Natural products of fine particles derived from *Neosartorya hiratsukae* against brown spot of rice cause by *Drechslera oryzae*

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Abstract Efficacy of *Neosartorya hiratsukae* was confirmed to control brown leaf spot of rice caused by *Drechslera oryzae*. In bi-culture antagonistic test, *Neosartorya hiratsukae* had highest significantly inhibited the colony growth and spore production which were 51.10% and 55.65% respectively. Crude methanol extract metabolites of *N. hiratsukae* gave significantly highest inhibition of colony growth and spore production of *D. oryzae* which ED_{50} was 168 µg/ml. Testing fine particles constructed from metabolites of *N. hiratsukae* showed that fine particles from methanol crude extact of *N. hiratsukae* gave significantly highest inhibition of colony growth and spore production of *D. oryzae* which ED_{50} of 4.11 µg/ml.

Keywords: *Neosartorya hiratsukae*, brown spot, rice disease.

Introduction

Rice is one of the most important staple food for increasing world population, especially in Asia. Asian farmers still account for 87% of the world's total rice production. Rice is the main export of Thailand. Disease damage to rice can greatly reduce yield. They are mainly caused by bacteria, viruses, or fungi. Brown spot is one of the important rice diseases in the world. It can be a serious disease causing a considerable yield loss. Brown spot causes both quantity and quality losses. On average, the disease causes 5% yield loss across all lowland rice production in South and Southeast Asia. Severely infected field can have as high as 45% yield loss (IRRI, 2019). The traditional chemical fungicides have been used for years and some case the pathogens become resistance to those chemical fungicides. However, there are many researcher were reported to use the biocontrol agents to control those disease.

This objective was to investgate the morphology of *Neosartorya hiratsukae* and *Drechslera oryzae*, pathogenicity test. Tesing crude extraxts and fine particles from *Neosartorya hiratsukae* to control brown leaf spot of rice var Chai Nat 1 were also conducted.

Materials and methods

Isolation of pathogen and Pathogenicity test

Drechslera oryzae causing by brown leaf spot of rice will be isolated form leaf rice var. Chai Nat 1 by tissue transplanting technique leaf of rice were soaked in sterilized water and followed by 1% sodium hypochlorite (NaClO) for 3 min and then sterilized water again. All seeds were transferred onto water agar (WA) medium for firstly observation of appearing mycelia and sub-cultured to PDA until get pure culture. Morphological identification was done by observation fungal characteristic under binocular compound microscope.

Pathogenicity test

The experiment will be designed in Completely Randomized Design (CRD) with four replications. The isolates of pathogen will be tested for pathogenicity using detached leaf method in pot followed Koch's Postulate. The mycelia of *Drechslera oryzae* will be removed into sterilized distilled water and conidia suspension which adjust to 1×10^5 conidia/ml. by using haemacytometer. Seedling of rice will be grown in pot for 21 days. And then sprayed on leaves. The inoculated leaves will be covered with plastic sheet and maintained to observe the infected leaves. The inoculated leaves with only spraying sterilize distilled water will be done to serve as controls.

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Bi-culture antagonistic test

Strain of antagonist used for experiments

Antagonists *Neosartorya hiratsukae* (EU06) which offered from Assoc. Prof. Dr. Kasem Soytong.

The assay for antagonism fungi will be performed on PDA on Petri dishes by the dual culture method (Fokkema, 1978). A 0.5 cm diameter sterilized cork borer will be used to remove agar plugs from the actively growing edge of cultures of the pathogenic fungus and of the antagonistic fungi and will be used to inoculate 9 cm diameter PDA plates: an agar plug of the pathogen will be placed on one the opposite site an agar plug of an antagonistic fungus. Plates will be inoculated with a single plug of an antagonistic fungus or of the pathogen acted as the controls. The plates will be incubated at room temperature for 30 days. The experiment is one with four replications of each treatment. The zone of inhibition will be recorded as the distance between the fungal pathogen and the area of antagonist growth after 7 days.

Observation of abnormal spores and nornal spore of pathogen from each treatment will be observed under compound microscope and take phoyograph for comparison.

The data were collected as colony diameter, number of conidia of pathogenic fungus. Percentage of growth and conidia inhibition of pathogen will be calculated using formula below:

Growth inhibition; $GI = \frac{R1-R2}{R1} \times 100$

R1 = colony diameter or conidia number of pathogen in control

R2 = colony diameter or conidia number of pathogen in control in dual culture plate

The data will be statistically computed for analysis of variance (ANOVA) and mean comparison was compared by using Duncan's New Multiple Range Test (DMRT) at P=0.01 and P=0.05.

In vitro test of crude extracts from antagonist against *Drechslera oryzae* causing brown spot of rice.

Crude extraction of antagonists

Extraction method

The effective isolates of antagonistic fungi from Bi-culture test will be used for bioactive substances testing. The crude extracts from antagonistic will be assayed for their biological mechanism against pathogen.

The antagonist *Neosartorya hiratsukae* will be cultured in potato dextrose broth (PDB) at room temperature for 30 days. The fungal biomass of endophytics will be removed from PDB, filtered through cheesecloth and air-dried overnight. Fresh weight and dry weight of fungal biomass will be weighted. The fungal biomass will be ground with electrical blender, and placed in triangular flask. And then will be dissolved with equal volum hexane 3-5 days at room temperature, and then, the biomass will be separated by filtration through Whatman filter paper. The solvents will be then evaporated in vacuo to yield crude hexane. The marc will be further extracted with ethyl acetate and methanol respectively using the same procedure as hexane and yield crude ethyl acetate (EtOAc) and crude methanol (MeOH) extracts.

Testing biological activity of crude extracts from antagonist against *Drechslera oryzae* causing brown spot of rice.

The crude extract substances will be assayed for inhibition of pathogen. The experiment will be conducted by using factorial experiment in Completely Randomized Design (CRD) with four replications. Factor A= different solvents, A1= hexane, A2= ethyl acetate and A3= methanol solvents. Factor B= different concentration of crude extract substances µg/ml which is consisted of B1 = 0, B2 = 10, B3 = 50, B4 = 100, B5 = 500 and B6 = 1000 ppm. Each crude extract will be dissolved in 2% of dimethyl sulfoxide (DMSO), and then mixed with PDA before autoclaved at 121^0C, 15lbs/inch2 for 30 minutes. The assay will be cut the colony margin on PDA by cork borer with 5 mm diameter and transferred on the centre of PDA Preti dishes (5 cm in diameter) which mixed with crude extract substance in each concentration and incubated at room temperature approximately 27-30 ^{0}C until the pathogen in control grown full plate. Abnormal and normal spores of pathogen from each treatment will be observed under binocular compound microscope and taken photograph for comparison.

Data will be collected as the colony diameter (cm), number of spore. The pathogen cell will be separately examined from each concentration under compound microscope. Percentage of inhibition will be computed as described in Bi-culture test. The data will be statistically computed analysis of variance. The comparison among treatment mean will be computed with DMRT at P=0.01 and 0.05. The effective dose (ED_{50}) will be computed by using Probit analysis. The effect of crude extracts to pathogen cells will be observed under microscopic compound. The experiment will be repeated in two times.

Test efficacy of fine particles from antagonist against *Drechslera oryzae* causing brown spot of rice.

Preparation of fine particles;-fine paticle will be done using the method of Dar and Soytong (2014) to get fine particle hexane, fine particle ethyl aectate and fine particle methanol. Experiment will be designed by using two factors factorial experiment in CRD with four replications. Factor A will be represented fine particle hexane, fine particle ethyl aectate and fine particle methanol and factor B will be represented concentrations at 0, 1, 3, 5, 7 and 10 ppm. Each fine particle dissolved in one drop 2% dimethyl sulfoxide (DMSO), and then mix into 30 ml PDA medium before autoclaving at 121℃, 15 lbs/inch 2 for 30 min. The culture of *Drechslera oryzae* will be cut at the edge of colony with sterilized cock borer (5 mm). Agar plug of pathogen will be transferred to the middle of PDA media in plate (5.0 mm diameter) incorporated with each fine particles. The transferred plates will be incubated at room temperature until the pathogen in control plates growing full. Abnormal and normal spores of pathogen from each treatment will be observed under binocular compound microscope and taken photograph for comparison.

Data will be collected as colony diameter (cm) and the number of spores that counted by using haemacytometer. Percentage of inhibition will be computed as described in Bi-culture test. The data will be statistically computed analysis of variance. The comparison among treatment mean will be computed with DMRT at P=0.01 and 0.05. The effective dose (ED_{50}) will be computed by using Probit analysis. The effect of crude extracts to pathogen cells will be observed under microscopic compound.

Results

Isolation of pathogen and Pathogenicity test

D. oryzae was isolated from leaf spot of rice var. Chai Nat 1 in this study and demonstrated to be pathogenic on this host. (Figure 1)

Figure 1. Pathogenicity test of *Drechslera oryzae* on rice. The inoculated control (A) and inoculated leaves (B) after 5 days.

Bi-culture antagonistic test

Result showed that *N. hiratsukae* could inhibit mycelial growth of *D. oryzae* which averaged colony of 4.40 cm when compared to control plate of 9.00 cm. It could inhibit mycelia 51.10 percent in 10 days. However, *N. hiratsukae* significantly inhibited spore production of *D. oryzae* 55.56 percent (Figure 1, Table 1).

Table 1. *N. hiratsukae* against *Drechslera oryzae* in bi-culture tests

	Colonydiameter (cm.)[1/]	Growth inhibition (%)	Spore number ($\times 10^5$ /ml)[1/]	Spore inhibition (%)
Control	9.00a	0.00	6.37a	0.00
Bi-culture	4.40b	51.10	2.75b	55.65
CV (%)	2.85		25.99	

1/: Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.05.

In vitro test of crude extracts from antagonist against Drechslera oryzae causing brown spot of rice.

Result showed that methanol crude extract from *N. hiratsukae* gave significantly highest inhibition of 37.50 % for the colony growth of *D. oryzae* at the concentration of 1,000 ppm when compared to the control (Tables 2). Moreover, methanol crude extract from *N. hiratsukae* gave significantly highest inhibition of 79.47 % for the spore production of *D oryzae* at the concentration of 1,000 ppm when compared to the controlwhich the ED_{50} was 168 (Table 2).

Table 2. Crude extracts of *N. hiratsukae* testing to inhibit *Drechslera oryzae*

Crude extracts	Concen-tration (ppm)	Colony-diameter (cm)1/	Growth inhibition (%)1/	Number of spore ($\times10^5$)1/	Spore inhibition (%)1/	ED_{50} (µg/ml)
Hexane	0	5.00a	0.00h	3.01a	0.00h	
	10	4.73abc	5.50fgh	1.94c	34.89f	
	50	4.50abc	10.00fgh	1.86c	36.73f	
	100	4.32bcd	13.50efg	1.78cd	38.78f	-
	500	4.22bcde	15.50def	1.68cde	43.49def	
	1000	3.52fgh	29.50abc	1.53cdef	48.72cdef	
Ethyl acetate	0	5.00a	0.00h	3.01a	0.00h	
	10	4.77ab	4.50gh	2.66ab	12.34gh	
	50	4.30bcd	14.00efg	2.50b	16.25g	
	100	3.92def	21.50cde	1.78cd	40.04ef	544.44
	500	3.55fgh	29.00abc	1.44cdef	50.83cdef	
	1000	3.35gh	33.00ab	1.28defg	55.88cde	
Methanol	0	5.00a	0.00h	3.01a	0.00h	
	10	3.75efg	25.00bcd	1.5cdef	50.25cdef	
	50	3.57fgh	28.50abc	1.2efg	58.55bcd	
	100	3.32gh	33.50ab	1.1fg	62.42bc	168
	500	3.17h	36.50a	0.8gh	72.76ab	
	1000	3.13h	37.50a	0.6h	79.47a	
	C.V. (%)	8.05	20.12	17.49	26.34	

1/: Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.05.

Test efficacy of fine particles from antagonist against Drechslera oryzae causing brown spot of rice.

Result showed that fine particle methanol from *N. hiratsukae* gave significantly highest inhibition of 64.00 % for the colony growth of *D. oryzae* at the concentration of 10 ppm when compared to the control (Tables 3).The fine particle methanol, fine particle ethyl aectate and fine particle hexane at the concentration of 10 ppm inhibited spore production by 94.87%, 93.32%, and 88.74%, respectively. These nanoparticles expressed antifungal activity against *Drechslera oryzae* with ED_{50} values of 4.11, 4.60, and 4.63 µg/ml, respectively (Table 3).

Table 3. Fine particles of *N. hiratsukae* testing to inhibit *Drechslera oryzae*

Fine particle	Concen-tration (ppm)	Colony-diameter (cm)1/	Growth inhibition (%)1/	ED_{50} (µg/ml)	Number of spore ($\times10^5$)1/	Spore inhibition (%)1/	ED_{50} (µg/ml)
Hexane	0	5.00a	0.00i		4.23a	0.00g	
	1	3.57b	28.50g		3.56b	15.71f	
	3	3.42c	31.50g	8.70	3.06c	27.12de	4.63
	5	2.97d	40.50f		2.80d	33.79d	
	7	2.52f	49.50d		1.30f	68.89b	
	10	2.27g	54.50c		0.47g	88.74a	
Ethyl	0	5.00a	0.00i		4.23a	0.00g	
acetate	1	2.97d	40.50f		3.35b	20.31ef	
	3	2.92de	41.50ef	5.28	3.34b	20.50ef	4.60
	5	2.92de	41.50ef		2.32e	44.84c	
	7	2.22g	55.50c		1.24f	70.66b	
	10	1.99h	60.00b		0.28g	93.32a	
Methanol	0	5.00a	0.00i		4.23a	0.00g	
	1	3.00d	40.00f		3.32b	20.95ef	
	3	2.97d	40.50f	4.73	3.00cd	28.57de	4.11
	5	2.80e	44.00e		2.40e	42.96c	
	7	2.29g	54.00c		1.43f	65.69b	
	10	1.79i	64.00a		0.22g	94.87a	
	C.V. (%)	3.09	5.02		7.85	14.14	

1/: Means four repeated experiments and followed by the same letter are not significantly differed by DMRT at P=0.05.

Discussion

The brown spot of rice caused by *Drechslera oryzae* was isolteds and proved pathogenicity as similar report of Chaijuckam *et al.* (2019).

In this study, *N. hiratsukae* could inhibit mycelial growth of *D. oryzae* as 51.10% similar report confirimed *Neosartorya* sp. (KUFC 6301) could inhibit 30% mycelium growth of Bipolaris maydis, *Collectrichum capsici* and *C. gloeosporioides* (Eamvijarn *et al*., 2009). Methanol crude extract from *N. hiratsukae* gave significantly highest inhibition of 37.50 % for the colony growth of *D. oryzae* at the concentration of 1,000 ppm this similar to the report of *N. pseudofischeri* KUFA0060 and *N. quadricincta* KUFA0064 crude extracts inhibited the mycelial growth of *P. palmivora* and *C. capsici* at a concentration of 100 ppm (Boonsang *et al.,* 2014). Jantasorn *et al*. (2016) also reported that *N. fischeri* Bodhi004 crude extract also recorded antifungal activity, 100% growth inhibition against *P. palmivora, P. grisea, Alternaria* sp. and *R. solani* at a concentration of 10,000 ppm. Fine particle methanol from *N. hiratsukae* gave significantly highest inhibition of 64.00 % for the colony growth of *D. oryzae* and 94.87% for inhibited spore production at the concentration of 10 ppm. This study was similar to the report *Chaetomium brasiliense* effectively against *Drechslera oryzae* at the concentration of 10 ppm (Vareeket, *et al.* 2014).

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