Bioformulations and nano product from *Chaetomium cupreum* CC3003 to control leaf spot of rice var. Sen Pidoa in Cambodia

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Abstract

Curvularia lunata was isolated from leaf spot of rice var. Sen Pidoa and tested for pathogenicity. *Chaetomium cupreum* CC3003 expressed antifungal activity against *C. lunata* in dual culture test. Hexane-crude extract, EtOAc-crude extract and methanol-crude extract from *C. cupreum* inhibited sporulation of *C. lunata* with ED_{50} of 6.41, 0.83 and 7.81 µg/mL, respectively. Pot experiment revealed that plant heights in treated with a spore suspension of *C. cupreum*, bioformulation of *C. cupreum*, nano product from *C. cupreum* and tebuconazole were not significantly different when compared to the inoculated control. Disease reduction compared to the inoculated control from treatment with a spore suspension of *Chaetomium*, bioformulation of *C. cupreum*, nano product from *C. cupreum* and tebuconazole ranged between 41.66% to 58.33%. Field experiment indicated that chemical method was decreased leaf spots infection by 60%, followed by organic method (40%) and GAP methods (40%) , respectively. The chemical and GAP methods were significantly higher in grain weight than the organic method when compared to the non-treated control. This is the first report using *C. cupreum* to control leaf spot of rice var. Sen Pidoa caused by *C. lunata* in Cambodia.

Introduction

Rice (*Oryza sativa* L) is one of the major food crops in Asia where it is the daily diet more than in other regions of the world. The major problems causing reductions in the quality and quantity of rice include pathogens and insect pests. Observation and preliminary disease diagnosis found that a leaf spot of rice caused by *Curvularia lunata* has become one of the most serious diseases of this crop in Cambodia especially in the rice var. Sen Pidoa.[1] It has been reported that *C. lunata* caused leaf spot for the first time in India, and that symptom showed brown leaf spot and finally blight. Moreover, it has been demonstrated that *C. lunata* caused many symptoms in rice *e.g.* grain discoloration,[2] leaf spot,[3] black kernel and seedling blight.[4] Sheath rot of rice was reported for the first time in Tamil Nadu, India.[5] *Curvularia lunata* causing leaf spots on *Sorghum bicolor* was also reported for the first time in Pakistan.[6] Biological control of plant diseases has widely contributed to the reduction of the use of toxic chemical fungicides by farmers that pollute the environment and Harmon-target organisms. *Chaetomium* sp., belonging to the Ascomycota, has been reported as a biocontrol agent against several plant pathogens.[7,8] *Chaetomium globosum* and *C. cupreum* have been successfully applied to control rice blast caused by *Pyricularia oryzae.*[9]

The objective was to evaluate *Chaetomium cupreum* CC3003 as a biocontrol agent to control leaf spot of rice var. Sen Pidoa caused by *Curvularia lunata*.

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Materials and Methods

Isolation of pathogen and pathogenicity test

The pathogen was isolated from leaf spots of rice var. Sen Pidoa in Cambodia by using the tissue transplanting technique which followed the method of Tann *et al.,*[10] and morphologically identified. A pure culture of the putativepathogen was tested for pathogenicity. A Completely Randomized Design (CRD) was performed with 4 replications. The rice var. Sen Pidoa was used for the pathogenicity test. The pathogen inoculums of *C. lunata* was cultured on potato dextrose agar (PDA) and incubated for 10 days at room temperature approximately (30-33ºC). The inoculums was adjusted to 1×10^6 spores/mL before spray-inoculating 20-day-old rice seedlings. Brown leaf spot symptoms were monitored and evaluated using a disease index as follows: 1=no symptoms 0%, 2=small blighted spots 1-25%, 3=dead cells in the area of blighted spots (1-2 mm) which turned brown 26-50%, 4=expanded oval-shaped lesions (1-2 cm) and cell death in the center of lesion 51-75% and 5=diseased area over 76 %.

Dual culture antagonistic test

Chaetomium cupreum CC3003 wasobtained from Assoc. Prof. Dr. Kasem Soytong. This promising antagonist was tested for inhibition of *C. lunata* causing brown leaf spot of rice var. Sen Pidoa. The experiment was done using the dual culture antagonistic test which was arranged in a CRD with 4 replications, following previous studied methods.[11,12] The antagonistic fungus and pathogen were cultured on PDA at room temperature (30-32ºC) for 7 days. A 0.5 cm diameter sterilized cork borer was used to remove an agar plug at the periphery of thepathogen and antagonistic fungus colonies. The agar plug of the pathogen was transferred to one side of a PDA plate and an agar plug of an antagonistic fungus to the opposite side. PDA plate with a single plug of an antagonistic fungus or thepathogen served as the controls. All plates were incubated at room temperature and abnormal spores and normal spores of pathogen in each treatment were recorded under a binocular compound microscope. Data included colony diameter (cm) and the number of pathogen spores which were counted using by haemacytometer. Percentage inhibition of colony growth andspore production of *C. lunata* were computed according to the following formula: % inhibition (colony diameter or spore production of pathogen in control plate – colony diameter or spore production of pathogen in the dual culture plate) / colony diameter or spore production of pathogen in control plate × 100. Colony diameter and spore production were statistically computed using analysis of variance. Treatment means were compared using Duncan's Multiple Range Test (DMRT) at P=0.05 and 0.01.

In vitro antifungal metabolites from Chaetomium cupreum CC3003 against Curvularia lunata

Fungal growth and crude extracts *C.cupreum* CC3003 was cultured in potato dextrose broth (PDB) and incubated at room temperature (28-30°C) for 4 weeks. Fungal biomasses were removed from the liquid by cheesecloth filtration and dried over night at 28-32°C. The extraction was performed by the method described by Kanokmedhakul *et al*.[13] The air-dried fungal biomass was ground and extracted with hexane (1:1 vol) and incubated by shaking for 24 hat room temperature. The solvent was separately taken out of the marc by filtration through filter paper (Whatman No.4). The marc from hexane extraction was extracted with ethyl acetate (EtOAc) and followed with methanol (MeOH) using the same procedure as hexane. The solvents were separately evaporated to yield crude hexane, EtOAc and MeOH extracts.

In vitro antifungal metabolites from *Chaetomium cupreum* CC3003 against *Curvularia lunata* was done by using the poison agar method.[14] The experiment was done by using a two-factor factorial experiment arranged in a CRD with four replications. Factor A represented crude hexane, EtOAc and MeOH extracts. Each crude extract was dissolved with 2% dimethyl sulfoxide (DMSO) to test for antifungal activity against the growth and spore production of *C. lunata* on potato dextrose agar (PDA) at concentrations of 0, 10, 50, 100, 500 and 1000 g/mL (Factor B). Agar plugs (3-mm-dia) of *C. lunata* was cut from the advance margin of a 7-days-old colony and subcultured to the middle of PDA plate containing each concentration of the crude extract and incubated at room temperature. Other data collected were colony diameter (cm) and spore production. Data were computed by analysis of variance (ANOVA), and treatment means were compared using the DMRT at P=0.01. The effective dose of ED_{50} values was computed using probity analysis.

Testing fungal metabolites from Chaetomium cupreum CC3003 to inhibit Curvularia lunata causing leaf spot of rice var. Sen Pidoa in a pot experiment

The experiment was performed by using Randomized Complete Block Design (RCBD) with four replications. Treatments were set up as follows: inoculated with *C. lunata* (T1), inoculated and applied spore suspension of *C. cupreum* CC3003 1 – 10^6 spore/mL (T2), biofungicide (*C. cupreum* CC3003) at 20 g/20 L of water (T3), nano-particle of *C. cupreum* CC3003 (T4) and chemical fungicide (tebuconasole) 0.1 mL/1 L of water (T5). Rice seeds var. Sen Pidoa were soaked in sterile water for 24 hours in moisten paper until germination, then planted into pots (3 seedlings per pot). The 15-day-old rice seedlings were inoculated by wounding leaves and applying a 1×10^6 spore/mL; three wounded leaves/seedlings were done. Each treatment was applied as mentioned above at every 15 days until harvest.

Preparation of nano-particles from Chaetomium cupreum CC3003

Nano-particles from *C. cupreum* CC3003 were obtained from Joselito Dar and Kasem Soytong (KMITL, Bangkok, Thailand) who firstly investigated these new nano-particles which were developed and characterized nano materials loaded with active compounds from *Chaetomium* sp. Crude extracts from *C. cupreum* CC3003 were used in this study. The extracts were incorporated into poly acetic acid and electro spin at 25-30 kv. The product from *C. cupreum* CC3003 was pale orange in color. Scanning electron microscope images revealed that the nano material from *C. cupreum* measured 171 nanometers.

Data were collected as plant height (cm), number of tillers. The leaf spot disease index (DI) consisted of the following rating scheme: 1=no symptoms 0%, 2=small blighted spots 1-25%, 3=dead cells in the area of blighted spot 1-2 mm which turned brown 26-50%, 4=expanded lesion in oval shape 1-2 cm and cell death in the center of lesion 51-75% and 5=diseased area over 76 %, which was modified from Soytong *et al*.[9] Data were computed by ANOVA and treatment means were compared using DMRT at P=0.05 and P=0.01.

Field experiment

The experiment was conducted by using a RCBD with 4 replications; and 4 treatments were done as follows: the non-treated control (T1), organic method (T2), GAP method (T3) and chemical method (T4).The non-treated control did not use any bio-products or chemicals. Organic method used nano-particles of *C. cupreum* (10 cc/20 L of water), applied organic fertilizer 4.5 kg/plot, liquid biofertilizer 40 cc/20 L, bioinsecticide (*Metarhizium* sp. and *Beauveria* sp.) (50 cc/20 L of water) every 20 days until harvest.

The GAP method (good agricultural practice) used the chemical-organic biofertilizer (12-3-3) 1.5 kg/plot, spraying bio-insecticide together with the nano-product of *Chaetomium* at the rate of 10 cc/20 L alternated with the chemical insecticide (buprofezin 25%WP 30 g/20 L) together with the chemical fungicide (tebuconazole 20 cc/20 L) every 20 days until harvest. The chemical method applied urea 46-0-0 (0.75 kg/plot) in the early stage and 15-15-15 before the flowering stage (0.75 kg/plot) and buprofezin 25%WP (30 g/20 L) together with tebuconazole (20 cc/20 L) every 20 days until harvest. Disease index of leaf spot was recorded as in the pot experiment. Other data collected were plant height (cm), number of tillers, panicle number/plant, panicle length (cm) and panicle weight (g), grain weight/plot (kg) and dried hay weight (kg). Data were computed by analysis of ANOVA and treatment means were compared using DMRT at P=0.05 and P=0.01.

Results

Isolation of pathogen pathogenicity test

Curvularia lunata was isolated from leaf spot of rice in var. Sen Pidoa in this study and demonstrated to be pathogenic on this host.

Dual culture antagonistic test

Chaetomium cupreum CC3003 significantly inhibited *C. lunata* causing leaf spot of rice in the dual culture test; spore production of pathogen was 151.38×10^6 spores/mL in dual culture and $256.72\times151.38\times10^6$ spores/mL in control plate which significantly inhibited by 41% when compared to the control plate.

In vitro antifungal metabolites from Chaetomium cupreum CC3003 against Curvularia lunata

Antifungal metabolites from *C. cupreum* CC3003 inhibited the growth and spore production of *C. lunata* causing leaf spot of rice as shown in Table 1.

The extraction of antifungal metabolites from *C. cupreum* using hexane, ethyl acetate (EtOAc) and methanol inhibited spore production at 1000 µg/mL by 95.14%, 94.93% and 87.91%, respectively. The ED_{50} values of hexane, EtOAc and methanol extracts from *C. cupreum* CC3003 were 6.41, 0.83 and 7.81 µg/mL, respectively (Table 1).

It was clearly shown under the compound microscope that the pathogen spores were abnormal due to antagonistic substances extracted with hexane, EtOAc and methanol extracts from *C. cupreum* CC3003 released into pathogen cells (Figure 1).

Testing fungal metabolites to inhibit Curvularia lunata causing leaf spot in rice var. Sen Pidoa in pot experiment

The results showed that the height of plants treated with a spore suspension of *C. cupreum*, bioformulation of *C. cupreum*, nano particles of *C. cupreum* and the chemical fungicide (tebuconazole) were significantly higher than the inoculated controls at 65 days after plant-

ing (Table 2) which were 28.49, 28.92, 28.04 and 27.16 cm, respectively when compared to the inoculated control (26.91 cm). It was clearly demonstrated that treatment with a spore suspension of *C. cupreum*, bio formulation of *C. cupreum,* nano particles of *C. cupreum* and the chemical fungicide (tebuconazole) gave higher number of tillers which were 9.25, 9.63, 9.88 and 9.94, respectively, than the inoculated controls (6.00). All treatments increased the number of tillers from 35.14% to 39.64% when compared to the inoculated control (Table 2).

Rice seedlings treated with a spore suspension of *C. cupreum*, bioformulation of *C. cupreum,* nano particles of *C. cupreum* and the chemical fungicide (tebuconazole) showed significantly lower disease indices (DI) of 1.75,1.50, 1.50 and 1.25, respectively, than the

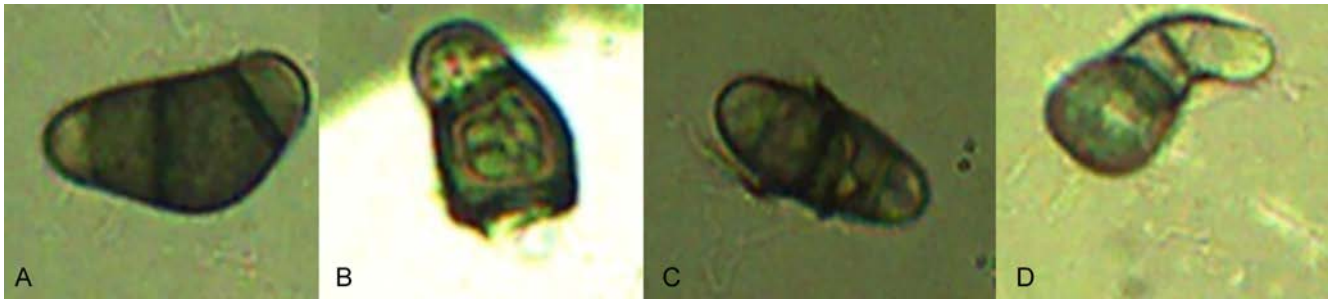

Figure 1. Effects of hexane, EtoAC and methanol extracts from *C. cupreum* CC3003 on spores of *C. lunata*. A) 0 µg/mL; B) Hexane-crude extract 1000 µg/mL; C) EtOAC-crude extract 1000 µg/mL; D) Methanol-crude extract 1000 µg/mL.

Table 1. Antifungal metabolites from *Chaetomium cupreum* CC3003 against *Curvularia lunata*.

Crude extracts	Conc. (µg/mL)	Colony diameter (cm)	Spore number (10^6 cfu/mL)	Colony inhibition*	Spore inhibition*	ED_{50} (µg/mL)
Hexane-crude extract	0	5.00^{a*}	77.00^{a}	0.00^{j}	0.00l	6.41
	10	4.38^{bc}	64.25^{c}	12.45^{hi}	16.56^{k}	
	50	4.23^{def}	46.88^{d}	15.40^{efg}	39.21^{j}	
	100	4.14^{fg}	30.00^{f}	17.20^{de}	61.06^{h}	
	500	4.05^{gh}	9.88^{j}	19.00^{cd}	87.20^{c}	
	1000	3.84^{i}	3.75^{l}	23.10^{b}	95.14^{a}	
Ethyl acetate-crude extract	0	5.00^{a}	71.50^{b}	0.00^{j}	0.00l	0.83
	10	4.48^{b}	21.50^{g}	10.50^{i}	69.91^{f}	
	50	4.34^{cd}	12.63^{i}	13.10^{gh}	82.35^{d}	
	100	4.28^{cde}	9.63^{j}	14.50^{fgh}	86.54^{c}	
	500	4.01^{h}	6.88^{k}	19.85^{c}	90.38^{b}	
	1000	3.60^{j}	3.63l	28.00^{a}	94.93^{a}	
Methanol-crude extract	0	5.00^{a}	78.5^{a}	0.00^{j}	0.00l	7.81
	10	4.46^{b}	37.38^{e}	10.70^{i}	52.38^{i}	
	50	4.30^{cde}	28.50^{f}	13.95^{fgh}	63.69^{g}	
	100	4.21^{ef}	18.63^{h}	15.75^{ef}	76.28^{e}	
	500	4.13^{fg}	14.00^{i}	17.45^{de}	82.15^{d}	
	1000	4.01^{h}	9.50^{j}	19.70^{c}	87.91^{c}	
C.V.(%)		1.74	4.47	10.74	2.37	

*Means of four replication, means followed by a common letter were not significantly different by DMRT at P=0.01.

Table 2. Number of tillers, plant height and disease index of rice var. Sen Pidoa in a pot experiment at 65 days.

Treatments	Number of tillers	% increase	Plant height (cm)	% increase	DI[2]	Disease reduction (%)
Inoculated control	6.00^{c}	0.00	26.91^{a}	0.00	3.00^{a*}	-
Spore suspension of *C. cupreum*	9.25^{ab}	35.14	28.49^{a}	5.55	1.75^{ab}	41.66
Bioformulation of *C. cupreum*	9.63^{ab}	37.69	28.92^{a}	6.95	1.50^{b}	50.00
Nano particles of *C. cupreum*	9.88^{a}	39.27	28.04^{a}	4.03	1.50^{b}	50.00
Tebuconazole - chemical fungicide	9.94^{a}	39.64	27.16^{a}	0.92	1.25^{b}	58.33
C.V.(%)	14.36	-	7.35	-	3.11	-

*Means of four replications. Means followed by a common letters are not significantly different by DMRT at P=0.01. Disease Index[2](DI) was rated using the following scheme: 1=no symptoms 0%, 2=small blighted spots 1-25%, 3=dead cells in the area of blighted spots 1-2 mm and turning brown 26-50%, 4=expanded oval-shaped lesions 1-2 cm and cell death in the center of lesion 51-75%, and 5=diseased area over 76%. Modified from Soytong & Quimio, 1989.9

Table 3. Disease index (DI) of leaf spot caused by *Curvularia lunata* on rice var. Sen Pidoa in field experiment.

Treatments	DI on leaves*	DI in grains and panicles	Disease reduction on leaves (%)	Disease reduction on grains (%)
Non treated control	5.00^{a**}	5.00^{a}	-	-
Organic method	3.00^{b}	3.00^{b}	40	40.00
GAP method	3.00^{b}	3.33^{b}	40	33.40
Chemical method	2.00^{c}	1.66^{c}	60	66.80
C.V.(%)	12.92	16.74		

*Disease index (DI) was rated using the following scheme: 1=no symptoms 0%, 2=small blighted spots 1-25%, 3=dead cells in the area of blighted spots 1-2 mm and turning brown 26-50%, 4=expanded oval-shaped lesions 1-2 cm and cell death in the center of lesion 51-75%, and 5=diseased area over 76%. **Means of four replications. Means followed by a common letters are not significantly different by DMRT at P=0.01. Modified from Soytong & Quimio, 1989.9

Table 4. Growth parameters of rice var. Sen Pidoa in the field trial at 80 days.

Treatments	Plant height	Tiller number	Panicle number/ plant	Panicle length (cm)	Panicle weight (g)
Non treated control	61.40^{b}	9^{b}	9^{c*}	16.85^{c}	1.65^{c}
Organic method	63.40^{ab}	16^{a}	15^{b}	19.45^{b}	2.10^{b}
GAP method	66.05^{ab}	21^{a}	21^{a}	19.67^{b}	2.25^{ab}
Chemical method	69.20^{a}	22^{a}	18^{a}	21.25^{a}	2.35^{a}
CV(%)	3.97	9.62	7.06	2.24	6.21

*Means of four replications. Means followed by a common letters are not significantly different by DMRT at P=0.01.

inoculated control (DI=3.00). All treatments reduced disease by 41.66% to 58.33% compared to the inoculated control (Table 2).

Field experiment

The results showed that the chemical method significantly reduced leaf spot disease caused by *C. lunata* by 60%, followed by organic method (40%) and GAP methods (40%), respectively (Table 3). It was found that plant height at 80 days were not significantly different among the organic, GAP and chemical methods when compared to the non-treated control (Table 4). The panicle number per plant in chemical and GAP methods were significantly higher than organic method when compared to the non-treated control. The panicle length in chemical method (21.25 cm) was significantly higher than GAP and organic methods which were 19.67 and 19.45 cm, respectively when compared to the non-treated control (16.85 cm). The panicle weights (2.35 g) in chemical method was significantly higher than organic and GAP methods which were 2.10 and 2.25 g, respectively when compared to the non-treated control (1.65 g) as seen in Table 4. With this, the chemical and GAP methods were significantly higher in grain weight (10.77 and 10.26 kg/20 m^2) than the organic method (7.37 kg/20 m^2) when compared to the non treated control (4.12 kg/20 m^2; Table 5). The dried weight of hay in chemical (17.21 kg/20 m^2) and GAP (16.42 kg/20 m^2) methods were also was significantly higher in organic method (9.40 kg/20 m^2) when compared to the non treated control (6.17 kg/20 m^2) as seen in Table 5.

Table 5. Grain weight and dried hay weight of rice var. Sen Pidoa in the field trial at 80 days.

Treatments	Grain weight (kg)/20 m^2	Dry hay weight (kg)/20 m^2
Non treated control	4.12^{c}	6.17^{c}
Organic method	7.37^{b}	9.40^{b}
GAP method	10.26^{a}	16.42^{a}
Chemical method	10.77^{a}	17.21^{a}
C.V.(%)	6.54	14.44

Means of four replications. Means followed by a common letters were not significantly different by DMRT at P=0.01.

Discussion and Conclusions

Curvularia lunata was found to seriously infected rice var. Sen Pidoa in the field in Cambodia and this research finding is confirmed by isolation of pathogenic isolate and proved pathogenicity test. It is reported for the first time in Cambodia. Ou stated that *C. lunata* is one of the most commonly found fungi in rice seeds leading to grain discoloration and,[15] Kamaluddeen *et al*. and Alcorn reported that *C. lunata* is caused leaf spot or leaf blight of rice and other hosts.[1,16] In this study showed that *C. cupreum* significantly inhibited *C. lunata* isolated from leaf spot of rice var. Sen Pidoa. Soytong *et al.*[17] reported that *C. cupreum* was antagonistic to the rice blast pathogen caused by *Pyricularia oryzae* in the Philippines.

Fungal metabolites released from *C. cupreum* CC3003 used isolate in this study was studied by Kanokmedhakul *et al*.,[13] who reported that it produces 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). Compounds 1, 3, 4, and 6 exhibited antifungal activity against *Candida albicans* with IC_{50} values of 10.5, 16.7, 24.3, and 0.6 ug/mL, respectively. It is suggested that *C. cupreum* could produce and release these bioactive compounds against *C. lunata*. Moreover, Tathan reported that crude extracts of *C. cupreum* CC3003 expressed antifungal activity against *Dreschera oryza* causing leaf blight of rice.[18] It was shown that the pathogen spores were abnormal due to metabolites from *C. cupreum* CC3003 extracted with hexane, EtOAc and methanol could destroy the pathogen cells. As a result, Soytong *et al*.[9] reported this phenomenon, namely antibiosis and lyses, that fungal-derived antagonistic substances could destroy the pathogen cells leading to loss of pathogenicity. Moreover,

Soytong *et al*.[7] stated that metabolites produced by *Chaetomium* spp. inhibited several plant pathogens including *C. lunata.* Similar result reported by Tathan,[18] stated that the metabolites from *Chaetomium* spp. could inhibit *Drechslera oryzae* which causes leaf spot of rice.

The results demonstrated that rice seedlings var. Sen Pidoa treated with a spore suspension of *C. cupreum*, bioformuation of *C. cupreum,* nano particles of *C. cupreum* and the chemical fungicide (tebuconazole) revealed significantly lower disease indices than the inoculated control. The plant heights treated with a spore suspension of *C. cupreum*, bio formulation of *C. cupreum*, nano particles of *C. cupreum* and the chemical fungicide (tebuconazole) were significantly higher than the inoculated controls at 65 days after planting. Similar report stated by Soytong *et al.*[19] that a bioformulation of *Chaetomium cochliodes* gave good control of brown leaf spot of rice caused by *Curvularia lunata*.

The field trial results showed that the chemical method gave better reduction of leaf spot disease caused by *C. lunata* than organic and GAP methods. But mostly growth parameters at 80 days were not significantly different among the organic, GAP and chemical methods when compared to the non-treated control. As a result, the chemical and GAP methods were significantly higher in grain weight than the organic method when compared to the non treated control. This result is contradicted to previous study by Tann *et al*.,[20] who reported that organic method trended to be higher yield than GAP and chemical methods. It is recommended that it could be affected by variable factors such as water management, weeding, soil type and soil fertility in different location.

Curvularia lunata is reported for the first time to cause leaf spots of rice var. Sen Pidoa in Cambodia. *Chaetomium cupreum* CC3003 can be significantly inhibited *C. lunata* in dual culture test. The antifungal metabolites from *C. cupreum* expressed antifungal activity against *C. lunata* at the ED_{50} values of hexane, EtOAc and methanol crude extracts were 6.41, 0.83 and 7.81 µg/mL, respectively . In pot experiment, it was shown that treatment with a spore suspension of *C. cupreum*, bio formulation of *C. cupreum*, nano particles of *C. cupreum* and the chemical fungicide (tebuconazole) gave higher growth parameters than the inoculated controls. Rice seedlings treated with a spore suspension of *C. cupreum*, bioformulation of *C. cupreum,* nano particles of *C. cupreum* and the chemical fungicide tebuconazole showed significantly lower disease incidence than the inoculated control. Field experiment showed that the chemical method was better reduction leaf spot disease caused by *C. lunata* than organic and GAP methods. The chemical and GAP methods gave higher in grain weight than the organic method when compared to the non-treated control.

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