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Application of *Chaetomium cochliodes* CTh02 to against durian root rot cause by *Phytophthora palmivora* RT01

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Abstract The durian is cultivated in tropical regions which are caused root rot by *Phytophthora palimovora* RT01. *P. palmivora* RT01 causing root rot of durian var. monthong was proved to be pathogenic isolate and confirmed morphological and phylogenetic identification. *Chaetomium cochliodes* CTh02 proved to be antagonized *P. palmivora* RT01 causing root rot of durain var. monthong through dual culture evaluation. Crude hexane, crude ethyl acetate and crude methanol extracts of *Ch. cochliodes* CTh02 significantly inhibited colony growth of *P. palmivora* RT01 at the ED_{50} values of 955, 402 and 240 ppm., respectively. Morevover, Crude methanol extracts gave significantly highest inhibited sporangia of *P. palmivora* RT01 which ED_{50} value of 25 ppm and followed by crude hexane, crude ethyl acetate extracts which the ED_{50} values of 163, and 339 ppm, respectively. The control mechanism *Ch. cochliodes* CTh02 against *P. palmivora* RT01 may possible act as antibiosis.

Keywords: *Chaetomium cochliodes, Phytophthora*, Durian root rot

Introduction

The pathogenicity of *Phytophthora palmivora* has infected a wide host range in Durian and Cocoa (Chee, 1969; 1974; Chan and Lim, 1986). Thailand is the leading producer and export durian in the world. Nowadays durian get high demand especially from China that makes durian become an importance economic crop. The most importance problem for durian cultivation is root rot disease cause by *Phytophthora* spp., especially *P. palmivora*. Withthis, Prommate *et al.* (2019) reported that *Phytophthora* rot of durian isolated from durian plantation is identified as *P. palmivora*. *Phytophthora* spp. can damage durian trees in any phase of cultivation, the symptoms of disease appear by the rot of root, leaves blight, stem blight and fruit rot. The application of chemical fungicides found the negative side effect to the environment and cause pathogen resistance. *Phytophthora* species are controlled by chemical

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fungicides such as phenylamides (metalaxyl and related compounds) that reported to be resistance to those fungicides (Erwin and Ribeiro, 1996). Disease management of *Phytophthora* diseases has developed to be eco-friendly control diseases as to reduce the application cost, and harm of fungicides. Biological control agents (BCAs) have become an importance research aspect and environmentally friendly agricultural control to consider among the most promising applications for sustainable agriculture to carry out all over the world (Naqvi, 2004; Sönmez and Mamay 2018). BCAs are increasingly investigated to control plant disease. Soytong (2010) reported that Chaetomium-biological fungicide was proved to be effective against *Phytophthora palmivora* causing stem and root rot of Durian (*Durio zebithenus* L.). *Chaetomium cupreum* strain CC6 and *Chaetomium globosum* strain CG7 significantly inhibited the growth of *P. palmivora* causiong durian rot. *Ch. globosum* and *Ch. aureum* were also reported to inhibit the growth of *Phytophthora infestans Fusarium culmorum* and *P. palmivora* causing pomelo root rot (Linkies *et al.,* 2020; Hung *et al.,* 2015). The research finding was aimed to evaluate the efficiency of *Chaetomium cochliodes* CTh02 to control *P. palmivora* RT01 causing root rot of durian var. monthong.

Materials and methods

Morphological study of Chaetomium cochliodes CTh02 and Phytophthora palmivora RT01

The islate of *Ch. cochliodes* CTh02 was reported by Phonkerd *et al.* (2008) to produce chaetoviridins E, chaetochalasin A which inbibited *Plasmodium falciparum* (malaria disease), and produced chchliodones C, chaetoviridins F, chaetochalasin A to inhibit Mycobacterium tuberculosis. Pure culture of *Ch. cochliodes* CTh02 was sub-cultured from holotype at Biocontrol Research Unit, Faculty of Agricultural Technology, King Mongkut's Institute of Technolopgy Ladkrabang (KMITL) into potato dextrose agare (PDA). *P. palmivora* was isolated from root rot of durian from Chantaburi province, characteristic under binocular compound microscope.

Molecular phylogenetic confirmation

Chaetomium cochliodes CTh02

DNAs was extracted by CTAB (cetyltrimethyl ammonium bromide) which modified the method of Doyle and Doyle (1987). DNA extract was then diluted in 50 µl TE buffer, stored at -20 ℃. T1/T2 primer pairs were used and

multiplied a 700 bp segments of the β-tubulin gene. The reaction was conducted in 5 µl consisted of 1 µl genomic DNA, 0.5 µl dNTPs, 1 µl of each primer, 0.2 µl Taq DNA polymerase in 2.5 µl PCR buffer. PCR products were seperated by 1.0% agarose gel in 1X TAE buffer. It PCR was purified using the PureLinkTM Quick Gel Extraction Kit (Invitrogen). The nucleotide sequences were obtained and searched from database to compare in the Genbank by BLAST. Nucleotide sequences of the related species based on β-tubulin gene that retrieved from GenBank. *Fusarium oxysporum* from GenBank accession No. MK 962470 was an outgroup. The sequences were assembled by BioEdit and aligned ClustalX. Phylogenetic relationship tree was constructed by performing heuristic search under Neighbor-joining (NJ).

***Phytophthora palmivora* RT01**

DNA was extracted using 800 µl of modified digestion buffer of 10 mM Tris/HCl pH 8.0, 50 mM EDTA, 0.5% SDS, 0.5% Triton X-100, 0.5% Tween 20, and 2 µl of 20 mg proteinase K/ml which poured to a 2 ml micro-tube contained the frozen ground fungal biomass, incubated at 55 ℃ for 30 min. The sample was added 800 µl chloroform/isoamyl alcohol (24:1, v/v), and centrifuged for 10 min at ~10,000 ×*g*. The supernatant was removed to a 2 ml tube and added chloroform/isoamyl alcohol. The precipitated DNA pellets were washed with 1 ml 70% (v/v) ethanol and 1 ml absolute ethanol and resuspended in 100 µl warm (55°C) TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA). The pellets were dissolved, and the DNA solution was cooled to 27 ℃, 2 µl 20 mg. Ribonuclease A/ml (Sigma, USA) was added and incubated at 37 ℃ for 30 min. DNA was extracted using the commercial DNeasy Plant Mini Kit and eluted in 50 µl of TE buffer. The sequences were retrived from Genbank by BLAST. *Aspergillus niger* from GenBank accession No. LC496500 was an outgroup. BioEdit and aligned ClustalX were used sequencing. Phylogenetic tree was constructed by performing heuristic search under Neighbor-joining (NJ).

Pathogenicity test

P. palmivora RT01 was tested pathogenicity using Kock's postulate method. The isolate was cultured on PDA for 7 days, sterilized cork borer of 0.3 cm was cut at peripheral colony and moved to the wounded leaves of durian var monthong, then incubated in moist chamber at 29-30 C for a few days to observe the lesion. The infected lesion was re-isolated *P. palmivora* by tissue transplating technique. The non-inoculated wounds on leaf were treated with only PDA agar plug served as controls. Each experiment was replicated four times.

Dual culture test between Chaetomium cochliodes CTh02 and Phytophthora palmivora RT01

Dual culture was evaluated using Completely Randomized Design (CRD) with four replications. *P. palmivora* RT01 and *Ch. cochliodes* CTh02 were separately cultured on PDA at room temperature (28-30 C) for 7 days. The agar plug (0.5 cm diameter) of each isolate was cut by sterilized cork borer from the actively growing edges and was transferred to 9 cm diameter PDA plate by placing an agar plug of the *P. palmivora* pathogen one side of the PDA plate and opposited an agar plug of *Ch. cochliodes* CTh02. The agar plugs on PDA plates either *P. palmivora* RT01 or *Ch. cochliodes* CTh02 were separatedly transferred to PDA served as the controls. The dual culture plates were incubated at room temperature (28-30 C°) for periodically observation within 30 days. The colony diameter (cm.) was recorded and the number of sporangia was counted by haemacytometyer. Inbibition (%) was calculated as the colony diameter or number of sporangia in control plate - the colony diameter or number of sporangia in dual culture plates/ the colony diameter or number of sporangia in control plate X 100. Analysis of variance (ANOVA) was computed to all data and treatment means were comprised with Duncan's New Multiple Range Test (DMRT) at P= 0.05 and 0.01.

Testing biological activity of fungal metaboliotes from Chaetomium cochliodes CTh02 against Phytophthora palmivora RT01

The extraction method was followed the protocal of Kanokmedhakul *et al.* (2006). *Ch. cochliodes* CTh02 was cultured in sterilized potato dextrose broth (PDB), and incubated at room temperature (28-30 C°) for 45 days. The dried biomass of *Ch. cochliodes* CTh02 was yielded by removing from PDB, filtered through cheesecloth and air-dried overnight. The dried biomass was ground with electrical blender and placed in flask with equal volume of hexane for 5 days at room temperature and filtered through Whatman filter paper No.4. The filtrate was then evaporated in vacuum evaporator to yield a crude hexane. The marc was further consequency extracted with ethyl acetate, and methanol by using the same procedure as hexane. The crude hexane, crude ethyl acetate (EtOAc) and crude methanol (MeOH) extracts were yielded. The crude metabolites *Ch. cochliodes* CTh02 were evaluated to inhibit *P. palmivora* RT01, The 3×6 factorial experiment in CRD with four replications was performed. Factor A was crude metabolites (hexane, ethyl acetate and methanol extracts), and factor B was concentrations (0, 10, 50, 100, 500 and 1,000 ppm). Each crude metabolite was dissolved a drop of 2% dimethyl sulfoxide (DMSO)

and mixed to 30 ml PDA before autoclaved at 121℃,15 Psi for 20 mins. 5 mm diameter agar plug of *P. palmivora* RT01 was transferred to the middle of PDA amened with each concentration of crude metabolite, incubated at room temperature (28-30 C ℃) until *P. palmivora* RT01 in control grew in full plate. The colony diameter was recorded and the number of sporangia was counted using haemacytometer. Data were statistically computed ANOVA and treatment means were compared by DMRT at P = 0.05 and 0.01. The effective dose (ED_{50}) was computed by probit analysis.

Results

Morphological study of Phytophthora palmivora RT01

The characteristics of colony were fast growing with aerial mycelium floccose, white colour, reaching 9 cm diameter in 5-7 days. Sporangia are papillate and ovoid with the widest part close to the base. Sporangiophores are proliferated, and oogonium with a single antheridium (Figure 1).

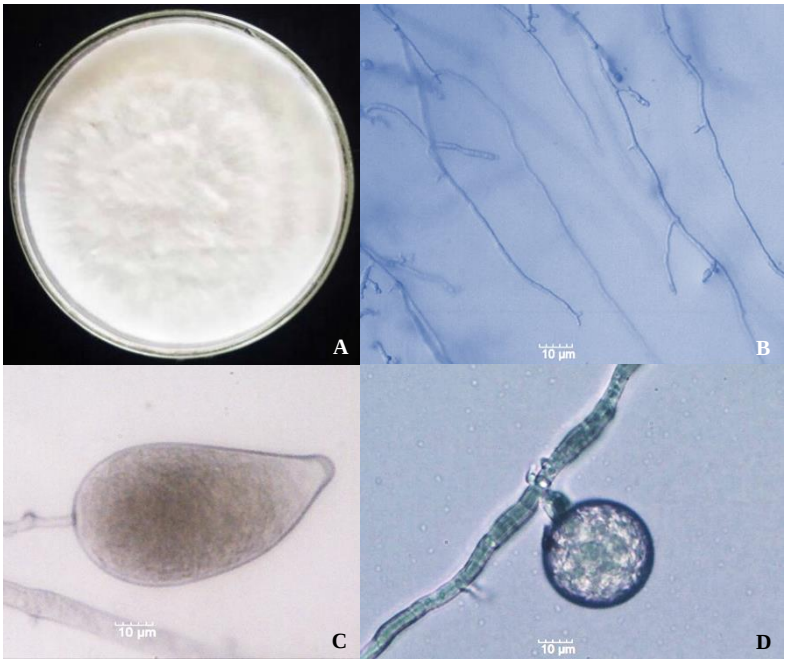

Figure 1. *Phytophthora pamivora* RT01, A=colony on PDA media, B= sporangia proliferation (40X), C=sporangium, D=oogonium

Morphological study of Chaetomium cochliodes CTh02

The culture of *Ch. cochliodes* was slow growing with olivaceous colour. Perithecium was superficial or subglobose with dark brown colour. Terminal hairs were verrucose and dark brown, and the tips, spirally coiled in the upper part, with coils regularly tapering, asci are clavate shape. Ascospores are dark brown colour when mature, then ascal wall disappers (Figure 2).

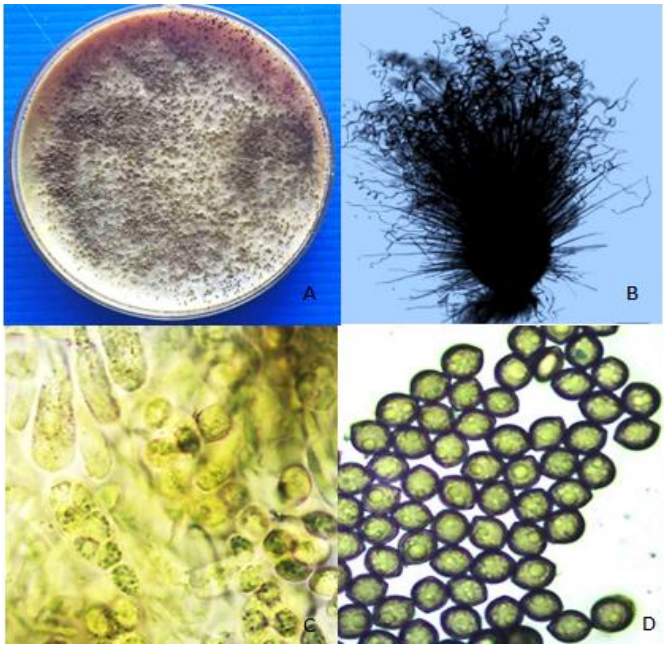

Figure 2. *Chaetomium cochliodes* CTh02, A= colony, B= ascocarps (10X), C= asci (40X) and D= ascospores (40X)

Molecular phylogenetic confirmation

Chaetomium cochliodes CTh02 was confirmed by molecular phylogenetic to species level. PCR prducts were sequenced in the tub2 gene for the identification of the species level. *Ch. cochliodes* CTh02 was comparted to *Ch. cochliodes* from GenBank accession No MH590621, HQ326556, KT895345, JN209864, JN209868 with 1q00 % similarity (Figure 3). Phylogenetic tree of *Chaetomium cochliodes* CTh02 was constructed by Neighbor-joining method based on the 5’ region of the tub2 gene.

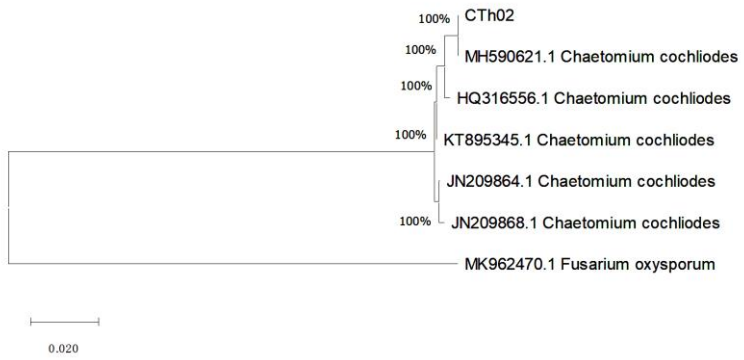

Figure 3. Phylogenetic tree of *Chaetomium cochliodes* CTh02

Phylogenetic tree of *Phytophthora pamivora* RT01 showed similarity cpmparison to *Phytophthora pamivora* which retrived from Genbank accession No. KR920754, KR920758, KR920759, HQ237479, MH219852, MH2!9839, MH219857. The *Aspergillus niger* with Genbank accession No. LC496500 was outgroup (Figure 4).

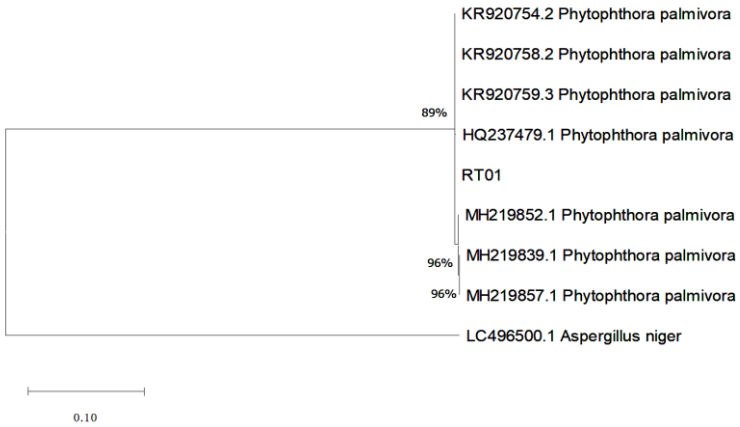

Figure 4. Phylogenetic tree of *Phytophthora palmivora* RT01

Pathogenicity test

Pathogenicity test was proved the pathogenic isolate, *P. palmivora* RT01 on detached leaves of durian var. monthong after 3 days inoculation. The inoculated leaves of durain bvar. Monthong under moist chamber condition showed brown symptom which enlargeg from the agar plug of the wounded leaves. The non- inoculated leaves of durian var monthong showed leaves remained healthy (Figure 5).

Figure 5. Pathogenicity test of *Phytophthora palmivora* RT01 A=non inoculated, B= inoculated

Dual culture test between Chaetomium cochliodes CTh02 and Phytophthora palmivora RT01

Ch. cochliodes CTh02 was proved its abilities to inhibit the growth of *P. palmivora* RT01 using dual culture test (Figure 6). Result showed that *Ch. cochliodes* CTh02 expressed antifugal activity against *P. palmivora* RT01 causing root ror of durian var. monthong which significantly inhibited colony growth and sporangial production of 60.11 and 68.55%, respectively.

Figure 6. *Chaetomium cochliodes* CTh02 inhibited colony growth of *Phytophthora palmivora* RT01 in dual culture test, A= *P. palmivora* RT01 alone, B= *P. palmivora* RT01 against *Ch. cochliodes* CTh02, and C= *Ch. cochliodes* CTh02 alone

Testing biological activity of fungal metaboliotes from Chaetomium cochliodes CTh02 against Phytophthora palmivora RT01

The active metabolites from *Ch. cochliodes* CTh02 were proved their abilities to inhibit the growth of *P. palmivora* RT01 causing root rot of durian var. monthong. Results demonstrated that crude ethyl acetate (EtOAc) extract and crude methanol showed significantly highest inhibited the colony of *P. palmivora* RT01 of 90.00% at the concentration of 1,000 ppm when compared to the control (Table 1, Figure 7). Meanwhile, ED_{50} values of crude ethyl acetate and crude methanol were 402 and 240 ppm. Crude hexane, crude ethyl acetate and crude methanol extracts expressed significantly highest inhibited sporangia of *P. palmivora* RT01 as 86.48%, 92.85% and 93.18% (Table 2) which ED_{50} values of 163, 339 and 25 ppm, respectively.

Table 1. Fungal crude metabolites testing *Chaetomium cochliodes* CTh02 against *Phytophthora palmivora* RT01 for 7 days

Crude metabolites	Concentrations (ppm)	Colony diameter (cm)	Growth inhibition (%)[/2]	ED_{50}(ppm)
	0	5[a1]	-	
	10	5[a]	-	
Hexane	50	5[a]	-	955
	100	5[a]	-	
	500	5[a]	-	
	1,000	1.82[f]	63.50[c]	
	0	5[a]	-	
	10	5[a]	-	
EtOAc	50	3.81[c]	23.75[ef]	
	100	2.62[e]	47.50[d]	402
	500	1.67[f]	66.50[c]	
	1,000	0.50[h]	90.00[a]	
	0	5[a]	-	
	10	4.18[b]	16.25[f]	
MeOH	50	3.43[d]	31.25[e]	
	100	2.46[e]	50.75[d]	240
	500	1.08[g]	78.25[b]	
	1,000	0.50[h]	90.00[a]	
C.V. (%)		6.67	14.85	

[1/]Means of four repeated experiments which followed by the same etter are not significantly differed by DMRT at P=0.05. [2]Inhibition (%)=R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

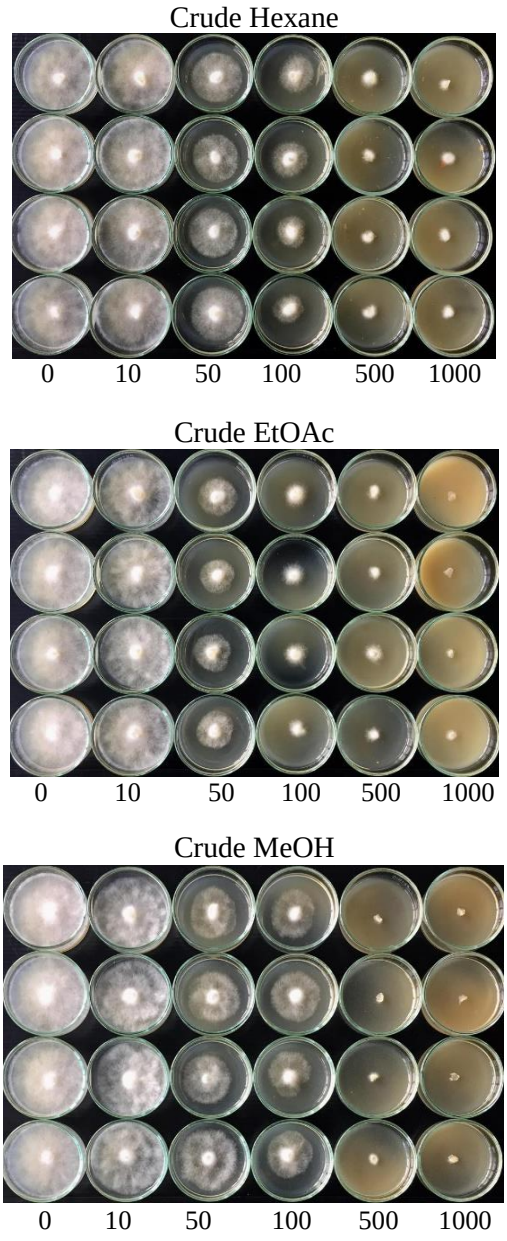

Figure 7. Fungal metabolites testing *Chaetomium cochliodes* CTh02 against *Phytophthora palmivora* RT01

Table 2. Sporangial inhibition of crude mertabolites from *Chaetomium cochliodes* CTh02 against *Phytophthora palmivora* RT01 for 7 days

Crude metabolites	Concentrations (ppm)	Number of sporagia (10^6) /1	Sporagia Inhibition (%)/2	ED_{50}(ppm)
Hexane	0	12.69[a1]	-	163
	10	10.15[bc]	19.27[ef]	
	50	6.55[ef]	46.96[c]	
	100	4.75[fg]	61.15[b]	
	500	3.75[gh]	69.04[b]	
	1,000	1.69[i]	86.48[a]	
EtOAc	0	12.69[a]	-	339
	10	11.54[ab]	8.58[fg]	
	50	9.67[bcd]	23.07[e]	
	100	8.52[cd]	32.05[de]	
	500	3.89[g]	68.72[b]	
	1,000	0.88[i]	92.85[a]	
MeOH	0	12.69[a]	-	25
	10	12.35[a]	2.88[g]	
	50	10.27[bc]	18.93[ef]	
	100	7.95[de]	37.17[cd]	
	500	1.91[hi]	84.93[a]	
	1,000	0.82[i]	93.18[a]	
C.V. (%)		17.87	22.37	

/Means of four repeated experiments which followed by the same etter are not significantly differed by DMRT at P=0.05. /2Inhibition (%) =R1-R2/R1x100 where R1 was colony diameter of pathogen in control and R2 was colony diameter of pathogen in treated plates.

Discussion

Phytophthora palmivora RT01 was morphological confirmation which the characteristics similar to report of Erwin and Ribeiro (1996). The molecular phylogenetic was confirmed to be *P. palmivora* as cpmpared to Genbank with the accession Nos.KR920754 and MH219852. *Chaetomium cochliodes* CTh02 was morphological and molecular phylogenetic identification which comfirmed that *Ch. cochliodes* CTh02 by 5' region of the compared to GenBank. Silmilarly, Wang *et al.* (2016a), stated that β-tubulin gene was more effective than the internal transcribed space gene sequence and the nucleotide sequences of tub2 gene were also useful to identify into species level of *Chaetomium* spp. (Wang *et al.*, 2016b).

Dual culture test *Ch. cochliodes* CTh02 expressed ability to inhibit colony growth and sporangial production of *P. palmivora* RT01 which similar reported by Kumar *et al.* (2020) who stated that *Ch. globosum* stain CG 5157 exhibited a broad spectrum antifungal activity against *Sclerotinia sclerotiorum*

at 73.80% and similar with report of Zhao *et al.* (2017) who statet that *Ch. globosum* CDW7 inhibited the growth of *Botrytis cinerea* (51.6%), *Phytophthora capsica* (55.8%), *Fusarium graminearum* (50.2%) and *S. sclerotiorum* (78.9%). Fungal metabolite extracts from *Ch. cochliodes* CTh02 against *P. palmivora* RT01 resulted that crude ethyl acetate and crude methanol extracts revealed the highest colony inhibition of *P. palmivora* RT01 and ED_{50} values were 402 and 240 ppm. It was similar to the study of Aggarwal *et al.* (2004) reported that crude extracts of from *Ch. globosum* Cg-2 inhibited the mycelial growth of *Cochliobolus sativus* over 87%. Crude hexane, ethyl acetate and methanol extrtacts were significantly highest inhibited sporangial production of *P. palmivora* RT01 from 80-93 %. It is similar reported by Tongon and Soytong (2016) who stated that crude extracts of *Chaetomium brasiliense* actively inhibited *Fusarium solani* and with the ED_{50} of 66 - 288 ppm. Moreover, Zhang *et al.* (2013) reported that *Ch. globosum* strain No.05 inhibited the northern corn leaf blight in maize.

Moreover, the control mechanism of *Ch. cochliodes* CTh02 against *P. palmivora* RT01 may possible act as antibiosis which Phonkerd *et al.* (2008) reported that *Ch. cochliodes* CTH02 produced chaetoviridins E, chaetochalasin A, chchliodones C, chaetoviridins F, chaetochalasin A like antibiotic substances against human pathogens. Similar report of Fatima *et al.* (2016) stated that the crude ethyl acetate extract of *Chaetomium* sp. NF15 showed promising antimicrobial activity against the clinical isolates of *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. The research finding is demonstrated that the active metabolites of *Ch. cochliodes* CTh02 gave significantly supressed *P. palmivora* RT01 causing root rot of duriamn var. monthong. Further research finging is towarded for phytoalexin induction of durian plants against *Phytophthora* rot using these active metabolites.

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