https://doi.org/10.17221/41/2019-PPS

Antifungal activity of microbial nanoparticles derived from *Chaetomium* spp. against *Magnaporthe oryzae* causing rice blast

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Citation: Song J.J., Soytong K., Kanokmedhakul S., Kanokmedhakul K., Poeaim S. (2020): Antifungal activity of microbial nanoparticles derived from *Chaetomium* spp. against *Magnaporthe oryzae* causing rice blast. Plant Protect. Sci., 56: 180–190.

Abstract: The *Magnaporthe oryzae* isolate PO2 was proven to cause rice blast var. PSL 2 in this study. *Chaetomium elatum*, *Chaetomium lucknowense* and *Chaetomium brasiliense* were antagonised to *M. oryzae*. The hexane extract of *C. brasiliense* gave the highest inhibition of the spore production with an ED_{50} of 35 ppm, the EtOAC extract of *C. lucknowense* inhibited the spore production at 57 ppm and the EtOAC extracts of *C. elatum* inhibited the spore production at 106 ppm. The nano-CLM (*C. lucknowense*) inhibited the spore production at 5.24 ppm, the nano-CBH (*C. brasiliense*) inhibited the spore production at 6.86 ppm and the nano-CEE (*C. elatum*) inhibited the spore production at 7.89 ppm. The rice leaves treated with nano-CBH from *C. brasiliense* produced Sakuranertin and Oryzalexin B as seen on the thin layer chromatography where the Rf value was 0.08 assumed to be Sakuranertin, and the R_f value of 0.28 supposed to be Oryzalexin B. It was found that the nanoparticles act as elicitors to induce immunity in rice plants through the production of phytoalexin.

Keywords: plant pathology; biological control; antagonistic fungi; blast disease

Rice blast caused by *Magnaporthe oryzae* B.C. Couch is of economic importance that causes losses in rice yields, especially in Asia (Ou 1985), which is a crucial food staple for more than half of the world (FAO 2009). Chemical fungicides have been applied to control the disease, but the pathogens have often developed resistance to those chemical fungicides (Soytong et al. 2001). Resistant varieties are usually broken down by resistant genes, none of these resistant genes, except Pi9, have durable broad-spectrum resistance. Those resistant genes often break down within 3–5 years due to the high variability and fast evolving populations of the fungus (Devi et al. 2015). In recent years, blast resistant genes were introgressed, such as the Luhui 17, G46B, Zhenshan 97B, Jin 23B, CO39, IR50, Pusa1602 and Pusa1603 lines through marker assisted selection. The genes for the resistance induced the occurrence of new

Supported by the King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand, Project No. KREF126004 and by Thailand Research Fund, Grant No. RTA5980002.

https://doi.org/10.17221/41/2019-PPS

races of blast fungus (Miah et al. 2013). The use of chemical fungicides resulted to increased deposits in rice products and residues in the soil, water and surrounding environment (Soytong et al. 2001). Scientists are increasingly investigating the biological control of plant pathogens to find effective agents for disease control.

An alternative method has been proposed to apply nanoproducts for plant disease control by inducing plant immunity. The application of nanotechnology in agriculture has expanded and advanced in recent years (Li et al. 2011). Nanotechnology serves as a new effective alternative method of plant disease control to reduce the pathogen inoculum and disease incidence. It involves the formation of organic materials into defined structures (Soutter 2012). Scientists are actively searching for organic nanomaterials to be used for biological properties (Elibol et al. 2003). Nano-particles can provide an efficient means for disease management in agriculture (Rai & Ingle 2012).

Phytoalexins were first discovered as phytoalexin-like substances in rice plants infected with the anamorph *P. oryzae,* an imperfect stage of *M. oryzae* using the drop-diffusate method by Uehara (1958). Fifteen phytoalexins were discovered in rice (*Oryza sativa* Linnaeus) that were infected by *M. oryzae* causing rice blast. Sakuranetin is a flavonoid and four groups of diterpenoids, such as phytocassanes A to E, oryzalexins A to F, momilactones A and B, and oryzalexin S, including phytoalexins like momilactones A and B (Kodama et al. 1992), oryzalexins A–F, oryzalexin S, phytocassanes A–D (Dillon et al. 1997). The research finding was to evaluate the fungal metabolites and nanoparticles derived from *Chaetomium elatum* Kunze (1818), *Chaetomium brasiliense* and *Chaetomium lucknowense* J.N. Rai & J.P. Tewari (1962) to inhibit the growth of the rice blast caused by the *M. oryzae* isolate PO2, and testing for the production of phytoalexin in the infected rice leaves.

MATERIAL AND METHODS

Pathogen and pathogenicity test. The blast symptoms on rice leaves of var PSL2 were cut into square pieces of 0.5 × 0.5 cm between the advanced margin, soaked in 10% sodium hypochlorite for 19 min, then cleaned with sterilised distilled water 3 times, left to dry on sterilised tissue paper before being transferred to a water agar. The hyphal tips from the advanced margin tissues were transferred to a potato dextrose agar until pure cultures were obtained. Each isolate was inoculated into the wounded leaves of 20 rice seedlings planted in pots by making a puncture of 0.5 cm in diameter five times. The controls were the non-inoculated ones. The lesion size was measured as the diameter (cm). An aggressive isolate was used for the experiment. The pathogenicity test was repeated four times following Koch's Postulates Method. The isolate was confirmed by the identification of the morphological and molecular phylogenetics that followed the instructions of Pornsuriya et al. (2011).

Biocontrol agents. *C. elatum* (ChE01), *C. brasiliense* (CB) and *C. lucknowense* (CL) were used as the biological control agents from the previous work (Song & Soytong 2016, 2017). *C. elatum* ChE01 has been reported to produce new chaetoglobosin V, prochaetoglobosin III and prochaetoglobosin III (ed). These compounds have been reported to cause cytotoxicity against the human breast cancer and cholangiocarcinoma cell lines (Thohinung et al. 2010). *C. brasiliense* have been reported to produce four new depsidones, mollicellins K–N (1–4), and six known depsidones, mollicellins B (5), C (6), E (7), F (8), H (9), and J (10). These pure compounds of 1–3, 5–7, and 10 have shown antimalarial activity against *Plasmodium falciparum* Welch, 1897. Mollicellins K expressed antimycobacterial activity against *Mycobacterium tuberculosis* and antifungal activity against *Candida albicans*. Compounds 1–10 have exhibited cytotoxicity against the KB, BC1, NCI-H187 cancer lines, and mollicellins K has exhibited cytotoxicity against cholangiocarcinoma cell lines (Khumkomkhet et al. 2009). *C. lucknowense* has been reported to actively produce a fungal metabolite, chaetomanone, against *Fusarium oxysporum* f. sp. *lycopersici* causing tomato wilt (Sibounnavong et al. 2011). The bi-culture test was undertaken by following the method of Song and Soytong (2016).

Molecular identification. The genomic DNA was separately extracted from the *M. oryzae* isolate PO2, *C. elatum, C. brasiliense* and *C. lucknowense*. Each fungus was cultured in a potato dextrose broth (PDB) for 5 days, then the mycelia were freeze-dried. The DNA (Deoxyribonucleic acid) was extracted by a modified CTAB (Cetyltrimethylammonium bromide), then cleaned with 25 mM EDTA (Ethylenediaminetetraacetic acid) by centrifugation. It was ground in liquid nitrogen to get a fine powder. The fungal cells were lysed in the CTAB buffer, β-mercaptoethanol, and mixed, then incubated at

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65 °C in a water bath for 1 hour. The lysates were extracted with chloroform/isoamyl alcohol (24 : 1), centrifuged at 14 000 rpm for 5 min at 4 °C. 2 µL Rnase (20 µg/mL) was added to the aqueous phase in a sterile tube for 30 min at 37 °C, mixed with 50 µL 10% CTAB. The mixture was then centrifuged at 14 000 rpm for 5 min at 4 °C. The DNA was precipitated in isopropanol and centrifuged at 4 °C for 20 min at 14 000 rpm. The pellets were washed twice with 70% and 95% ethanol, air dried and dissolved in a 100 µL TE buffer at 37 °C.

The extracted DNAs were monitored by electrophoresis in a 1% agarose gel to check the DNA bands. The polymerase chain reaction (PCR) amplification of each fungal DNA was performed by the ITS ribosomal DNA regions amplified by PCR using the universal primers, ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The 25 µL reaction mixture contained 2.5 µL of the PCR buffer, 0.625 µL of each dNTP (1.25 mM), 0.5 µL of $MgCl^2$, 1 µL of each primer (20 pmol/µL), 2 ng of DNA and 0.2 µL of the Taq DNA polymerase (1 U). The PCR conditions for the ITS regions were programmed with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C and 56 °C for 1 min, 72 °C for 2 min, and a final extension at 72 °C for 5 minutes.

The amplified products (5 µL) were visualised on 1% (w : v) agarose gel. The PCR products were purified with a PCR purification kit. The amplified products were sequenced DNAs and aligned by comparison with the sequences at the GenBank and by a basic local alignment search tool (BLAST) analysis (Altschul et al. 1997) in the National Center for Biotechnology Information (NCBI) databases. The sequences from the closely related species were downloaded and aligned through ClustalW using MEGA software (version 6.0) (Tamura et al. 2007). The phylogenetic trees were performed by the neighbour-joining method using the same software.

Testing metabolites against pathogen. ChE01, CB and CL were separately cultured in the PDB and incubated at 28 °C for 30 days to obtain the fungal biomass. The extraction of metabolites from each isolate was undertaken using hexane, ethyl acetate and methanol as the solvents following the method of Thohinung et al. (2010) and yielded thefungal substances of crude hexane, crude ethyl acetate and crude methanol extracts. The experiment was conducted by using a factorial experiment in a Completely Randomised Design (CRD), where factor A stood for the crude extracts of fungal substances and factor B stood for the tested concentrations (0, 10, 50, 100, 500 and 1000 ppm). The experiment was repeated four times. The colony diameter (cm) and sporulation were measured and counted using a haemocytometer. The data were analysed for variance and the means were compared using Duncan's Multiple Range Test (DMRT) at $P = 0.05$ and 0.01 levels of significance.

Testing nanoparticles against pathogen. The nanoparticles were prepared from crude hexane, ethyl acetate, and the methanol extracts of ChE01, CB and CL separately incorporated into the polylactic acid (PLA)-based nanoparticles through electrospinning by following the method of Dar and Soytong (2014). The crude extracts from ChE01, CB and CL were constructed nanoparticles to get the nano-CEH, nano-CEE and nano-CEM. These nanoparticles were tested to inhibit the *M. oryzae* isolate PO2 which was conducted by a factorial experiment in the CRD with four replications. Factor A was for the nanoparticles and factor B was for the tested concentrations (0, 3, 5, 10 and 15 ppm). The experiment was repeated four times. The data were collected as the colony diameter (cm) and the sporulation was counted using a haemocytometer. The data were subjected to ANOVA and the means were compared using DMRT at $P = 0.05$ and 0.01 levels of significance.

Control mechanisms. All the nanoparticles treated onto the pathogen cells were examined under a binocular compound microscope. The normal and abnormal spores of *M. oryzae* were observed. The abnormal spores from the treated nanoparticles were inoculated to the wounded leaves in the 20-day old rice seedlings var PSL2, then the disease incidence was recorded.

Testing nano-CB for phytoalexin production. The determination of the phytoalexin in the rice was preliminary investigated by thin layer chromatography (TLC). The phytoalexin production was investigated using the treated nanoparticles CB and the non-treated ones served as controls. Twenty-day old rice seedlings of var PSL 2 were inoculated with *M. oryzae* and sprayed with the nano-CBH at a concentration of 15 ppm. The leaf samples were taken at 3, 6 and 9 days after inoculation. Each fresh leaf sample was weighed to 1.2 g and cleaned in methanol, then cut into small pieces and ground, soaked in 10 mL methanol, put in a water bath at 50 °C for 10 min, passed through a filter paper (No. 4, Whatman, UK) The filtrate was then

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evaporated with a rotary vacuum evaporator to get the crude extract. 3 mL of methanol and 1 mL of ethyl acetate was added to the crude extract and kept for the trials. The detection of the phytoalexin by TLC was undertaken by preparing a solvent of benzene and ethyl acetate at the ratio of 10 : 1. Two mL of the TLC solvent was added to the TLC tank. The TLC plate was spotted with a crude extract sample and a standard one for comparison, then observed under UV light at 365 nm, soaked in an anisaldehyde solvent, then dried and heated until spots appeared. The R_f value was calculated and compared with the standard one. The experiment was repeated three times. The R_f value is calculated as (Equation 1):

$$R_f = \frac{\text{distance spot travels}}{\text{distance mobile phase travels}} \quad (1)$$

Where: R_f – retention factor

RESULTS

Pathogen and pathogenicity test. *M. oryzae* was isolated from the rice leaves showing blast symptoms and proved to be pathogenic. The culture grown on the PDA (potato dextrose agar) was greyish brown when mature, producing conidia in opera shape with 2 septa, on the conidiophore as the imperfect stage (Figure 1). The isolate was confirmed by molecular phylogenetic identification as seen in

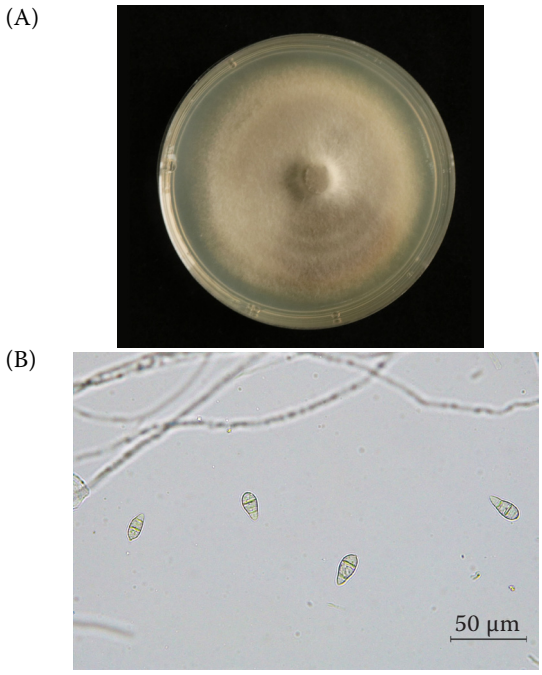

Figure 1. The *Magnaporthe oryzae* isolate PO2: (A) the pure culture on the RFA medium and (B) spores

Table 1. The colony growth of *Magnaporthe oryzae* on the antagonistic bi-culture tests

Antagonists	*Magnaporthe oryzae*	
	colony (cm)	inhibition of colony (%)
Control	9.00[a1]	–
Chaetomium elatum	2.56[c]	71.55[a1]
Chaetomium brasiliense	2.75[b]	69.44[b]
Chaetomium lucknowense	2.65[b]	70.55[a]
CV%	1.20	2.11

[1]the average of four replications; the means followed by a common letter are not significantly different by DMRT at $P = 0.05$

Figure 2. The *M. oryzae* isolate PO2 was confirmed to be a pathogenic isolate to cause rice blast in the rice var PSL2. The pathogenicity test showed blast symptoms on the inoculated leaves with roundish to slightly elongated, necrotic and grey spots.

Biocontrol agents. *C. elatum, C. brasiliense* and *C. lucknowense* were subjected to morphological and molecular studies. *Chaetomium* spp. were used as the biological control agents from the previous work. These isolates were confirmed by morphological and molecular phylogenetic identification. The antagonistic activities of *C. elatum*, *C. lucknowense* and *C. brasiliense* against *M. oryzae* gave preliminary results of the control mechanism. *C. elatum* expressed the highest growth inhibition of the rice blast pathogen at 71.55%, followed by *C. lucknowense* and *C. brasiliense*, which inhibited the pathogen fungal growth by 70.55 and 69.44%, respectively (Table 1 and Figure 3).

Molecular phylogenetic confirmation. The fungi were separately cultured in potato dextrose broth (PDB) and incubated at room temperature (30 °C) for 5 days. The *M. oryzae* isolate PO2 was confirmed by molecular phylogenetic identification.

The cluster revealed that *M. oryzae* was in the same clade with the sequences of *M. oryzae* MH859782, MH859782 (2), MH443357, MF669475, MF678836, MF668692, MF668691 recorded in the database supported by the 99% bootstrap value (Figure 2). The separated phylogenetic confirmations of *C. elatum* is closely related to MH856469, MH859003, MH864188, MN534815, MN534813, MN534809, which the database supported by the 100% bootstrap value. *C. lucknowense* is closely related to MH080360, JX280827, which the database supported by the 99% bootstrap value and *C. brasiliense* is closely related

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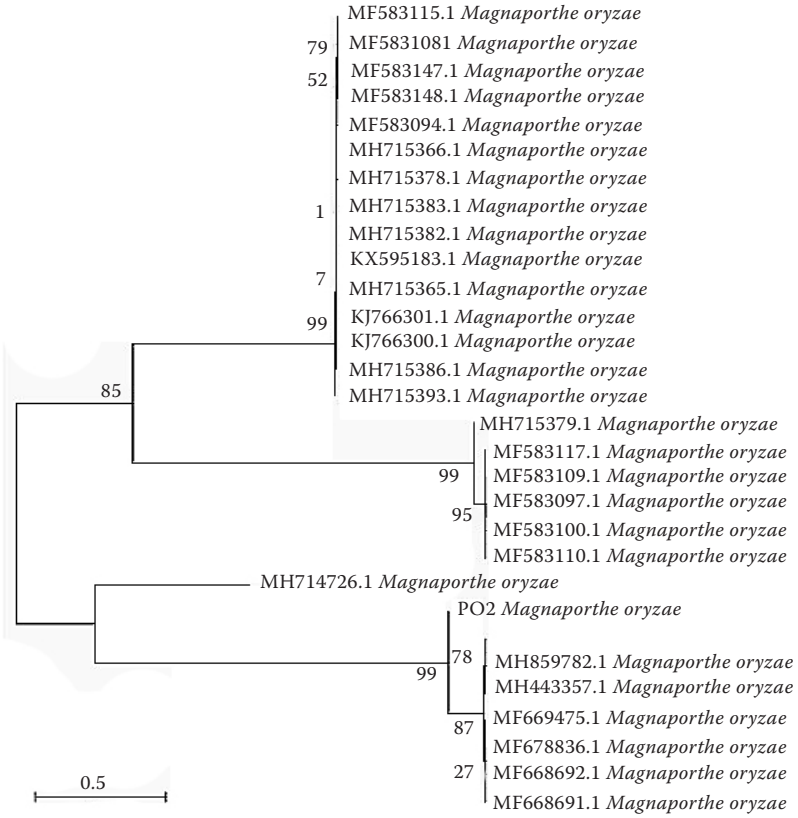

Figure 2. The phylogenetic tree of *Magnaporthe oryzae* from the GenBank including the *Magnaporthe oryzae* isolate PO2 constructed after the distance-based analysis of the ITS1, 4 and 5.8S regions of the rDNA

The numbers at the branches indicate the percentage of the bootstrap values after 1 000 replications

to KF 680267, KT357687, KF680266, KX146504, KT357682, KT357646, JX966545, which the database supported by the 100% bootstrap value (Figure 4). All the isolates were deposited at the Department of Plant Pest Management, Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok, Thailand.

Testing metabolites against pathogen. The EtOAC crude extracts of *C. elatum* showed significantly higher spore inhibition with an ED_{50} value of 106 ppm, followed by the crude MeOH and crude Hexane with ED_{50} values of 188 and 266 ppm, respectively. The hexane crude extract of *C. brasiliense* gave highly significant inhibition of the spore production with the ED_{50} value of 35 ppm, which was higher than the EtOAC crude extract and MeOH crude extract with the ED_{50} values of 55 and 119 ppm., respectively. The EtOAC crude extract of *C. lucknowense* gave the significantly highest spore production with the ED_{50} value of 57 ppm, followed by the Hexane crude extract and MeOH crude extract with the ED_{50} values of 103 and 422 ppm, respectively (Table 2).

Testing nanoparticles against pathogen. The characteristics of the nanoparticles from *C. elatum*: nano-CEH, nano-CEE, nano-CEM; *C. brasiliense*: na-

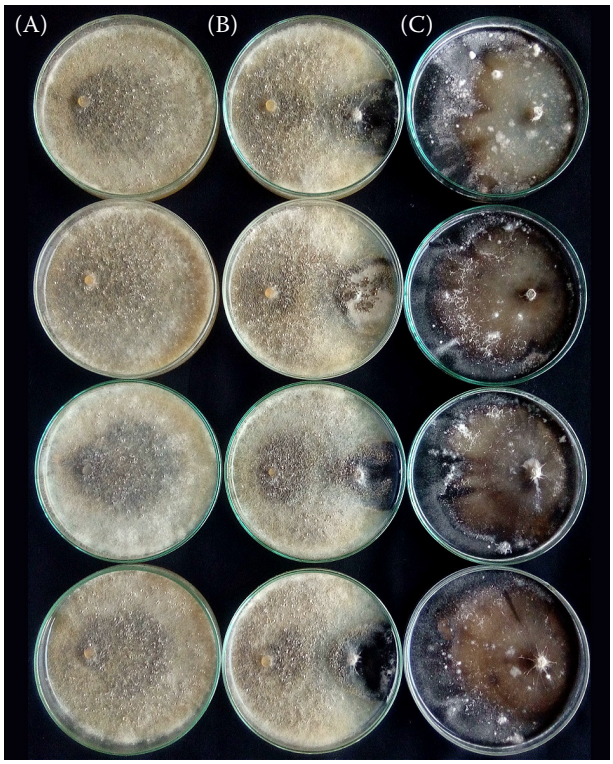

Figure 3. The bi-culture of *Chaetomium elatum* against the *Magnaporthe oryzae* isolate PO2. (A) *Chaetomium elatum,* (B) bi-culture plates and (C) *Magnaporthe oryzae* isolate PO2

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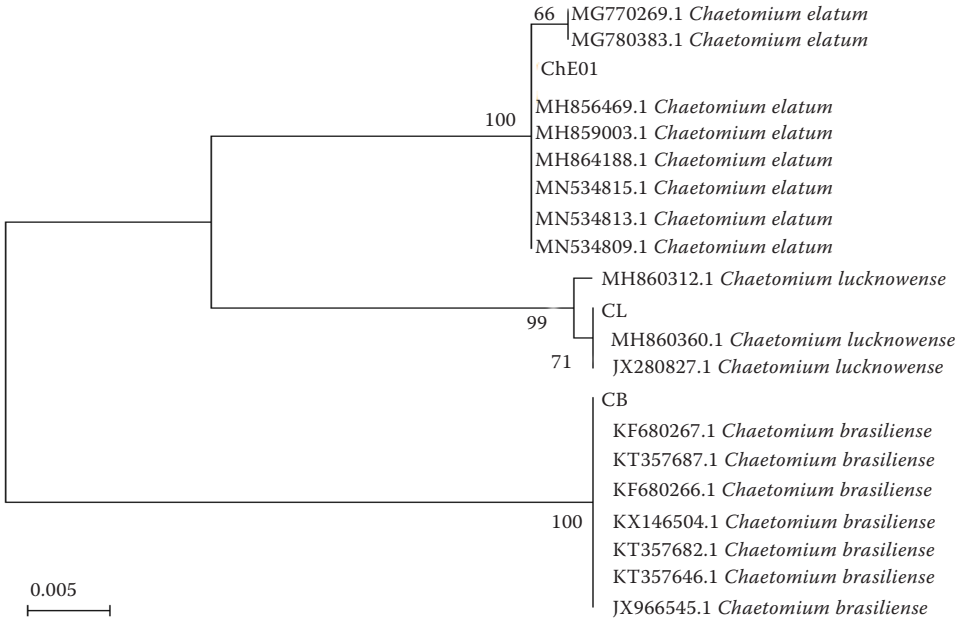

Figure 4. The phylogenetic tree of *Chaetomium* spp. from the GenBank including *Chaetomium elatum*, *Chaetomium lucknowense* and *Chaetomium brasiliense* constructed after the distance-based analysis of the ITS1, 4 and 5.8S regions of the rDNA

The numbers at the branches indicate the percentages of the bootstrap values after 1000 replications

Table 2. The crude extracts of *Chaetomium elatum*, *Chaetomium brasiliense* and *Chaetomium lucknowense* testing for the spore inhibition of *Magnaporthe oryzae*

Crude extracts	Conc. (ppm)	Crude *C. elatum*			Crude *C. brasiliense*			Crude *C. lucknowense*		
		No. of spores (10^5)[1]	spore inh. (%)[1]	ED_{50} (ppm)	No. of spores (10^5)[1]	spore inh. (%)[1]	ED_{50} (ppm)	No. of spores (10^6)[1]	spore inh. (%)[1]	ED_{50} (ppm)
Hexane	0	57.25^{a}	–		39.00^{a}	–		52.00^{a}	–	
	10	49.00^{b}	14.87^{j}		22.50^{cd}	42.28^{h}		40.25^{bc}	22.49^{hi}	
	50	42.25^{cd}	27.83^{h}	266	19.50^{de}	50.08^{g}	35	27.25^{ef}	47.59^{ef}	103
	100	33.75^{fg}	40.43^{f}		17.75^{ef}	54.55^{fg}		24.25^{fg}	53.31^{de}	
	500	24.75^{h}	57.09^{d}		9.00^{hi}	77.01^{c}		19.75^{gh}	62.02^{c}	
	1 000	19.50^{i}	66.66^{c}		2.75^{j}	93.20^{a}		14.75^{h}	71.82^{b}	
EtOAc	0	57.25^{a}	–		39.00^{a}	–		52.00^{a}	–	
	10	45.75^{bc}	20.80^{i}		26.00^{c}	33.26^{i}		35.75^{cd}	31.22^{g}	
	50	36.75^{ef}	36.29^{fg}	106	22.00^{cd}	43.65^{h}	55	28.00^{ef}	46.10^{f}	57
	100	29.75^{g}	48.82^{e}		16.25^{efg}	58.57^{ef}		21.25^{fgh}	59.04^{cd}	
	500	17.50^{ij}	70.24^{bc}		12.50^{gh}	68.10^{d}		15.50^{h}	70.22^{b}	
	1 000	8.75^{k}	85.74^{a}		3.00^{j}	92.46^{a}		8.00^{i}	84.92^{a}	
MeOH	0	57.25^{a}	–		39.00^{a}	–		52.00^{a}	–	
	10	45.75^{bc}	20.12^{ij}		34.00^{b}	12.87^{j}		43.00^{b}	17.03^{i}	
	50	39.25^{de}	31.48^{gh}	188	24.75^{c}	36.53^{i}	119	39.25^{bcd}	24.24^{h}	422
	100	33.25^{fg}	41.66^{f}		18.50^{de}	52.50^{g}		33.25^{de}	35.86^{g}	
	500	25.00^{h}	58.27^{d}		14.00^{fg}	64.14^{de}		27.25^{ef}	47.59^{ef}	
	1 000	14.50^{j}	75.55^{b}		6.00^{ij}	84.98^{b}		19.00^{gh}	63.53^{c}	
CV%		8.19	5.17		11.26	5.07		10.91	6.45	

[1]the average of four replications; the means followed by a common letter are not significantly different by Duncan's multiple range test (DMRT) at $P = 0.01$; inh. – inhibition; conc – concentration

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no-CBH, nano-CBE, nano-CBM and *C. lucknowense*: nano-CLH, nano-CLE, and nano-CLM are shown in Figure 5. The results showed that the nano-CEE expressed antifungal activity against *M. oryzae* causing rice blast, with a highly significant spore inhibition with an ED_{50} value of 7.89 ppm, followed by the nano-CEM and nano-CEH with the ED_{50} values of 8.66vppm and 16.7 ppm, respectively. Nano-CBH gave the highest significance in the spore production with the ED_{50} value of 6.86 ppm, followed by the nano-CBE and nano-CBM with the ED_{50} values of 9.76 and 13.42 ppm, respectively. The nanoparticles of *C. lucknowense* exhibited antifungal activity against the conidial stage of *M. oryzae* causing rice blast. The nano-CLM gave highly significant spore inhibition with an ED_{50} value of 5.24 ppm, followed by the nano-CLE and nano-CLH with the ED_{50} values of 7.01 and 10.72 ppm, respectively (Table 3).

Control mechanisms. The effect of the nanoparticles, nano-CE from *C. elatum*, nano-CL from *C. lucknowense* and nano-CB from *C. brasiliense* against *M. oryzae* (rice blast pathogen) showed the control mechanism as being lysis in nature. The spores treated with the nanoparticles showed broken and abnormal cells as can be seen in Figure 6. The spore suspensions of *M. oryzae* that were separately treated with nano-CE, nano-CL and nano-CB lost their pathogenicity thereafter; with those inoculated to the rice seedlings var PSL2, there were no signs and symptoms of rice blast.

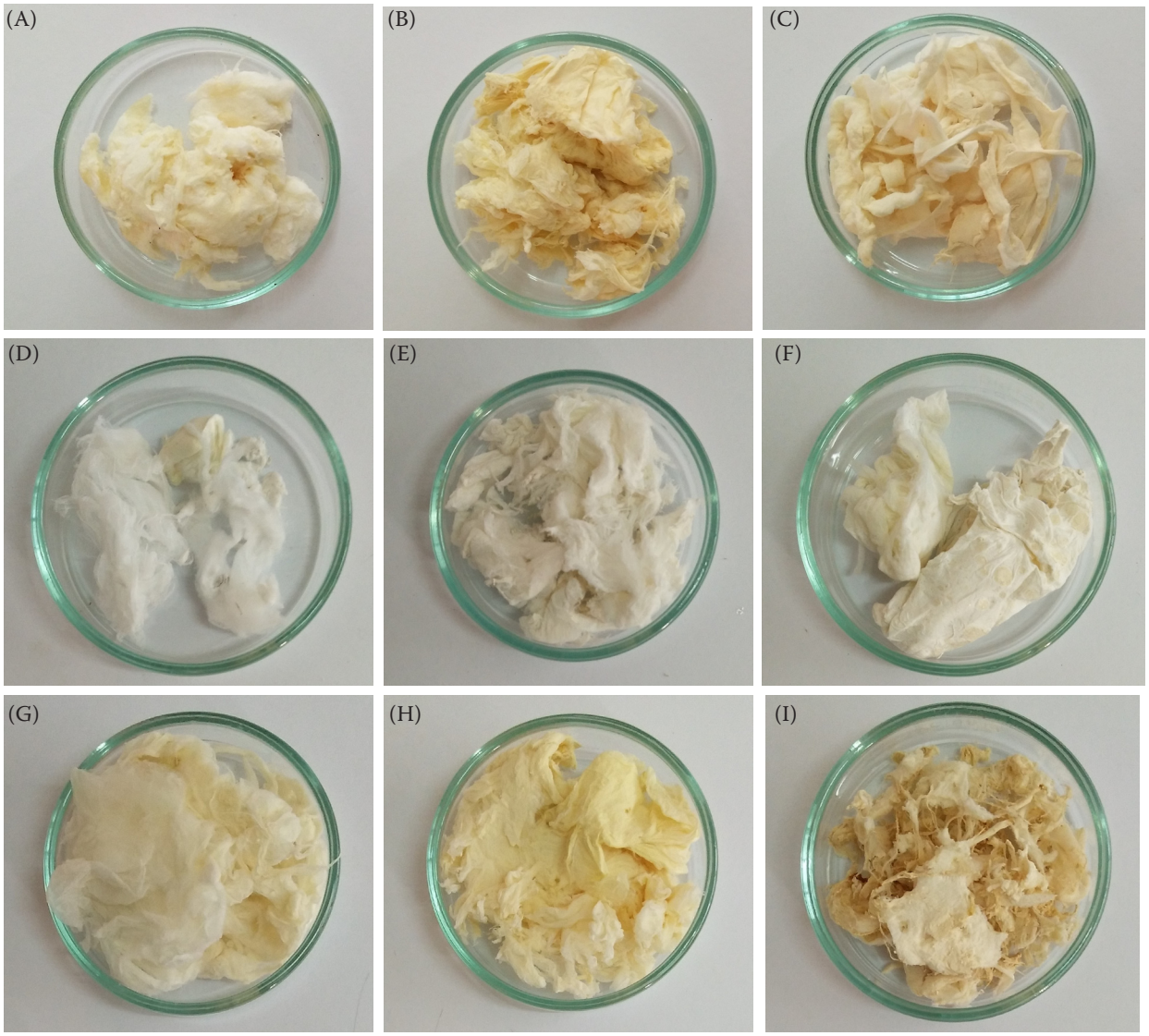

Figure 5. The characteristics of the nanoparticles from *Chaetomium elatum, Chaetomium brasiliense* and *Chaetomium lucknowense*: (A) nano-CEH, (B) nano-CEE, (C) nano-CEM, (D) nano-CBH, (E) nano-CBE, (F) nano-CBM, (G) nano-CLH, (H) nano-CLE and (I) nano-CLM

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Table 3. The nanoparticles of *Chaetomium elatum, Chaetomium brasiliense* and *Chaetomium lucknowense* testing for the spore inhibition of *Magnaporthe oryzae*

Nano particles	Conc. (ppm)	Nano-*C. elatum*			Nano-*C. brasiliense*			Nano-*C. lucknowense*		
		No. of spores (10^5)[1]	spore inh. (%)[1]	ED_{50} (ppm)	No. of spores (10^5)[1]	spore inh. (%)[2,3]	ED_{50} (ppm)	No. of spores (10^5)[1]	spore inh. (%)[1]	ED_{50} (ppm)
Hexane	0	32.50^{a}	–	16.70	17.25^{a}	–	6.86	40.50^{a}	–	10.72
	3	30.50^{ab}	5.69^{g}		14.50^{bc}	15.75^{e}		34.00^{b}	15.32^{j}	
	5	27.00^{bc}	16.45^{ef}		10.75^{d}	37.57^{d}		28.75^{cd}	28.53^{h}	
	10	22.50^{de}	30.63^{cd}		5.50^{f}	68.05^{b}		23.50^{ef}	41.81^{f}	
	15	15.75^{f}	51.85^{b}		3.50^{g}	79.74^{a}		14.50^{g}	64.18^{cd}	
EtOAc	0	32.50^{a}	–	7.89	17.25^{a}	–	9.76	40.50^{a}	–	7.01
	3	29.75^{ab}	7.91^{fg}		14.00^{bc}	18.47^{e}		31.25^{bc}	22.41^{i}	
	5	20.50^{e}	37.06^{c}		11.75^{d}	31.72^{d}		25.00^{def}	37.82^{fg}	
	10	15.50^{f}	52.73^{b}		7.50^{e}	56.36^{c}		15.00^{g}	63.00^{d}	
	15	6.50^{g}	80.46 ^{a}		4.50^{fg}	73.89^{ab}		10.50^{gh}	73.87^{ab}	
MeOH	0	32.50^{a}	–	8.66	17.25^{a}	–	13.42	40.50^{a}	–	5.24
	3	30.25^{ab}	6.80^{fg}		15.75^{ab}	8.34^{f}		26.50^{de}	34.25^{g}	
	5	25.00^{cd}	23.01^{de}		13.75^{c}	20.03^{e}		21.25^{f}	47.53^{e}	
	10	15.50^{f}	52.43^{b}		11.50^{d}	33.29^{d}		12.75^{gh}	68.65^{bc}	
	15	7.25^{g}	77.78^{a}		7.25^{e}	57.92^{c}		8.75^{h}	78.52^{a}	
CV%		12.45	19.01		10.23	8.14		11.75	7.75	

[1]the average of four replications; means followed by a common letter are not significantly different by Duncan's multiple range test (DMRT) at $P = 0.01$; inh. – inhibition; conc. – concentration

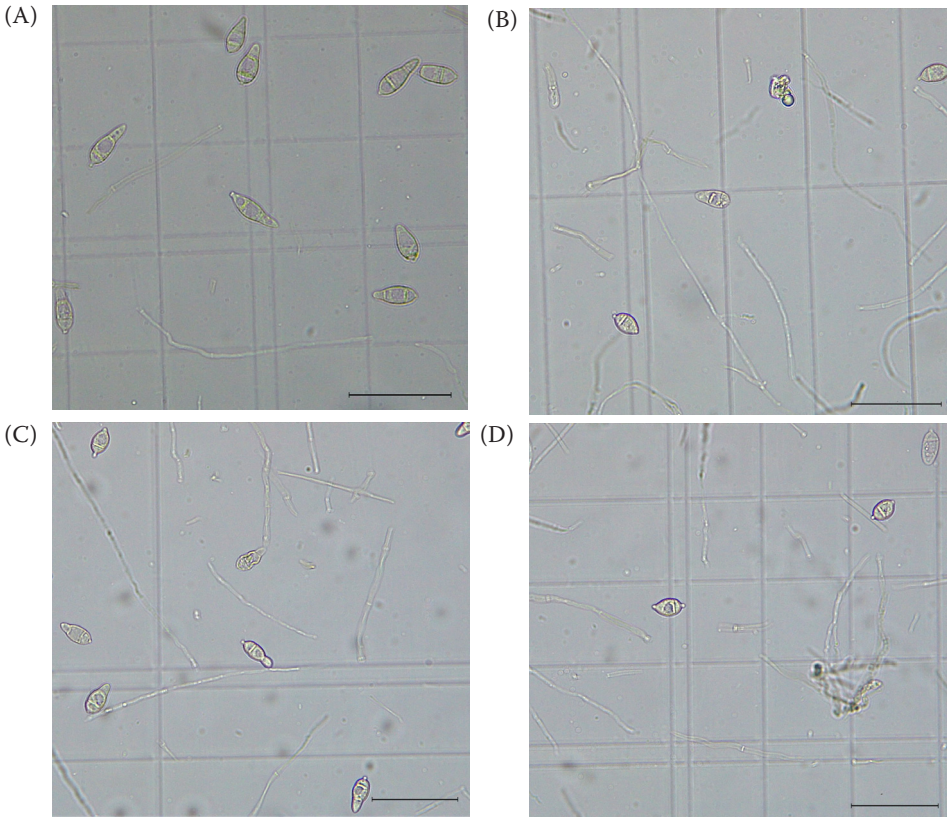

Figure 6. The effect of the nanoparticles: (A) normal spore, (B) nano-CB from *C. brasiliense,* (C) nano-CL from *C. lucknowense* and (D) nano-CE from *C. elatum* against the *M. oryzae* isolate PO2

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Testing nano-CBH for phytoalexin production. The rice leaves treated with nano-CBH from *C. brasiliense* were examined for the phytoalexin production by thin layer chromatography. The treated nano-CBH found spots on the TLC plates when using a combination of benzene: ethyl acetate (10 : 1) under 365 nm UV light for leaves with the Rf value of 0.08 supposing to be Sakuranertin, and spots on the TLC plates with the R_f values of 0.28 supposing to be Oryzalexin B. The inoculated rice var PLS2 with *M. oryzae* was treated with nano-CBH, the rice seedlings were expected to produce phytoalexins against the rice blast disease (Figure 7).

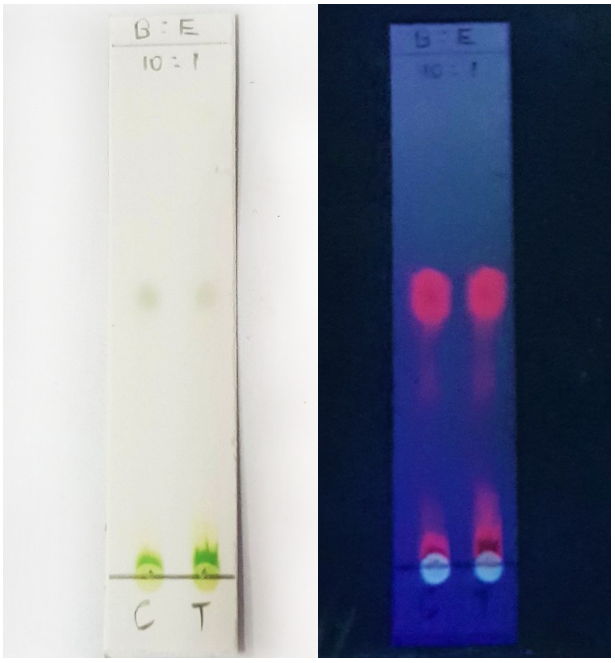

Figure 7. The presence of possible phytoalexins of Sakuranetin at an R_f value of 0.08 and Oryzalexin B at an R_f value of 0.28 in the TLC plates when using a combination of benzene:ethyl acetate (10 : 1) under a 365 nm UV light for the leaves

DISCUSSION

M. oryzae PO2 is an aggressive isolate that causes rice blast disease as reported by Ou (1985). The symptoms normally show up with the presence of roundish to slightly elongated, necrotic, grey spots as reported in another study (Song & Soytong 2016). The molecular phylogeny was confirmed as *M. oryzae,* which was in the same clade with the sequences of *M. oryzae* MF669475, MF678836, MF668692, MF668691 recorded in the database supported by the 99% bootstrap value consistent with Jagadeesh et al. (2018). *C. elatum* was confirmed in the phylogenetic tree that is closely related to MH856469, MH859003, MH864188, MN534815, MN534813, MN534809, which the database supported by the 100% bootstrap value and *C. lucknowense* is closely related to MH080360, JX280827, which the database supported by the 99% bootstrap value. *C. brasiliense* is closely related to C. *brasiliense* JX966545 which the database supported by the 100% bootstrap value (Yew et al. 2014). Pornsuriya et al. (2011) stated that the phylogenetic analysis of the ITS rDNA sequence data also supports *Chaetomium siamense* as being distinct.

The tested *Chaetomium* spp. showed activity against *M. oryzae* in the bi-culture with similar effects to the antibiosis mechanism of the control reported by Soytong (1992) where the *Chaetomium cupreum* isolated from upland rice field soils in the Philippines, was antagonistic to the anamorph *Pyricularia oryzae* (T.T. Hebert) M.E. Barr in the biculture test. Coating the rice seeds of the blast susceptible rice line, IR442-2-58 resulted in the control of the rice blast disease.

The crude EtOAC, crude MeOH and crude hexane extracts of *C. elatum* inhibited spore production with the ED_{50} values of 106, 188 and 266 ppm, respectively. The results were similar to the previous work (Song & Soytong 2016). The crude hexane extract of *C. brasiliense* inhibited the spore production of *M. oryzae* with the ED_{50} value of 35 ppm when compared to the EtOAC and MeOH crude extracts with the ED_{50} values of 55 and 119 ppm, respectively. The research finding was similar to that of the work of Soytong (2014). Moreover, the crude extracts from EtOAC of *C. lucknowense* inhibited the spore production of *M. oryzae* PO2 with the ED_{50} value of 57 ppm. This was a similar result to the work of Soytong et al. (2013). The hexane and MeOH crude extracts *C. lucknowense* inhibited the tested pathogen with ED_{50} values of 103 and 422 ppm, respectively.

The highlight of the research findings is that that nanoparticles constructed from *Chaetomium* spp. can be used at lower concentrations than the crude extracts to inhibit *M. oryzae* that cause rice blast. Previous research on nanoparticles from *Chaetomium* sp. were characterised to be nano-materials loaded with active compounds. The crude extracts from *Chaetomium* spp. were incorporated into a polyacetal acid and electrospun at 25–30 kv. The scanning electron microscope images revealed that the nano-materials from *Chaetomium* spp. measured

https://doi.org/10.17221/41/2019-PPS

171 nm (Tann & Soytong 2016). The results showed that the nano-CEE, nano-CBH and nano-CEM inhibited the spore production of the rice blast pathogen, *M. oryzae* with the ED_{50} values of 7.89, 8.66 and 16.7 ppm, respectively. The nanoparticles of *C. brasiliense,* nano-CBH, nano-CBE and nano-CBM suppressed the spore production of *M. oryzae* with the ED_{50} values of 6.86, 9.76 and 13.42 ppm, respectively. The nano-CLM, nano-CLE and nano-CLH resulted in the spore inhibition of *M. oryzae* with the ED_{50} values of 5.24, 7.01 and 10.72 ppm, respectively. Tan and Soytong (2016; 2017) reported that nanoparticles loaded with *Chaetomium globosum* KMITL0805 actively worked against *Curvularia lunata* that cause leaf spot of the rice var Sen Pidoa, and the nanoproduct from *Chaetomium cupreum* CC3003 could control the leaf spot of the rice var. Sen Pidoa. Moreover, the *in vivo* test showed these nano-particles treated onto the rice seedlings could control the leaf spot of the rice var. Sen Pidoa and decreased the leaf spot disease of the rice which supported the stability of the nanoparticles after applying it to the rice seedlings.

The control mechanism of the nanoparticles derived from *Chaetomium* spp. was proven to have an antibiosis and lysis mechanism as shown in previous reports by Tann and Soytong (2016) and Vilavong and Soytong (2017). It was demonstrated that rice seedlings inoculated with the treated spores of *M. oryzae* with the nano-CE, nano-CL and nano-CB resulted in the loss of pathogenicity. A similar result was reported by Sibounnavong et al. (2011). The result from the TLC plates found Sakuranetin at R_f value of 0.08 which was similar to Hasegawa (2014) who stated that Sakuranetin extracted from rice leaves showed spots in the TLC to detect the presence of Sakuranetin at an R_f value of 0.09 under a 365 nm UV light. It is indicated the spots to be Oryzalexin B on the TLC with an R_f value of 0.28 when using a combination of benzene:ethyl acetate (10 : 1) under a 365 nm UV light for the leaves. The research findings revealed the same as Akatsuka (1983) who found Oryzalexin B as the phytoalexin against rice blast in the TLC at the same R_f value.

Acknowledgement: I would like to acknowledge the King Mongkut's Institute of Technology Ladkrabang (KMITL) for my PhD Scholarship and research fund as supported by the Faculty of Agricultural Technology, KMITL and additional research fund supported by KMITL, Bangkok, Thailand. The financial support from Thailand Research Fund is also gratefully acknowledged.

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Received: March 25, 2019
Accepted: March 10, 2020
Published Online: May 15, 2020