

Application of A New Bio-Formulation of *Chaetomium cupreum* For Biocontrol of *Colletotrichum gloeosporioides* Causing Coffee Anthracnose on Arabica Variety in Laos

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ABSTRACT

The anthracnose pathogen was isolated from coffee leave and bean symptoms. Morphological and molecular phylogenetic data confirmed the species as *Colletotrichum gloeosporioides*. The pathogenicity of the isolate was also confirmed by detached leaf method which inoculated the virulent isolate into coffee leaves. The crude extracts with hexane, ethyl acetate and methanol solvents from *Chaetomium cupreum* CC3003 resulted significantly inhibited *C. gloeosporioides* that the ED₅₀ values of 13, 11 and 28 ppm, respectively. The bioactive substances of *C. cupreum* CC3003 expressed antifungal activity against *C. gloeosporioides* as can be seen in the abnormal appearance of spores. A powder bio-formulation of *C. cupreum* significantly resulted to reduce anthracnose disease of 54.77 %. The application of nano-rotiorinol, nano-trichotoxin and a spore suspension of *C. cupreum* reduced anthracnose incidence of 46.23, 42.71 and 18.59 %, respectively while the inoculated control had high anthracnose disease. The application of bio-formulation of *C. cupreum* in powder form, nano-rotiorinol, and nano-trichotoxin to reduce coffee anthracnose was reported for the first time in Lao PDR.

Keywords: anthracnose; coffee; nano-elicitors; plant immunity

INTRODUCTION

Coffee var. Arabica is distributed in Laos to the growers in the uplands in 1920 which the sea level elevation of 800 and 1,200 m (Clifford & Helm, 1985). Coffee becomes one of the primary sources of income and export product of 50 coffee producing countries in Latin America, Africa and Asia (Ridler, 1983). Coffee production is the most important perennial crop in the Lao People's Democratic Republic (PDR), mainly grown in the

Phouphieng Paksong area. There are 41,000 ha of coffee plantations, especially in the Saravan, Sekong, Champasak provinces in the Lao PDR. The government has promoted the production of this crop to support local and international needs (Ministry of Agriculture and Forestry, 1997). However, the export price of coffee is 10 % lower than the international market price due to its lower quality, poor agronomic practices and early and manual harvest processing for drying. The important reason for poor quality is limited knowledge by coffee growers to improve quality, control of insects and diseases on coffee beans. The coffee growers have been repeatedly applied chemical fungicides to eliminate anthracnose disease and the pathogen become resistant to chemical fungicides (Soyong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001).

Application of biocontrol agents for disease control has increasingly extended to plant pathologists. The effective biocontrol agents can reduce the pathogen inoculum and disease incidence. *Chaetomium* was reported as biological fungicide to control several plant pathogens. The need to find alternative effective methods of disease control to safe human health and protect the environment is evident. Nanotechnology involves the building and re-structuring materials at the molecular level. Molecular nanotechnology refers to build up the organic or inorganic particles into defined nanometers by molecule (Li, Huang, & Wu, 2011). Agricultural applications of nanotechnology are recently explored and interested by growers (Soutter, 2012). Plant cells are easily and vastly absorbed the nanoparticles through cuticles and tissues (Ditta, 2012). The nanospheres, and nanocapsules are the popular shapes of nano-particles to be used for biocide (Perlatti, de Souza Bergo, Fernandes da Silva, Fernandes, & Forim, 2013).

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This approach enables safe, economical, effective and rapid disease control in crop production (Soutter, 2012). The metabolites from *Chaetomium* spp. were published to control the plant pathogens (Soyong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001). Previous research stated that nano-particles loaded with active metabolites of *C. cupreum* with polylactic acid and electropun at 25-30 kV showed pale orange color. The nano-particle size from *C. cupreum* is averaged as 171 nanometers after loaded in scanning electron microscope. Moreover, nanomaterial containing bioactive metabolites from *Trichoderma harzianum* PC01 at 5-10 ppm exhibited antifungal activity against *C. capsici* causing chili anthracnose (Dar & Soyong, 2014). The current research aimed to isolate the causal agent of coffee anthracnose and evaluate bio-formulations to control anthracnose of coffee var. Arabica.

MATERIALS AND METHODS

Coffee Anthracnose Pathogen and Pathogenicity Test

The causal agent isolated from anthracnose symptoms on leaf and bean of coffee var. Arabica by the tissue transplanting method. The advanced margin of symptom between disease and healthy parts were cut to small pieces (5 × 5 mm), and surface disinfected with 10 % clorox for 1 minute, washed in sterilized water for two times, and moved onto water agar (WA). The hyphal tips growing from infected tissue were moved by sterilized needle and placed onto potato dextrose agar and incubated at 28-30 °C for 7 days. Pure culture derived from single spore isolation techniques. The isolate was morphologically and molecular phylogenetic identified into species level. The molecular phylogenetic identification was conducted by DNA extraction using polymerase chain reaction (PCR). The extraction of genomic DNA from culture growing in potato dextrose broth (PDB) was done with DNA easy Plant Mini kit (Qiagen, Hilden, Germany). PCR amplification of the internal transcribed spacer, ITS1, 5.8S and ITS2 regions was used the primer pairs PN3 and PN16 (modified from Neuvéglise, Brygoo, Vercambre, & Riba, 1994). The 25 µl volumes contained 1 µl genomic DNA, 0.5 µl dNTPs, 1 µl of each primer and 0.2 µl *Taq* DNA polymerase in 2.5 µl buffer were reacted. The initial denatured step of 95 °C for 5 minutes, 35 cycles of 94 °C for 1 minutes, 60 °C for 2 minutes, then 72 °C for 3 minutes and a final extension of 72 °C for 5 minutes

were amplified. The separation of PCR products were shown in agarose gel to yield the purified for DNA for sequencing by the U-gene gel Extraction Kit II (U-gene Biotechnology Co., P.R. China). The related species based on the ITS including 5.8S gene of DNA sequencing database was retrieved from GenBank.

Pathogenicity was done by the plug inoculation method. The healthy coffee leaves were sterilized by 10 % sodium hypochlorite and air-dried. The surface sterilized leaves were made wounds by a sterilized needle, 0.5 cm-diameter wound was done per leaf. PDA medium was used to culture the pathogen and incubated at room temperature for 15 days. The plugs of the pathogen (0.3 cm-diameter) were cut from peripheral colony by sterilized cork borer, and placed to the wounds on the leaves. The control was wounded and transferred a PDA plug without the pathogen. The leaves were inoculated that kept in moist box at room temperature. The experiment was done using Completely Randomized Design (CRD) and repeated 4 times. Data was recorded as lesion diameter (mm). The statistical analysis was computed analysis of variance. Duncan's Multiple Range Test (DMRT) at P = 0.05 and 0.01 was used to compare the different.

Evaluation Bioactivity from *Chaetomium* sp. Against Coffee Anthracnose Pathogen

The crude extracts of *Chaetomium cupreum* was prepared as the method of Kanokmedhakul et al. (2006). *Chaetomium* sp. was cultured in PDB at 28-30 °C for 30 days and filtered through cheese cloth to get the specimen and dried overnight. Then, specimen was put into an electrical blender to get ground specimen. It was processed to extract in a solvent of 200 ml hexane, shaken for 24 hours, then filtrated with filter paper to get filtrate. The extracted marc with hexane was serially also done with the solvents of hexane and methanol to get crude extracts. Data were collected as fresh and dry weight (g), and kept in refrigerator at 5 °C for experiment. All crude extracts were evaluated to inhibit the tested pathogen. The experiment was designed as 3 x 6 factor factorial CRD, and repeated four times. Factor A was hexane, ethyl acetate and methanol crude extracts of *C. cupreum*. Factor B was 0, 10, 50, 100, 500 and 1,000 ppm. The 2 % dimethyl sulfoxide used to dissolve each crude extract in PDA, then sterilized in autoclave for 30 minutes. The colony margin of pathogen culture at 5

Somlit Vilavong and Kasem Soyong : *Bio-formulation of Chaetomium cupreum to control coffee anthracnose*.....

days was cut by a sterilized borer (3 mm-diameter) to get agar plugs. The pathogen's agar plug was moved to the center of a 5.0 cm-diameter PDA plate, and maintained at 28-30 °C room for 4 days. Data collection was done as diameter of colony and count the conidia using haemocytometer. Probit analysis was computed the effective dose (ED_{50}). Thenormal and abnormal conidia were recorded under a binocular compound microscope.

Testing Bio-formulations to Control Coffee Anthracnose in Pot Experiment

One-year-old coffee var. Arabica plants were inoculated with a 1×10^6 spore ml^{-1} suspension of the anthracnose pathogen. Ten wounds on leaves/ seedling with the fifth leaf from the top; a wound was puncture with sterilized needles 10 times. The experiment was designed as Randomized Complete Block Design (RCBD) and repeated four times. The following treatments were applied at 15-day intervals: T1 was inoculation with anthracnose pathogen, T2 was a spore suspension of *C. cupreum* CC3003 at a concentration of 1×10^6 spore ml^{-1} , T3 was a bio-formulation in powder of *C. cupreum* CC3003 at a concentration of 10 g.20 L⁻¹ of water, T4 was nano-trichotoxin-A50 and T5 was nano-rotiorinol. Nano-trichotoxin-A50 and nano-rotiorinol were produced by Dr. Kasem Soyong and Joselito Dar at the Biocontrol Research Laboratory, KMITL, Bangkok, Thailand, which designed from metabolites of *C. cupreum* CC3003 producing rotiorinol (Kanokmedhakul et al., 2006), and *Trichoderma harzianum* PC01 producing trichotoxin A50 (Suwan et al., 2000) as previous works. The data were statistical analyzed and compared treatment difference by DMRT.

Disease index (DI) scored as level 1 was no symptoms, level 2 was 21-25 % blight, level 3 was 26-50 % blight, level 4 was 51-75 % blight and level 5 was over 75 % blight.

Reduction of disease (%) was calculated as

$$DI \text{ in inoculated control} - \frac{DI \text{ in treatment}}{DI \text{ in inoculated control}} \times 100$$

RESULTS AND DISCUSSION

Coffee Anthracnose Pathogen and Pathogenicity Test

Colletotrichum sp. designated as "CC" was isolated from coffee leaves and beans of var. Arabica exhibiting anthracnose symptoms (Fig. 1). The pathogenicity of the isolate was confirmed; the isolate produced typical anthracnose symptoms on coffee leaves 20 days after inoculation (Fig. 2).

The non-inoculated leaves remained anthracnose-free. The molecular phylogeny of the coffee anthracnose pathogen was determined the species of *Colletotrichum* isolate CC as *Colletotrichum gloeosporioides*. *Chaetomium globosum* was compared as the outgroup. Bio Edit, version 7.0.2 was used for sequencing assembly, aligned with Clustal X, version 1.83. The phylogenetic tree was performed a heuristic search by using neighbour joining. The search was done by using PAUP* 4.0b8 (Fig. 3). The previously reports were confirmed through molecular and morphological characters that anthracnose on coffee var. Arabica in Laos was caused by *C. gloeosporioides* (Bailey & Jeger, 1992; Sutton, 1980).

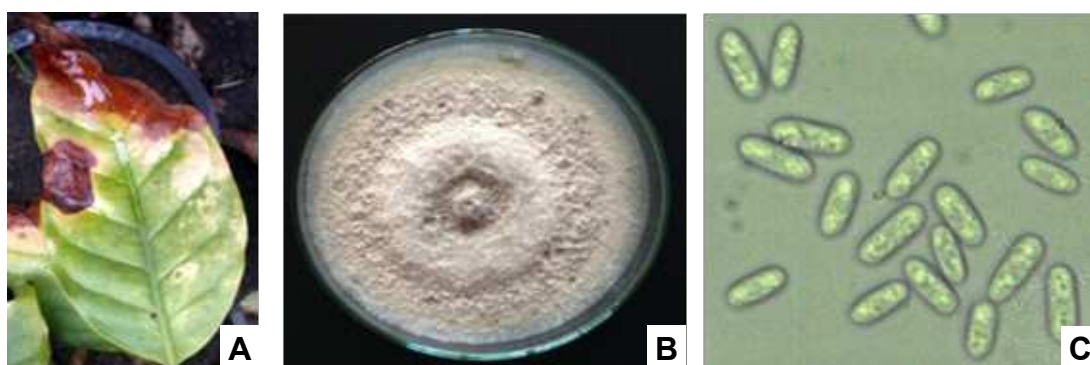


Fig. 1. Leaf anthracnose of coffee var. Arabica caused by *Colletotrichum gloeosporioides* (A); pure culture on PDA at 20 days (B); and conidia, 400 X (C)



Fig. 2. Pathogenicity test of *Colletotrichum gloeosporioides* on Arabica variety coffee. The inoculated control (left) and inoculated leaves (right) after 20 days.

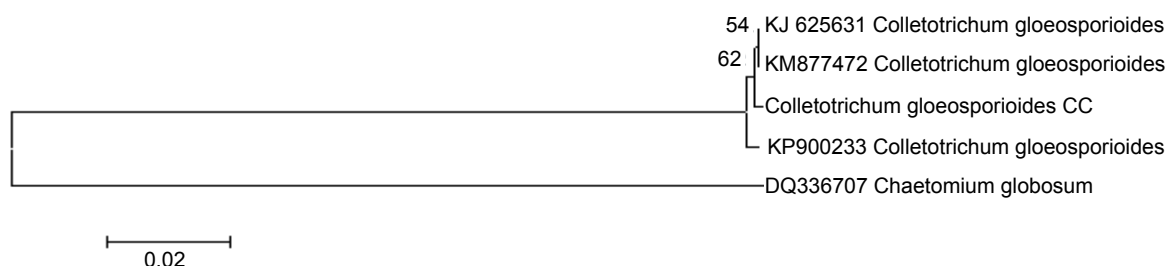


Fig. 3. Phylogenetic tree of *Colletotrichum gloeosporioides* isolate CC from coffee anthracnose compared to GenBank data. Distance-based analysis construction of the ITS1 and 5.8S regions of rDNA. Numbers at the branches indicated the percentage of bootstrap values after 1000 replications. *Chaetomium globosum* was out group

Evaluation Bioactivity from *Chaetomium* sp. against Coffee Anthracnose Pathogen

The results showed that the crude ethyl acetate extract of *C. cupreum* CC3003 showed the highest inhibition of *C. gloeosporioides* with an ED_{50} of 11.03 ppm, followed by the crude hexane and methanol extracts which exhibited ED_{50} of 23.42 and 28.26 ppm, respectively. Colony growth and conidial production were inversely related to bioactive substance concentration. Crude ethyl acetate extract at 1,000 ppm showed significantly highest inhibited sporulation of *C. gloeosporioides* (96 %), followed by the crude methanol (94 %) hexane extracts (89 %) (Table 1). The crude ethyl acetate extract significantly inhibited colony growth by 61 %, while the crude hexane and methanol extracts produced a 21 % reduction (Fig. 4).

Previous research indicated that crude extracts of *C. cupreum* CC3003 significantly inhibited *C. gloeosporioides* causing anthracnose of chili (Soyong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001). The bioactive substances of *C. cupreum* CC3003 extracted by hexane, ethyl acetate and

methanol clearly showed that antifungal control mechanism was lysis and antibiosis as can be seen in the abnormal appearance of spores under the compound microscope. All concentrations of bioactive substances of *C. cupreum* CC3003 caused abnormal spore morphologies which were apparently related to loss of pathogenicity (Fig. 5). *C. cupreum* was reported to produce antagonistic substances that break down the pathogen cells resulting in loss of viability and pathogenicity (Heye & Andrews, 1983). Moreover, the mechanism of plant disease control by *C. cupreum* CC3003 involves the production of antibiotics including rotiorinol A, and C, rotiorinol and epi-isochromophilone II that expressed to inhibit the growth of *Candida albicans* which IC_{50} were 10.5, 16.7, 24.3 and 0.6 ppm, respectively (Kanokmedhakul, et al., 2006; Soyong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001). Moreover, *C. cupreum* CC3003 is reported to inhibit *C. capsici* causing chilli anthracnose (Ratanacherdchai, Wang, Lin, & Soyong, 2010) and other antagonistic *Chaetomium* sp reported to inhibit *Botrytis cinerea* (Köhl, Molhoek, van der Plas, & Fokkema, 1995) and *Helminthosporium victoriae* (Tveit & Moore, 1954).

Somlit Vilavong and Kasem Soyong : *Bio-formulation of Chaetomium cupreum to control coffee anthracnose*.....

Table 1. The effect of crude extracts of *Chaetomium cupreum* CC3003 against *Colletotrichum gloeosporioides* causing coffee anthracnose at 7 days after inoculation

Crude extracts	Concentration (ppm)	Colony diameter (cm)	Spore number	% colony inhibition	% spore inhibition	ED ₅₀ (ppm)
Hexane	0	5.0a	24.3a	0.0i	0.0i	23.42
	10	4.8ab	17.6c	3.7ghi	27.7h	
	50	4.7ab	8.1ef	4.2gh	66.6ef	
	100	4.7bc	3.8gh	5.2fg	84.1bcd	
	500	4.5de	3.0hi	9.8de	87.7abc	
	1000	3.9g	2.6hi	21.4b	89.2abc	
Ethyl acetate	0	5.0a	21.5b	0.0i	0.0i	11.03
	10	4.8ab	11.1d	3.1ghi	47.6g	
	50	4.5cde	6.5fg	9.0def	69.8e	
	100	4.4e	4.2gh	10.6d	80.4cd	
	500	4.2f	2.8hi	15.5c	86.2abc	
	1000	1.9h	0.8i	61.5a	96.0a	
Methanol	0	5.0a	24.2a	0.0i	0.0i	28.26
	10	4.9a	16.5c	0.5hi	31.7h	
	50	4.6bcd	10.0de	6.3efg	58.8f	
	100	4.5cde	6.3fg	9.1def	73.8de	
	500	4.1f	2.0hi	17.5c	91.7ab	
	1000	3.9g	1.3hi	21.9b	94.3ab	
C.V. (%)		2.27%	14.93%	18.1%	8.85%	

Remarks: Statistical analysis and treatment means of four replications in each column are shown significantly different with common letters by DMRT at P = 0.01



Fig. 4. Effects of metabolites of *Chaetomium cupreum* on the colony growth of *Colletotrichum gloeosporioides*

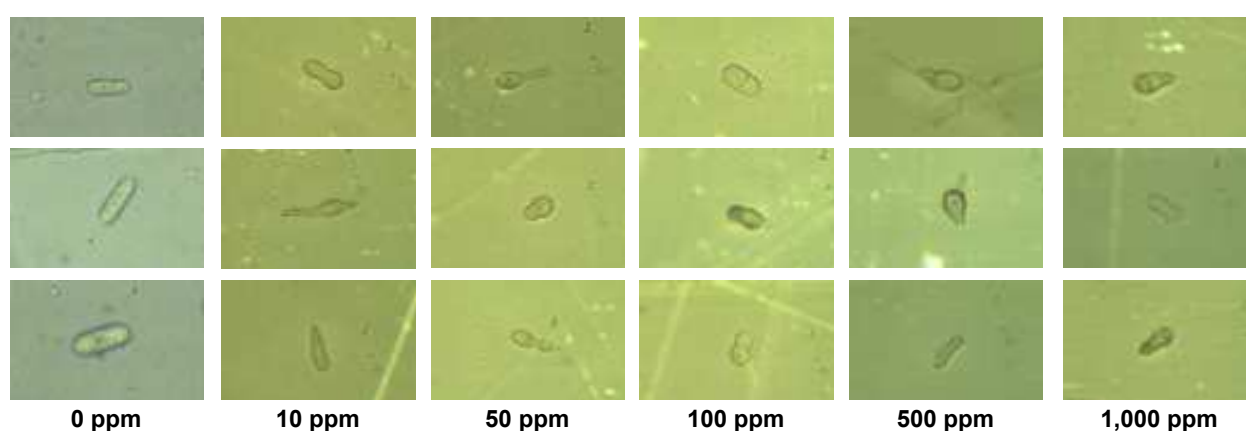


Fig. 5. Abnormal spore morphologies of *Colletotrichum gloeosporioides*. Crude hexane (top), crude ethyl acetate (middle) and crude methanol (bottom) of *Chaetomium cupreum*



Fig. 6. The effect of bio-formulation of *Chaetomium cupreum* to control coffee anthracnose caused by *Colletotrichum gloeosporioides* at 60 days after inoculation, where T1 was inoculated with *C. gloeosporioides*, T2 was a spore suspension of *C. cupreum* CC3003 (1×10^6 spore ml^{-1}), T3 was a powder bio-formulation of *C. cupreum* (10 g.20 L^{-1} of water), T4 was nano-trichotoxin-A50 and T5 = Nano-rotiotinol

Table 2. Effect of bio-formulation of *Chaetomium cupreum* CC3003 on control of leaf anthracnose caused by *Colletotrichum gloeosporioides* in coffee var. Arabica at 30 days after inoculation

Treatments	Disease index	Disease reduction (%) ²
Control	1.99a ¹	0.00
Spores(<i>C. cupreum</i>)	1.62ab	18.59
Bioformulation (<i>C. cupreum</i>)	0.90b	54.77
Nano-trichotoxin	1.14b	42.71
Nano-rotiorinol	1.07b	46.23
C.V. (%)	33.39%	-

Remarks: ¹ Statistical analysis and treatment means of four replications in column was shown significantly different with common letters by DMRT at $P = 0.01$. ² Disease reduction (%) = $\text{DI in inoculated control} - \text{DI in treatment} / \text{DI in inoculated control} \times 100$

Testing Bio-formulations to Control Coffee Anthracnose in Pot Experiment

The powder bio-formulation of *C. cupreum* CC3003 at a concentration of 10 g.20 L^{-1} of water gave the significantly highest to control coffee anthracnose caused by *C. gloeosporioides* with a disease index (DI) of 0.90. Thenano-rotiorinol, nano-trichotoxin and spore suspension of *C. cupreum* showed DI of 1.07, 1.14 and 1.62, respectively when compared to the control (1.99). With this, the powder bio-formulation of *C. cupreum* CC3003 revealed the highest disease reduction of 54.77 %. The application of nano-rotiorinol reduced anthracnose of 46.23 %. Thenano-trichotoxin and spore suspension of *C. cupreum* reduced the disease of 42.71 and 18.59 % when compared to the control (Fig. 6 and Table 2). In the current research bio-formulations *C. cupreum* CC3003 showed effective reduction of coffee anthracnose. In addition, a microbial elicitor from *Chaetomium* was shown to induce immunity against anthracnose caused by *Colletotrichum*

capsici in chili by production of the phytoalexin (Soyong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001). It is interesting that new roles for nano-rotiorinol and nano-trichotoxin-A50 in plant disease control were also identified. But our research found that nano-rotiorinol and nano-trichotoxin were less effective in disease suppression than the powder bio-formulation *C. cupreum* CC3003. This may due to the concentration used and/or physiological conditions. Further study should examine possible phytoalexin production in coffee by bio-formulations of *C. cupreum* CC3003 in coffee and concentration optimization.

CONCLUSION

The molecular phylogeny of the coffee anthracnose pathogen was determined the species of *Colletotrichum* isolate CC to be *C. gloeosporioides*. The pathogenicity of the isolate was confirmed; the isolate produced typical anthracnose symptoms on coffee leaves for 20

Somlit Vilavong and Kasem Soyong : *Bio-formulation of Chaetomium cupreum to control coffee anthracnose*.....

days after inoculation. Crude ethyl acetate extract at 1,000 ppm resulted the highest spore inhibition of *C. gloeosporioides* (96 %), followed by the crude methanol (94 %) crude hexane extracts (89 %). The bioactive substances of *C. cupreum* showed antifungal control mechanism as can be seen in the abnormal appearance of spores. In pot experiment, the powder bio-formulation of *C. cupreum* showed the highest disease reduction (54.77 %). The tested nano-rotiorinol, nano-trichotoxin and spore of *C. cupreum* reduced anthracnose 46.23, 42.71 and 18.59 %, respectively, when compared to the control.

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Somlit Vilavong and Kasem Soyong : *Bio-formulation of Chaetomium cupreum to control coffee anthracnose*.....

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