectively, and the ED_{50} at 491.57, 42.40 ppm, respectively (Table 5). 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 2 Crude extracts of *Nigrospora* spp. testing for growth inhibition of *Colletotrichum capsici* at 7 days

Crude extracts	**Concentration (ppm)**	**Colony diameter (cm)/1**	**Growth inhibition(%)/2**
Crude Hexane	0	5.00^{a}	
	10	4.41^{c}	11.75^{g}
	50	4.43^{c}	11.25^{g}
	100	2.83^{h}	43.25^{b}
	500	3.47^{f}	30.50^{d}
	1000	3.28^{g}	34.25^{c}
Crude EtOAc	0	5.00^{a}	
	10	4.93^{a}	1.25^{i}
	50	4.72^{b}	5.5^{h}
	100	4.55^{c}	9.00^{g}
	500	3.63^{e}	27.25^{e}
	1000	3.44^{f}	31.00^{d}
Crude MeOH	0	5.00^{a}	
	10	4.89^{a}	2.00^{i}
	50	4.85^{ab}	3.00^{hi}
	100	4.10^{d}	18.00^{f}
	500	3.51^{ef}	29.75^{de}
	1000	2.56^{i}	48.75^{a}
C.V.(%)		1.78	7.92

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

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

Table 3 Spore production inhibition of crude extracts from *Nigrospora* spp. to *Colletotrichum capsici* at 30 days and effective dose (ED_{50}) values

Crude extracts	**Concentration (ppm)**	**Number of spores /1 (10^6)**	**inhibition(%)/2**	**ED_{50}**
Crude Hexane	0	4.87^{a}		259.56
	10	4.25^{b}	12.72^{j}	
	50	3.68^{bcd}	24.40^{hi}	
	100	3.50^{cd}	28.16^{gh}	
	500	2.06^{gh}	57.84^{e}	
	1000	1.06^{ijk}	78.36^{bc}	
Crude EtOAc	0	4.87^{a}		130.90
	10	3.93^{bc}	19.18^{ij}	
	50	3.25^{de}	33.43^{g}	
	100	2.81^{ef}	42.40^{f}	
	500	1.50^{hi}	69.39^{d}	
	1000	0.93^{ijk}	80.93^{ab}	
Crude MeOH	0	4.87^{a}		41.51
	10	3.62^{cd}	25.84^{hi}	
	50	2.50^{fg}	48.74^{f}	
	100	1.31^{ij}	73.40^{cd}	
	500	0.62^{k}	87.26^{a}	
	1000	0.81^{jk}	83.50^{ab}	
C.V.(%)		10.77	7.38	

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

[2]/Inhibition (%) = R1-R2/R1x100 where R1was number of pathogen spores in control and R2 was number of pathogen spore in treat

Table 4 Crude extracts of *Fusarium* spp. testing for growth inhibition of *Colletotrichum capsici* at 7 days

Crude extracts	**Concentration (ppm)**	**Colony diameter (cm)/1**	**Growth inhibition(%)/2**
Crude Hexane	0	5.00[a]	
	10	4.73[b]	5.25[j]
	50	4.69[b]	6.00[ij]
	100	4.56[cd]	8.75[ghi]
	500	4.56[cd]	9.00[ghi]
	1000	4.61[bc]	7.75[hij]
Crude EtOAc	0	5.00[a]	
	10	4.62[bc]	7.50[hij]
	50	4.49 [cde]	32.50[e]
	100	4.38[ef]	10.00[fgh]
	500	4.44[de]	12.25[ef]
	1000	4.30[f]	11.00[fg]
Crude MeOH	0	5.00[a]	
	10	4.41 [ef]	11.75[efg]
	50	4.12[g]	17.50[d]
	100	3.96[h]	20.75[c]
	500	3.56[i]	28.75[b]
	1000	3.27[j]	34.50[a]
C.V.(%)		1.48	10.60

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

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

Table 5 Spore production inhibition of crude extracts from *Fusarium* spp. to *Colletotrichum capsici* at 30 days and effective dose (ED_{50}) values

Crude extracts	**Concentration (ppm)**	**Number of spores /1 (10^6)**	**inhibition(%)/2**	**ED_{50}**
Crude Hexane	0	14.56[a]		491.57
	10	10.81[b]	25.12[de]	
	50	10.00[bc]	32.23[d]	
	100	6.62[def]	54.45[bc]	
	500	5.06[ef]	65.18[ab]	
	1000	9.25[bcd]	36.06[d]	
Crude EtOAc	0	14.56[a]		42.40
	10	4.68[ef]	68.03[a]	
	50	9.25[bcd]	35.86[d]	
	100	9.12[bcd]	36.26[d]	
	500	6.81[cdef]	53.56[bc]	
	1000	11.68[ab]	16.61[e]	
Crude MeOH	0	14.56[a]		47.39
	10	10.00[bc]	30.54[d]	
	50	7.00[cde]	51.36[c]	
	100	4.81 [ef]	67.01[a]	
	500	4.18 [ef]	70.85[a]	
	1000	3.68[f]	74.52[a]	
C.V.(%)		17.63	12.52	

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

[2]/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 the tested endophytic fungi, *Nigrospora* spp. from *Chrysalidocarpus lotescens* and *Fusarium* spp. from *Mascarena Lagencuulis* were proved biological activity against *C. capsici* causing chili anthracnose. The results demonstrated that crude methanol of *Nigrospora* spp. showed the best inhibition of colony growth and spore production of *C. capsici* at concentration of 1000 ppm. Both crude ethyl acetate and crude methanol of endophytic fungus *Fusarium* spp. expressed significantly inhibition of spore production of *C. capsici* with low effective dose (ED_{50}) values as 42.40 and 47.39 ppm, respectively.

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