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Biological Control of Brown Leaf Spot Disease Caused by *Curvularia lunata* and Field Application Method on Rice Variety IR66 in Cambodia

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ABSTRACT

Curvularia lunata was found to cause a serious rice brown leaf spot in Cambodia. This is the first report of brown leaf spot on rice in Cambodia. All isolates were tested for pathogenicity. Dual culture antagonistic tests showed that *Chaetomium cupreum* inhibited sporulation of *C. lunata* when compared to the control. In a pot experiment, *C. cupreum* significantly reduced the incidence of brown leaf spot caused by *C. lunata*. After application of a spore suspension of *C. cupreum*, *Chaetomium*-biofungicide and chemical fungicide (tebuconazole) to rice seedlings inoculated with *C. lunata*, the disease was reduced by 68.79 %, 75.80 % and 72.41 %, respectively. In a field trial, the chemical method gave the best results in all plant parameters, followed by the good agricultural practice (GAP) and organic methods. The chemical method gave the highest panicle/plant, panicle length, panicle weight, grain weight/plant which were different from the GAP and organic methods. The chemical method also gave the best results in filled grain panicle^{-1}, unfilled grain panicle^{-1}, grain weight plot^{-1}, dry hay weight plot^{-1}, biomass weight plot^{-1} and harvest index, and was significantly better than the GAP and organic methods.

Keywords: biological control; *Chaetomium cupreum*; *Curvularia lunata*; rice

INTRODUCTION

Rice (*Oryza sativa* L.) belongs to Gramineae and is the most economically important food crop in many developing countries (Matsuo, Kumazawa, Ishii, Ishihara, & Hirata, 1995). Rice is a stable food to billions of people around the world. It is about 2.5 billion people consumers and the growing areas are mostly in Asia. It serves about 21 % of global human per capita energy and about 15 % of per capital protein.

The world's rice production is traded only 6-7 % of the world market. Thailand, Vietnam, China and the United States are the largest exporters. The United States reported to produce approximately 1.5 % of the world's rice crop in Arkans as, California and Louisiana. Rice production in the world is directly consumed about 85 % (Rockwood, 2001). About 57 % of rice is grown on irrigated land, 25 % in rain-fed lowland, 10 % in the uplands, 6 % in deep-water, and 2 % in tidal wetlands (Chopra & Prakash, 2002). Rice is cultivated in many different environmental conditions in Asia (Alford & Duguid, 1998; Chaudhary, Tran, & Duff, 2001). In Cambodia, rice planted in rain-fed lowland areas without irrigation. Total cultivated area for rice production in 24 provinces in 2009 was 2,719,080 ha and in 2010 was 2,795,892 ha, but the harvested area in 2009 was 2,674,603 ha and in 2010 was 2,777,323 ha only. The total yield production of rice in 2009 was 7,175,473 t, average yield was 2.84 t ha^{-1} and the total yield production of rice in 2010 was 8,249,452 t, and average yield was 2.97 t ha^{-1} (Nesbitt, 1996; Bell, Pracilio, Cook, Chhay, & Vang, 2006; Maeder et al., 2002; MAFF, 2010).

Brown leaf spot is one of the problems associated with rice disease of many varieties in Cambodia, and is caused by *Curvularia lunata* especially in the last few years. The pathogen not only infects leaves but also infects rice seeds (Kamaluddeen, Simon, & Lal, 2013).

Other problems for rice production are pathogen resistance to chemical fungicides and their toxic residues, which can negatively impact the environment and human health. Biological

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control of plant pathogens has successfully provided a recent strategy for integration with other control measures. It helps to reduce the heavy application of chemical fungicides, build up agro-ecosystem and reserve natural balances. There are several reports on the potential use of biological control agents against plant pathogens (Kaewchai, Soytong, & Hyde, 2009). *Chaetomium cupreum* is a strictly sapro-phytic antagonist which is effective against several plant pathogens (Soytong & Quimio, 1989)e.g. *Phyto-phthora palmivora* (Pecchprome & Soytong,1997) and *Colleto trichumgloeosporioides* (Noiaium & Soytong, 1999) and *Pyricularia oryzae* (Soytong & Quimio, 1989; Soytong, 1992a; 1992b). It is challenging to find alternative methods which are safe for agricultural inputs like bio-fertilizer and bio-pesticides to be used instead of toxic chemicals on rice production representing of good agricultural practice (GAP) and organic agriculture (Soytong, Kanokmedhakul, Kukongviriyapa, & Isobe, 2001).

The objectives of this research were to study biological control of rice brown leaf spot caused by *C. lunata*, and application methods in a pot and in field experiment using cultivated rice variety IR66 in Cambodia.

MATERIALS AND METHODS

Isolation, Identification and Pathogenicity Test of the Rice Pathogen

Brown leaf spot of rice variety IR66 was isolated from leaf symptoms by the tissue trans-planting method (Soytong & Quimio, 1989). The mycelia on water agar (WA) were transferred onto potato dextrose agar (PDA) until pure cultures were obtained. All isolates were identified by morphologically observation under a compound microscope. All isolates tested for pathogenicity followed Koch's Postulates. The pathogen inoculums were prepared as a spore suspension of 1 x 10^6 spores ml^{-1}. Twenty-days-old rice seedlings planted in pots were inoculated by spraying then covered with plastic bags to maintain moisture content. The pathogen was re-isolated from symptomatic tissue, returned to pure culture and identified morphologically to confirm species.

Dual Culture Antagonistic Test Against Rice Pathogen

Chaetomium cupreum was tested against *C. lunata* in dual culture plates. The test used the method of Soytong (1992a). The fungal antagonists and a virulent isolate of *C. lunata* were cultured on potato dextrose agar (PDA), and incubated at the room temperature (28-30 ºC). The edge of actively growing colony was cut into 5 mm diameter by the sterilized cork borer and one agar plug of each fungus was transferred to the opposite sides on PDA plates of 9 cm diameter and separately cultured *C. cupreum* and *C. lunata* served as controls, then incubated at room temperature (28-30 ºC) for 4weeks. Data was collected as colony diameter (cm) and number spore production, which counted using a Haemacytometer under a compound microscope. The analysis of variance (ANOVA) was computed, and treatment means were compared using Duncan's Multiple Range Test (DMRT) at α = 0.05 and 0.01.

Efficacy of *C. cupreum* for Controlling Brown Leaf Spots Caused by *C. lunata* on Rice Variety IR66 in Pot Experiment

The experiment used a randomized complete block design (RCBD), four replications and treatments were done as follows: the inoculated control with *C. lunata*, spore suspension of *C. cupreum* 1 x 10^6 spores ml^{-1}, biofungicide (*C. cupreum*) 20 g L^{-1} of water, chemical fungicide (tebuconazole) 0.1 ml 1 L^{-1} of water. Rice seeds of variety IR66 were soaked in sterile water for 24 hours, put in moisten paper until germination, and then planted into pots (3 seedlings per pot). The 15 day sold seedlings of rice variety IR66 were inoculated by *C. lunata* with 1 x 10^6 spores ml^{-1} (three-wounded leaves $seedling^{-1}$) to all treatments and immediately applied the products as mentioned above every 15 days until harvest. The collected data included plant height (cm), number of tillers, disease index, and disease reduction.

Application of *C. cupreum* to Control Brown Leaf Spots on Rice Variety IR66 in the Field

The field experiment was conducted at Toek Vil Agriculture Research Station, located in Siem Reap province, Cambodia where in the area of disease epidemic or infestation to the rice. The experiment used the infected rice by *C. lunata* naturally. The experiment was conducted by using Randomized Complete Block Design (RCBD) with four replications and treatments as follows: the non-treated control, organic method which applied organic fertilizer 4.5 kg $plot^{-1}$, liquid biofertilizer 40 cc $20L^{-1}$, bioinsecticide (*Meta-*

rhizium and Beauveria) at the rate of 40 cc 20L^{-1} of water, biofungicide (*C. cupreum*) at the rate of 10 g 20L^{-1} of water every 20 days until harvest. Good Agriculture Practice (GAP) method applied the chemical-organic, biofertilizer (12-3-3) at the rate 1.5 kg plot^{-1}, for disease and insects were controlled by alternated spraying with a bioinsecticide plus biofungicide and a chemical insecticide (Buprofezin 25 % WP 30 g 20L^{-1}) plus chemical fungicide 20 cc 20L^{-1}) every 20 days until harvest. The chemical method applied urea 46-0-0 at the rate of 0.75 kg plot^{-1} in the early stage and 15-15-15 before the flowering stage at the rate of 0.75 kg plot^{-1} and spraying with chemical insecticide (Buprofezin 25 % WP 30 g 20L^{-1} plus chemical fungicide 20 cc 20L^{-1}) every 20 days until harvest. The plot size was 6 x 5 m^2 (30 m^2).

Each replication was separated by a 0.5 m bund. Twenty-days-old seedlings of rice variety IR66 were transplanted at a spacing of 25 × 25 cm. Fertilizer application was done according to the above-mentioned treatments for individual experimental plots. Manual weeds control was used in all treatments. The necessary water management was maintained for rice cultivation. At the panicle initiation phase to ripening stage, a water level of 5 -10 cm was maintained and drained off from the field 10 days before harvesting. Harvested plants were left in the field for 4-5 days for sun drying. Threshing was done manually, and grains were obtained and weighed at a 14 % moisture content. The collected data included plant height (cm), number of tillers per plant, length and weight of panicles, number and weight of grains per panicle, numbers of filled and unfilled grains and grain and straw yields.

RESULTS AND DISCUSSION

Isolation of Rice Pathogen and Pathogenicity Test

C. lunata was found to be the causal agent of brown leaf spot on rice variety IR66 in Cambodia. *C. lunata* was tested for pathogenicity to 20 days old seedlings by inoculating with a spore suspension at a concentration of 1 x 10^6 spores ml^{-1}. The rice seedlings showed clear symptoms of brown leaf spot and the pathogen was re-isolated to confirm species. The most virulent isolate was used for further experiments. Ou (1985) stated that *C. lunata* is one of the most commonly encountered fungal genera which may infect rice varieties up to 80 %. The research finding is reported by Kamaluddeen, Simon, & Lal (2013) who discovered *C. lunata* causing blight disease on rice in Uttar Pradesh in India. The symptoms were observed on leaves, brown spots, and the maximum infection was recorded on the leaf sheath.

Dual Culture Antagonistic Test

C. cupreum actively expressed antifungal activity against *C. lunata* isolated from rice variety IR66 in dual culture after 28-days incubation. In dual culture the *C. cupreum* significantly inhibited spore production of *C. lunata* at 28.55 % when compared to the control plate. *C. lunata* on dual culture with *C. cupreum* plate produced 183.44 spores ml^{-1} compared to control plate at 256.72 spores ml^{-1}. *C. cupreum* significantly inhibited colony growth of *C. lunata* in dual culture plate by 21.78 % at 28 days. The colony diameter of *C. lunata* in dual culture plate was 7.04 cm compared to the control plate at 9.00 cm in Table 1.

Table 1. *C. cupreum* to inhibit colony growth and spore production of *C. lunata* which isolated from rice variety IR66 in dual culture at 28 days

Treatments	Colony diameter (cm)	2) Colony diameter (cm) (%)	Number of spore production (10^6 spore ml^{-1})	Spore production Inhibition (%)
Control	9.00 a 1)	-	256.72 a	-
Dual culture	7.04 b	21.78	183.44 b	28.55

Remarks: 1) Number followed by a common letter are not significantly different by DMRT at α = 0.01; 2) % inhibition of colony diameter (cm) or spore production = (R1 - R2) /R1 x 100; R1 = colony diameter or spore production of *C. lunata* in control plate and R2 = colony diameter or spore production of *C. lunata* in dual culture plate

Similar reports, *C. cupreum* has been recorded to control rice blast caused by *Pyricularia oryzae* in the Philippines (Soytong, 1992b). Moreover, the *C. cupreum* isolate used in this study was reported by Kanokmedhakul et al. (2006) to produce rotiorinols A, C, rotiorin, and rubrorotiorin expressed antifungal activity to inhibit *Candida albicans* with IC_{50} values of 10.5, 16.7, 24.3, and 0.6 µg ml^{-1}, respectively. It was concluded that the control mechanism of *Ch. cupreum* could be antibiosis. However, Soytong (2014) reported that *Chaetomium cochliodes* actively against brown leaf spot on rice var. Pittsanulok 2 caused by *Drechslera oryzae* in Thailand. It showed good inhibition of mycelia growth of 38.18 % and inhibited inoculums production by 71.55 %.

Efficacy of *C. cupreum* to Control Brown Leaf Spot of Rice Variety IR66 Caused by *C. lunata* in a Pot Experiment

Rice seedlings treated with biofungicide (*C. cupreum*), chemical fungicide (tebuconazole) and spore suspension of *C. cupreum*, showed a significant lower disease index (DI) of 1.75, 2.00, and 2.25, respectively than the inoculated control with *C. lunata* of 7.25. With this spraying the spore suspension of *C. cupreum*, chemical fungicide and biofungicide to inoculated rice seedlings with *C. lunata* the disease reduced of 68.97, 75.86 and 72.41 %, respectively Table 2.

After application with a spore suspension of *C. cupreum*, biofungicide (*C. cupreum*) and chemical fungicide on rice seedlings inoculated with *C. lunata*, the results showed that plant heights were not significantly different which were 18.77 cm, 18.34 cm and 18.94 cm, respectively but different compared to the inoculated control (14.16 cm). Moreover, the number of tillers was also not different significantly after application with a spore suspension of *C. cupreum*, biofungicide and chemical fungicide which were 4.94, 5.50 and 4.94 respectively but different significantly when compared to the inoculated control (3.25) Table 3.

With this, Soytong (2014) reported that testing *C. cochliodes* in different formulations resulted to control brown leaf spot on rice caused by *Drechslera oryzae*. The results of that study showed that a biopowder formulation was the most effective to control leaf spot and highest plant growth compared to the non-treat control, followed by crude extract of *C. cochliodes*, and spore suspension of *C. cochliodes.* Moreover, the bio-powder formulation resulted a significant increased plant growth of over 44 %, followed by a crude extract of *C. cochliodes*, spore suspension of *C. cochliodes* and benlate. The current research represents the first report of control of brown leaf spot of rice caused by *C. lunata* by application of *Chaetomium* sp.

Table 2. Effect of treatment on disease index, and disease reduction on rice variety IR 66 at 95 days

Treatments	1) Disease index	2) Disease reduction (%)
Inoculated control with *Curvularia lunata*	7.25 a3)	---
Spore suspension of *Chaetomium cupreum*	2.25 b	68.97
Biofungicide (*Chaetomium cupreum*)	1.75 bc	75.86
Chemical fungicide (tebuconazole)	2.00 bc	72.41

Remarks: 1) Disease index was modified from Soytong (2014) which level 1 = leaf spot 0 %, 2 = leaf spots 1-10 %, 3 = leaf spots 11-20 %, 4 = leaf spots 21-30 %, 5 = leaf spots 31-40 %, 6 = leaf spots 41- 50 %, 7 = 51-60 %, 8 = 61-70 %, 8 = 71-80 %, 9 = 81-90 % and 91-100 %. 2) Disease reduction (%) was disease index of inoculated control - disease index in each treatment / disease index of inoculated control x 100. 3) Average of four replications. Means followed by a common letter in each column are not significantly different by DMRT at α =0.01.

Table 3. Efficacy of treatments on plant height and number tillers of rice variety IR66 at 35 days in a pot experiment

Treatments	Plant height (cm)	Number of tillers
Inoculated control with *C. lunata*	14.16 a	3.25 b
Spore suspension of *C. cupreum*	18.77 a	4.94 ab
Biofungicide (*C. cupreum*)	18.34 ab	5.50 ab
Chemical fungicide (tebuconazole)	18.94 a	4.94 ab

Remarks: Number followed by a common letter in each column are not significantly differed by DMRT at α =0.01

Application of *C. cupreum* to Control *C. lunata* Caused Brown Leaf Spot of Rice Variety IR66 in the Field

The organic, good agricultural practice (GAP) and chemical methods were tested for rice cultivation of variety IR66 in a field trial in Cambodia. Result showed that the chemical method gave the highest plant height at 80 days which was 72.55 cm, followed by the GAP method (67.2 cm) and organic method (62.35 cm) which were significantly different from the non-treated control (53.19 cm). The chemical and GAP methods gave the best results in number of tillers at 80 days which were 15 tillers and 14 tillers, respectively, followed by organic method (12 tillers) which was significantly different from the non-treated control (6 tillers) (Table 4).

The chemical method also gave the best results in panicle plant^{-1}, panicle length (cm), panicle weight (g), grain weight plant^{-1} (g) were 13 panicles plant^{-1}, 26.09 cm, 4.70 g and 4.05 g, respectively, which were significantly different when compared to GAP were 18 panicles plant^{-1}, 25.38 cm, 4.24 g and 3.60 g, respectively, and the organic method were 11 panicles plant^{-1}, 24.83 cm, 3.36 g and 2.90 g (Table 5).

Number of filled grain and unfilled grain per panicle, grain and dry hay weight (kg) per plot (20 m^2) at 14 % MC were gathered. Chemical method gave the best results in filled grain panicle^{-1}, unfilled grain panicle^{-1}, grain weight (kg) plot^{-1}, dry hay weight (kg) plot^{-1}, biomass weight (kg) plot^{-1} and Harvest Index (5 %) were 111 filled grain panicles^{-1}, 15 unfilled grain panicles^{-1}, 10.55 kg, 25.97 kg, 41.04 kg and 0.31, respectively which were significantly different from GAP were 106 filled grain panicles^{-1}, 12 unfilled grain panicles^{-1}, 9.65 kg, 28.49 kg, 35.62 kg and 0.27, respectively and the organic method which 104 filled grain panicles^{-1}, 7 unfilled grain panicles^{-1}, 6.34 kg, 16.52 kg, 22.61 kg and 0.27, respectively (Table 6).

This experiment revealed that chemical and GAP application gave better result than organic method. This contradicts to the previous experiment of Tann, Soytong, Makhonpas, & Adthajadee (2011) reported that the organic method revealed a better rice straw weight than non-treated control, and followed by GAP and chemicals at harvesting of 115 days. The organic method can be increased in plant height and number of tillers per plant by 3.06 % and 57.69 %, respectively in 60 days after planting. The GAP method increased in plant height and tiller number by 11.23 % and 69.44 %, respectively while the chemical method increased plant height and tiller number by 6.73 % and 62.71 %. The grain weight (yield) increased by the GAP, chemical and organic methods by 59.15 %, 55.38 % and 44.23 %, respectively. This may due to different location of experimental sites, soil fertility, disease and different tested variety (Stanhill, 1990; Maeder et al., 2002). The organic method requires evaluation of many factors for completely successful cultivation (Paull, 2011).

Table 4. Efficacy of treatments on plant height and number of tillers per plant of rice variety IR66 at 80 days in the field trial

Treatments	Plant height (cm)	Number of tillers per plant
Non-treated control	53.19 f	6 c
Organic method	62.35 d	12 ab
GAP method	67.2 bc	15 a
Chemical method	72.55 a	14 a

Remarks: Number followed by a common letter in each column are not significantly different by DMRT at α =0.01

Table 5. Efficacy of treatments on panicles and grains of rice variety IR66 at 80 days in the field trial

Treatments	Panicle per plant	Length of panicle (cm)	Panicle weight (g)	Grain weight (g)
Non-treated Control	6 c	23.25 bc	2.56 c	2.25 c
Organic method	11 b	24.83 ab	3.36 b	2.90 b
GAP method	18 a	25.38 a	4.24 a	3.60 ab
Chemical method	13 b	26.09 a	4.70 a	4.05 a

Remarks: Number followed by a common letter in each column are not significantly differed by DMRT at α =0.01

Table 6. Efficacy of treatments on grains, dry hay, biomass and harvest index of rice variety IR66 per plots (20 m^2 planted area) at 14 % moisture content

Treatments	Filled grain per panicle	Unfilled grain per panicle	Grain weight per plot (kg)	Dry hay weight per plot (kg)	Bio mass weight per plot (kg)	Harvest Index (5 %)
Non-treated Control	79 c	16 a	4.35 c	8.89 d	13.24 d	0.33 a
Organic method	104 b	7 c	6.34 b	16.52 c	22.61 c	0.27 b
GAP method	106 b	12 b	9.65 a	28.49 a	35.62 b	0.27 b
Chemical method	111 a	15 a	10.55 a	25.97 b	41.04 a	0.31 ab

Remarks: Numbers followed by a common letter in each column are not significantly different by DMRT at α =0.01

CONCLUSION

Brown leaf spot on rice variety IR66 caused by *C. lunata* was found to be the first report in Cambodia. Based on dual culture test, *C. cupreum* inhibited spore production of *C. lunata*. The brown leaf spot was reduced by application of a spore suspension of *C. cupreum*, biofungicide(*C. cupreum*)and chemical fungicide to rice seedlings inoculated with *C. lunata* in pot experiment.

In field, the chemical method gave the best results in all plant parameters, followed by the GAP and organic methods. It concluded that spraying a spore suspension of *C. cupreum*, biofungicide(*C.cupreum*) and chemical fungicide to rice seedlings inoculated with *C. lunata* reduced the disease. The experiment showed that chemical and GAP application gave better result than organic method.

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