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Antagonism of *Chaetomium* spp and their ability to control citrus root rot caused by *Phytophthora parasitica* in Vietnam

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Phytophthora parasitica was isolated from root rot of citrus trees in the fields in Hung Yen and BacGiang provinces in Vietnam. It proved to be virulent isolate caused root rot of citrus seedling using root-dipped method.Many isolates of *Chaetomium* spp were isolated by baiting technique. It was morphological identified as *Chaetomium cupreum* (CC) and *Chaetomium globosum* (CG). The ED50 values of CG- methanol to inhibit *P. parasitica* was 16 ppm, and followed by CC-hexane 88 ppm, CC-ethyl acetate 97 ppm, CC-methanol 165 ppm, CG-hexane 185 ppm and CG -ethyl acetate 4487 ppm. It is proved the control mechanism as antigiosis. Further investigation would formulate as biological fungicide and teast in the fields.

Introduction

Citrus is one of the major plants cultivated in Vietnam. Citrus orchards are also one of the major fruit commodities to support the household economic for small farmers, which are directed at increasing income, employment opportunities and nutritional status through high value of commercial and nutritional crop (Kean *et al*., 2010). The citrus orchards have been applied chemicals for years and now are being damaged by diseases, especially root rot disease caused by Phytophthiraparasitica. Root rot becomes serious disease causing symptoms of basal stem rot, brown-rot, gummosis, root rot or similar types of gum disease which are caused by *Phytophthora* spp. (Ohazuruike and Obi (2000) which also reported in many countries wherever citrus is grown (Timmer and Menge, 2000). Other diseases are helongjiang disease, greening and tristeza virus diseases which found to be associated with Phytophthora rot as a disease complex (Kean *et al*., 2010). In the case of these diseases has

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become seriously infected citrus. The only choice is needed to remove and replant. However, infection is invaded widespread that many mother plants and nursery stocks. Then, citrus trees are usually found as nutrient deficiency (Molina *et al.,* 1998). Application chemical fungicides have resulted in an increased degree of pathogen resistance (Levy *et al.,* 1983). Many reports stated that disease control problem in citrus can be solved by applying biological fungicide eg Ketomium (Soytong *et al*., 2001) or Trichoderma harzianum and Trichoderma viride to control *Phytophthora* sp. (Singh, 1986; Mukerji and Garg, 1988). The possible promising biofungicide formulated from effective isolates of *Chaetomium* spp. are reported by Soytong *et al*. (2001). It can degrade cellulolytic plant debris to increase high organic matter in soil and specific isolate can inhibit several pathogens (Soytong and Quimio, 1989). *Chaetomium globosum* and *Chaetomium cochlioides* are reported to inhibit the growth of *Fusarium* spp. and *Helminthosporium* spp. (Tveit and Moore, 1954). *Ch. globosum* produces specific metabolites that inhibit the growth of *Pythium ultimum* which causes damping-off of sugar beet (Di-Pietro *et al*., 1991), *Rhizoctonia solani* (Walter and Gindrat, 1988), leaf blight of brassicas caused by *Alternaria brassicicola* (Vannacci and Harman, 1987) and can reduce the pathogen inocula of *Botrytis cinerea* on deadly lily leaves in the field (Kohl et al., 1995). *Chaetomium cupreum* has been reported to control soybean plant pathogens e.g. *Phomopsis* and *Colletotrichum* spp. (Manandhar *et al.,* 1986).

Ch. cupreum and *Ch. globosum* are reported to reduce leaf spot disease of corn caused by *Curvularia lunata*, rice blast caused by *Magnaporthe grisea* (*Pyricularia oryzae*) and sheath blight of rice caused by *Rhizoctonia oryzae* (Soytong, 1989, 1992). The research objectives were to isolateand identifyroot rot of citrus and to isolate the local strains of *Chaetomium* spp inVietnam and to evaluate their abilityto control root rot pathogen of citrus in term of control mechanism.

Materials and methods

Isolation and identification of Phyththora root rot of Citrus

Soil and disease samples of Phytophthora rot of citrus were collected from infested citrus trees in the fields in Hung Yen and BacGiang provinces in Vietnam. Samples were taken from healthy citrus trees and infected root rot of citrus trees. Samples were then separately placed in plastic bags and brought to laboratory. The infected roots were isolated by tissue transplanting method. The roots were surface-disinfected with 10% sodium hypochlorite for 5 minutes, and washed with sterilized distilled water for three times before placing onto water agar (WA), observed and isolated into pure culture. Observation was

periodically done until the hypha growing and transferred to WA and followed by potato dextrose agar (PDA) to be pure cultures. Fungal colonies were cultured and incubated at room temperature (28-32° C) for 5-10 days. Isolate of Phytophthorais kept in PDA slants for further experiment.

Pathogenicity test for Phytophthora root rot

The experiment was designed in Completely Randomized Design (CRD) with four replications. Pathogenicity test was conducted in pot experiment to confirm pathogenicity using Koch's postulation. The tests were performed using one year seedlings of citrus. The isolate was cultured on PDA for 5-7 days at room temperature (28-32° C) then, the mycelium were removed and filtered through two layers cheesecloth to obtain sporangial suspension which adjusted to $2x10^6$ sporangia/ml using haemacytometer. Seedlings were grown in mix potting soil for 30 days. The root-dipped method was used for inoculation followed the method of Marlatt et al. (1996). Dirt and excess soil was removed from the roots of seedlings and washed with tab water. Root tips of seedlings were cut with sterilized scissors of 5 mm and then dipped into sporangial suspension for 15 minutes before transplanting into 8 cm diameter plastic pots containing a sterilized soil (soil mixture consists of loam soil: fine coconut shield: sand = 2:1:1) which autoclaved at 121°C 15 lbs/inch2 for 1 hour. Seedling roots in control were cut and dipped into sterilized distilled water without inoculum. Four replications per isolate (four plants/replication) were used in the experiment. Inoculated plants were maintained in greenhouse at 25-32° C after inoculation. Disease severity index (DSI) was scored at 30 days after inoculation. DSI was analyzed using analysis of variance (ANOVA). The mean comparison were compared by using Duncan's New Multiple Range Test (DMRT) at P=0.05 and P=0.01.

Isolation and identification of Chaetomiumspp

Each soil sample (ca 10 g) was placed to sterilized Petri dishes and moistened with sterile distilled water before being baited with small pieces of sterilized rice straws, filter paper, tissue paper and pineapple leaves. The fungal colonies or fruiting structures on baits were observed by stereomicroscope and then a small piece of mycelia or fruiting structure was taken and placed on WA in a 9-cm-diameter Petri dish, incubated at room temperature. The hyphal tips were transferred to PDA plates and isolated into pure culture. All isolates were screened for biological control activity against Phytophthoraparasitica causing root rot of citrus.

Antagonism test of Chaetomium spp by biological active substance test

The antagonistic substances were extracted from *Chaetomium* spp as crude extracts. These antagonistic substances were tested for their abilities to inhibit the growth of Phytophthoraparasitica. The crude extraction from antagonistic fungi was performed using the method of Kanokmedhakul *et al.* (2006). *Chaetomium* spp was separately cultured in PDB at room temperature (28-30˚C) for 30 days. Fungal biomass were removed from PDB, filtered through cheesecloth and air-dried overnight. Fresh weight and dry weight of fungal biomass were weighed. Dried fungal biomass were ground with electrical blender, extracted with 200 ml hexane (H) and shaken for 24 hour at room temperature. The ground biomass was separated by filtration through Whatman No. 4 filter paper. The marc was extracted again with hexane using method described above. The filtrates were evaporated in vacuo to yield crude extract. The marc was further extracted with ethyl acetate (EtOAc) and methanol (MeOH) respectively using the same procedure as hexane. Each crude extract was weighed, then kept in refrigerator at 5°C until use. *Ch. globosum* cultured in PDB 31 L for 30 days, brought to filter then yielded 2,400 g. It exposed in air dry room tempearature 27-30 C for 5 days yielded 284 g. Fungal biomass was the soaked in hexane 1,000 ml for 5 daysfiltered to separate marc, the filtrate was performed in rotary vacuum evaporator yielded crude Hexane (CG-H) 1.03 g (0.36%). The marc was then soaked in ethyl acetate 1,000 ml for 5 days and filtered to get filtrate and passed through rotary vacuum evaporator yielded crude ethyl acetate (CG-Et) 10.12 g (3.92%). Marc was further then soaked in methanol 1,000 ml for 5 days, then filtered and filtrate and passed through rotary vacuum evaporator yielded crude methanol (CG-M) 31.43 g (13.09%). *Ch. cupreum* cultured in PDB 30 L for 30 days, brought to filter then yielded 2300g. It exposed in air dry room tempearature27-30 C for 5 days yielded 284 g. Fungal biomass was the soaked in hexane 1,000 ml for 5 daysฆfiltered to separate marc, the filtrate was performed in rotary vacuum evaporator yielded crude Hexane (CG-H) 5.32 g (1.99%)ฺThe marc was then soaked in ethyl acetate 1,000 ml for 5 days and filtered to get filtrate and passed through rotary vacuum evaporator yielded crude ethyl acetate (CG-Et) 18.45 g (6.83%)ฺMarc was further then soaked in methanol 1,000 ml for 5 days, then filtered and filtrate and passed through rotary vacuum evaporator yielded crude methanol (CG-M) 9.41 g (3.76%).

Fungal metabolites from Chaetomium spp to inhibit Phytophthora parasitica causing root rot of citrus

The experiment was conducted by using 3x6 factorial in Completely Randomized Design (CRD) with four replications. Factor A represented crude extracts which consisted of hexane crude, ethyl acetate crude and methanol crude and factor B represented concentrations of 0, 10, 50, 100, 500, and 1,000 µg/ml. Each crude extract was dissolved in 2% dimethyl sulfoxide (DMSO), then mixed into PDA before autoclaving at 121°C, 15 lbs/inch2 for 30 minutes. The tested pathogen was cultured on PDA and incubated at room temperature for 5 days, then colony margin was cut by 3 mm diameter sterilized cork borer. The agar plug of Phytophthoraparasitica was transferred to the middle of PDA plate (5.0 cm diameter) in each concentration and incubated at room temperature (28-30°C) for four days. Data were collected as colony diameter and number of sporangia. Percentage of inhibition was computed as described above. Data was statistically computed analysis of variance. Treatment means were computed with DMRT at P= 0.05 and P=0.01. The effective dose (ED_{50}) was computed by using probit analysis.

Results and discussions

Isolation and identification of Phytophthora root rot of Citrus

Phytophthora parasitica was isolated from root rot of citrus trees in the fields in Hung Yen and BacGiang provinces in Vietnam. It proved to be virulent isolate caused root rot of citrus seedling using root-dipped method. The result was similar reported by Kean *et al* (2010).

Isolation and identification of Chaetomium spp.

Many isolates of Chaetomiumspp were isolated by baiting technique. It was morphological identified as *Chaetomium cupreum* and *Chaetomium globosum* by following literatures of Soytong (1989, 1990). The molecular phylogeny of effective isolates of *Chaetomium*spp was confirmed by molecular phylogenetic to species level. DNA extraction, polymerase chain reaction (PCR) and data analysis were done. Genomic DNA was extracted from mycelial culture grown in PDB by using Dneasy Plant Mini kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) amplification of the internal transcribed spacer (ITS1, 5.8S and ITS2 regions) was performed with primer pair PN3 and PN16 (Neuvéglise*et al*., 1994).The reaction was carried out in 25 µl volumes containing 1 µl genomic DNA, 0.5 µl dNTPs, 1 µl of each primerand 0.2 µl *Taq* DNA polymerase in 2.5 µl PCR buffer.

Amplifications were performed with an initial denaturation step of 95°C for 5 min followed by 35 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 3 min and a final extension of 72°C for 5 min. The PCR product was separated on an agarose gel and purified for DNA sequencing by the U-gene gel Extraction Kit II (U-gene Biotechnology Co, P.R China). DNA sequences of the related species based on the ITS including 5.8S gene data were retrieved from GenBank (Table 1). *Ch. nigricolor* was used as outgroup. All of the sequences were assembled using BioEdit, version 7.0.2 and aligned using ClustalX, version 1.83. Phylogenetic relationship was constructed by performing heuristic search under neighbor joining (NJ). The search was carried out using PAUP* 4.0b8. Tree was drawn using Treeview.

Table 1.*Chaetomium* species and isolates used in this study

Isolates	**ITS GenBank accession No.**	**Host/substrate**	**Geographic origin**
Chaetomiumatrobrunneum	FJ595483	Chinese child	China
Chaetomiumbostrychodes	EU520130	-	China
Chaetomiumfunicola	EU139246	Macrotermitinae nest	France
Chaetomiumglobosum	FJ538594	Chrysanthemum	USA
Chaetomiumhispanicum	DQ069021	*Piceaabies*	Lithuania
Chaetomiumlongicolleum	GQ922525	-	Netherlands
Chaetomiummurorum	GQ376100	Deteriorated painting	Greece
Chaetomiumnigricolor	EU543258	*Dioscoreazingiberensis*	China
Chaetomiumpachypodioides	GQ922526	-	Netherlands

Fugal metabolites from Chaetomium globosum to inhibit Phytophthora parasitica causing root rot of citrus

Result showed that CG-H crude extract inhibited highest colony growth of *Phytophthora parasitica* at concentration of 500 ppm which was 4.92 cm significantly different when compared to the control, and followed by 100 50 and 10 ppm which colony diameter were 5.175.25 and 5.25 cm, respectively. However, at 500 ppm gave the best colony inhibition of 7.08 %, and spore suspension was 0.31×10^7spited spore per ml which inhibited spore production of 75.60 %. At concentrations of 100, 50 and 10 ppm were inhibited spore production of 57.85, 41.46 and 41.32 %, respectively. CG-H crude extract

inhibited spore production at ED_{50} of 185 ppm. CG-Et gave the best colony inhibition of *P. parasitica* at 50 ppm which colony diameter was 5.07 cm significantly differed from the control (0 ppm), and followed by 100 50 and 10 ppm which colony diameter were 5.10, 5.22 and 5.25 cm, respectively. At 500 ppm gave the best colony inhibition of 4.25%, and the number of spore production was 0.16×10^7spore per ml which inhibited spore production of 75.13 5 and followed by 100, 50 and 10 ppm, respectively which inhibited spore production of 74.28 , 70.53 and 48.66 %, respectively which ED_{50} was 4,487 ppm. CG-M gave the best colony inhibition of *P. parasitica* at 50 ppm which colony diameter was 5.07 cm significantly differed from the control (0 ppm), and followed by 100 50 and 10 ppm which colony diameter were 5.27 5.30 and 5.30 cm, respectively. At 500 ppm gave the best colony inhibition of 4.28 % and the number of spore production was 0.11 $x10^7$ spore per ml which inhibited spore production of 87.76 and followed by 100, 50 and 10 ppm, respectively which inhibited spore production of 76.06 58.80 and 45.43 %, respectively which ED_{50} was 16 ppm (tables 2 and 3). As a result, Pechprome and Soytong (1997) reported *Ch. globosum* gave a good control of *P. palmivora* causing root rot of durian and Kean *et al.* (2010) stated that C. globosum inhibited *P. parasitica* causing root rot of citrus. Kanokmedhakul *et al.* (2002) stated that *Ch. globosum* is reported to ptroduce new comounds, chatomanone, chaetoglobosin-c and echinuli which also expressed inthibition to *Mycobacterium tuberculosis* causing human disease.

Fungal metabolites from Chaetomium cupreum to inhibit Phytophthora parasitica causing root rot of citrus

CC-H crude extract inhibited highest colony growth of *P. parasitica*at concentration of 500 ppm which was 4.65 cm significantly different when compared to the control, and followed by 100 50 and 10 ppm which colony diameter were 4.67 4.73 and 5.0 cm, respectively. However, at 500 ppm gave the best colony inhibition of 7.0 % and spore suspension was 0.23 $x10^7$spited spore per ml which inhibited spore production of 69.33 %. At concentrations of 100, 50 and 10 ppm were inhibited spore production of 54.39 43.11 and 24.46 %, respectively. CC-H crude extract inhibited spore production at ED_{50} of 88 ppm. CG-Et gave the best colony inhibition of *P. parasitica* at 50 ppm which colony diameter was 3.15 cm significantly differed from the control (0 ppm), and followed by 100 50 and 10 ppm which colony diameter were 3.65 4.53 and 4.55 cm, respectively. At 500 ppm gave the best colony inhibition of 37.0 % and the number of spore production was 0.81 x 10^7spore per ml which inhibited spore production of 68.66 % and followed by 100, 50 and 10 ppm, respectively which inhibited spore production of 74.28 , 70.53 and 48.66 %,

respectively which ED_{50} was 97 ppm. CG-M gave the best colony inhibition of *P. parasitica* at 50 ppm which colony diameter was 3.68 cm significantly differed from the control (0 ppm), and followed by 100 50 and 10 ppm which colony diameter were 4.55 4.55 and 4.60 cm, respectively. At 500 ppm gave the best colony inhibition of 26.5 % and the number of spore production was 1.20 $x10^7$spore per ml which inhibited spore production of 42.26 % and followed by 100, 50 and 10 ppm, respectively which inhibited spore production of 38.64 33.22 and 30.06 %, respectively which ED_{50} was 165 ppm (Tables 2 and 3). *Ch. cupreum* was reported by many literatures to control *P. parasisiata* causing root ror of citrus (Usuwan and Soytong, 1999 and Soytong *et al.*, 1999 and Kean *et al.*, 2010). Moreover it was reported to inhibit *P. palmivara* causing black pepper roor rot (Sodsaart and Soytong,1999).

It is concluded that The ED50 values of CG- methanol to inhibit P. parasitica was 16 ppm, and followed by CC-hexane 88 ppm, CC-ethyl acetate 97 ppm, CC-methanol 165 ppm, CG-hexane 185 ppm and CG -ethyl acetate 4,487 ppm. Kanokmedhakul *et al.* (2002) also pointed that three new azaphilones named rotiorinols A-C (**1**-**3**), two new stereoisomers, (-)-rotiorin (**4**) and *epi*-isochromophilone II (**5**), and a known compound, rubrorotiorin (**6**), were isolated from the fungus *Ch.cupreum* CC3003. Compounds**1**, **3**, **4**, and **6** exhibited antifungal activity against *Candida albicans* (human pathogen) with IC50 values of 10.5, 16.7, 24.3, and 0.6 *í*g/mL, respectively.

Table 2. Per cent inhibition of spore production of *Phytophthoraparasitica* by crude extracts

Crude extracts	% inhibition [1/]				C.V.(%)
	10(ppm)	50(ppm)	100(ppm)	500(ppm)	
CG-H	41.32b[2]	41.46b	54.85ab	75.60a	14.33
CG-Et	48.66b	70.53a	74.28a	75.13a	9.98
CG-M	45.43d	58.80c	76.06b	87.76a	7.49
CC-H	24.46c	43.11b	54.39ab	69.33a	23.34
CC-Et	36.84d	47.77c	54.28b	68.66a	2.66
CC-M	30.06b	33.22a	38.64a	42.26a	25.29

[1/]Inhibition (%) = number of spore in control – number of spore in treatment/number of spore in control

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

Table 3. ED_{50} of crude extracts from *Chaetomium* to inhibit *Phytophthora parasitica*

Crude extracts	ED_{50} (ppm)
CG-H	185
CG-Et	4487
CG-M	16
CC-H	88
CC-Et	97
CC-M	165

CG= *Chaetomiumglobosum*, CC = *Chaetomiumcupreum*, H=hexane, ET=ethylt acetate, M= methanol

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