International Journal of Agricultural Technology 2018 Vol. 14(1): 83-98
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Control of Rice Blast Disease Using Antagonistic Yeasts

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Kunyosying, D., To-anun, C. and Cheewangkoon, R. (2018). Control of rice blast disease using antagonistic yeasts. International Journal of Agricultural Technology 14(1):83-98.

Abstract Sixty three epiphytic yeasts isolated from various vegetable fruit surfaces and rice leaves were selected for antagonistic screening against rice blast disease caused by the fungus *Pyricularia oryzae*. In vitro screening was undertaken to assess the antagonistic potential of the yeasts. Preliminary testing showed that eight isolates inhibited growth by more than 50%. Then the five most antagonistic isolates: CMY047, CMY018, CMY045, CMY113 and CMY057, were selected to conduct further growth inhibition tests using the dual culture method. These isolates suppressed growth of the fungus by 62.86, 55.17, 54.28, 53.21 and 51.43%, respectively. Then the antagonistic yeasts were tested for their pathogen spore germination inhibition by the slide culture technique. Spore germination was observed under a microscope at 3, 6, 12 and 24h following test initiation. All antagonistic yeasts delayed but did not completely inhibit spore germination. Furthermore, antagonistic yeast isolates CMY045 and CMY018 significantly reduced appressorium formation and length of the germ tube when compared to the control treatment. In a greenhouse experiment, the yeast isolates CMY045 and CMY018 significantly reduced disease incidence to 15.20 and 17.06% respectively when compared with the control treatment (80.14%). Similar results were obtained in a field experiment.

Keywords: Biocontrol, Antagonist yeasts, Rice blast disease, *Pyricularia oryzae*

Introduction

Rice (*Oryza sativa* L.) is one of the most important staple crops for a large part of the world's population, but mainly in East, Southeast and South Asia (Plodpai *et al.*, 2013). Losses due to diseases and pests are major constraints for rice production. Rice blast disease is the most serious disease of cultivated rice and poses a serious threat to global food security, and is caused by the ascomycetous fungus *Pyricularia oryzae* Cavara [synonym *Pyricularia grisea Sacc.* the telemorph of Magnaporthe grisea (Herbert)] Couch and Kohn (2002). This disease occurs in almost all the rice growing areas. Each year rice blast disease is responsible for a 10–30% loss of the rice harvest (Talbot, 2003).

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The disease can be managed by use of fungicides, resistant cultivars, agronomic practices, biotechnological methods, and their integration (Ribot *et al*., 2008). However, the management of blast disease by using synthetic fungicides may be harmful to humans and the environment. The use of resistant cultivars has been known to be the most effective strategy; however, *Pyricularia oryzae* rapidly develops new races that may breakdown the rice resistance (Castano *et al*., 1990; Khan *et al*., 2001; Haq *et al*., 2002). Therefore, biological control using microbial agents has been reported among several alternatives to be an effective approach to the use of synthetic chemical fungicides (Droby *et al*., 2009; Spadaro and Gullino, 2004).

In recent years, considerable attention has been focused on the application of antagonists for the inhibition of plant disease (Couch *et al*., 1999; Tian *et al*., 2002a; El-Ghaouth *et al*., 2003). Among these antagonistic microorganisms, yeasts have been efficacious as biological control agents (Irtwange, 2006; Qing and Shiping, 2000). Utilization of antagonistic yeasts as an alternative appears to be a promising technology (Wilson and Chalutz, 1989; Droby *et al*., 1991; Elad *et al*., 1994; Ippolito *et al*., 2000; Fan *et al*., 2002). Several mechanisms have been reported to play significant roles in the biocontrol activity of antagonistic yeasts, and these have been examined by studying the interactions between yeasts and postharvest pathogens. It has been suggested that attachment of the yeast to fungal hyphae and extensive production of an extracellular matrix by yeasts may play key roles by either enhancing nutrient competition or by some other undetermined mechanisms (Wisniewski *et al*., 1991; Jijakli and Lepoivre, 1998; Wan and Tian, 2002).

The objective of this study was to test the efficacy of antagonistic yeast isolated from various fruits and rice leaves in controlling blast disease in vitro and in vivo.

Materials and methods

The fungal pathogen

Eight rice samples with leaf and neck infections were collected. Blast lesions were surface sterilized with 10% sodium hypochloride for 1 min and placed on clean glass slides kept in sterile Petri dishes padded with moist cotton. The Petri dishes were incubated for 48 h at room temperature. Single conidia were identified from the sporulating lesions using a stereomicroscope and aseptically transferred to potato dextrose agar (PDA) slants for maintenance. The causal organism was identified as *Pyricularia oryae* based on its spore morphology.

Yeast isolation

Samples were collected from surfaces of fruit and rice leaves from agricultural fields. Ten grams of sample were used for serial dilutions in sterile distilled water. After that, 0.1 ml of each dilution was spread on yeast malt extract agar (YM agar). The plates were incubated at 28 ºC for 48 h. Yeast colonies were examined under the microscope and different morphological colonies were selected. The yeast isolates were re-streaked on YM agar to obtain pure cultures and they were maintained on YM agar stored at 4 ºC until use.

Morphological Characteristics of Antagonistic Yeasts

Yeast antagonists were characterized morphologically (texture, color, surface, elevation, margin, cell arrangement and type of spore). The yeast cells were cultivated on YM agar. The cultures were incubated for 48-72 h at 25 °C. Characteristics of cells and colony patterns were recorded.

Screening for Yeast Antagonists against P. oryzae

The dual culture technique followed the method of Rabindran and Vidyasekaran (1996); a 0.5 mm-diameter agar plug from a10–day-old culture of *P. oryzae* was put on one side of PDA plate. The plate was incubated at 25 ºC for 4 d. Next, a 48 h culture of the yeast isolate was streaked on the opposite side of the agar 3 cm away from the pathogen. Each inoculation was done in four replicates. The inoculated plates were incubated at 28 ºC for 20 d until fungal mycelia completely covered the agar surface in the control plate. The radius of the pathogen colony was compared with the control. The inhibition zone and mycelial growth of the pathogen, and percent inhibition of pathogen growth was calculated using this formula:

$$\text{Percent inhibition of radial growth} = \frac{R_1 - R_2 \times 100}{R_1}$$

R_1 = radial of pathogen in control treatment
R_2 = radial of pathogen in treatment

Inhibitory effect of antagonistic yeast on conidial germination and of Pyricularia oryzae on PDA

A conidial germination assay was conducted to evaluate the antagonistic activity of yeast isolates against *P. oryzae* at room temperature according to methods of Zhang *et al*. (2007), using five of the most promising yeasts from the screening experiment.

One hundred µL of a 1×10^9 cells/mL^{-1} yeast suspension were mixed with 100 µL of a 1×10^5 conidia/mL suspension of *P. oryzae* and spread in Petri dishes containing potato dextrose agar (PDA). The yeast spore suspension was replaced by 100 µL of sterile distilled water in the control treatment. Conidial germination, was examined by observing 100 conidia of each treatment under a compound microscope 3, 6, 9 and 12 h after inoculation with the conidia. The conidial germination was calculated according to the formula described by Manici *et al.* (1997):

$$\text{Percentage of germination} = \frac{\text{Number of germinated spores} \times 100}{\text{Total number of spores}}$$

Appressorium formation and penetration assays

Conidia harvested from 10-day-old Oat meal agar cultures, were suspended at a concentration of 5×10^5 conidia/ml in sterile water, and used for appressorium formation assays with glass cover slips according to described by (Xue *et al*., 2002). Penetration assays were conducted with onion epidermal cells (Kankanala *et al*., 2007).

Infection assays with detached rice leaves

Conidia of *P. oryzae* were harvested from 10-day-old on Oat meal agar cultures and suspended at a concentration of 5×10^5 conidia/mL in sterile distilled water. Detached leaves from 2-week-old seedlings of rice cultivar Hommali 105 were used in the infection assays as described by Park *et al*., 2004. The yeast cell suspension (1×10^9 CFU/mL) was sprayed on the seedlings 1 h before inoculation assay initiation as described by Li *et al*., 2004. Lesion formation was examined 7 d post inoculation. The disease incidence was calculated according to the formula described by (Hajano *et al*., 2011):

$$\text{Disease incidence} = \frac{\text{Number of plants infected with the disease}}{\text{Total number of plants studied}} \times 100$$

Greenhouse experiment

Effect of antagonistic yeast isolates on blast disease incidence in the greenhouse

In the greenhouse experiment, each treatment (Table 1) had 15 replications arranged in a randomized complete block design (RCBD).

Table 1. Greenhouse treatments

Treatment	Antagonistic yeasts
1	Sterile water
2	*P. oryzae*
3	CMY045
4	CMY113
5	CMY057
6	CMY018
7	CMY045

Rice stems from 20-day-old plants were collected from the field and cut into small pieces of 1 cm-in-length. Fifteen pieces were placed in 50 ml Erlenmyer flasks and sterilized at a pressure of 1.4 kg/cm2 for 1.5 h. Each flask was inoculated with two 5-mm-diameter mycelial agar plugs of *P. oryzae* and incubated for 15 d at room temperature. The stem piece was placed in a test tube containing 1ml of sterile water, and shaken well to dislodge the conidia (Priya *et al*., 2013). The conidial concentration was 5×10^7 conidia/ml to produce yeast suspensions yeasts were activated in 10 mL of a yeast extract broth medium (3 g yeast extract, 5 g peptone per liter) in 250 mL flasks on a rotary shaker at 150 r/min at 27 ºC for 72 h, and adjusted to a final concentration of 1×10^9 CFU/mL with a haemocytometer. The yeast cell suspensions were sprayed on 25 day-old rice plants 1 h before spray inoculation with the pathogen and sprayed two additional times at 5-day intervals. The disease incidence was recorded after 15 d. The disease incidence was calculated according to the formula described by Hajano *et al*., (2011):

$$\text{Disease incidence} = \frac{\text{Number of plants infected with the disease}}{\text{Total number of plants studied}} \times 100$$

Field experiment

Each field experiment treatment (Table 2) had five replications arranged in an RCBD. The disease incidence was calculated according to the formula described by Hajano *et al*., (2011)

Table 2. Field treatments

Treatment	Antagonistic yeasts
1	Control
2	CMY045
3	CMY113
4	CMY057
5	CMY018
6	CMY045

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) and means were separated according to the least-significant-difference test (LSD) ($P \leq 0.05$).

Results

Symptoms and morphological characteristics of the pathogen

Pyricularia oryzae was isolated from leaf lesions of *Oryza sativa* in Chiang Mai Province. The isolated fungus was purified by the single spore isolation technique and identified by morphological characteristics. The fungal strains were grown on PDA at 26 ºC for 20 d.

Typical symptoms on leaves were diamond-shaped, white to gray lesions. When grown in pure culture, the fungal colony appears white to light gray. When observed under a microscope, the conidiophores are light brown and simple to rarely branched that are moderately long and septated (130–)125–120(118–) × 3–4 µm. Conidia are found sympodially and at the tip (17–)16–21(23–) × (8–)7.6–10(11–)µm, and generally pyriform to obclavate. The the conidia are pale olive to hyaline and they are two septate (Figure 1).

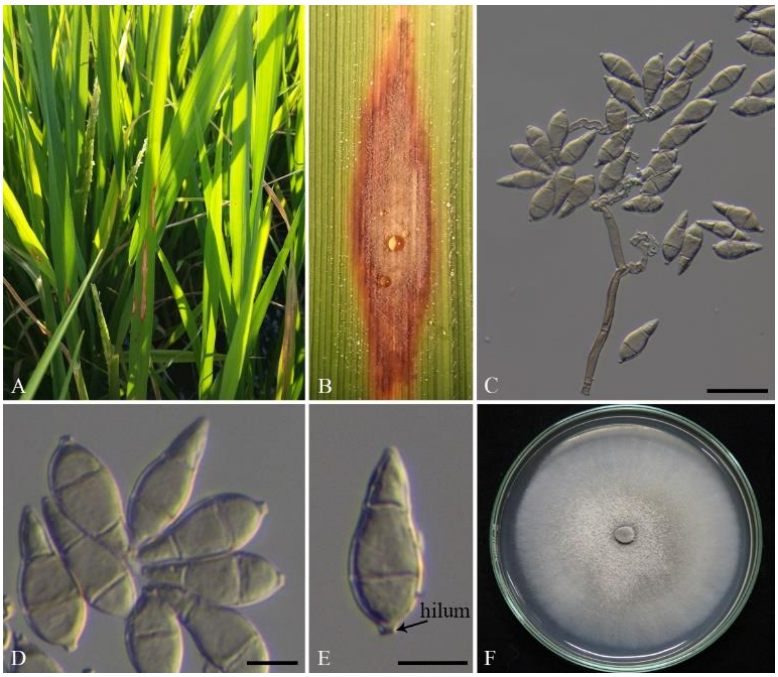

Figure 1. Morphology of *Pyricularia oryzae* which causes rice blast disease: (A–B) Symptoms; (C-E) conidia and conidiophores of *P. oryzae*; (F) Colony of *P. oryzae* on PDA; Scale bars: C - 30 µm, D-E - 10 µm.

Yeast isolation and selection

A total of 63 epiphytic yeasts isolated from various vegetable fruit surfaces and rice leaves were obtained from the microorganism collection of the Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand and kept at 4^{o}C.

Screening of yeasts against Pyricularia oryzae

A preliminary test showed that eight isolates inhibited growth of the fungus by more than 50%. Then the five most antagonistic isolates, CMY047, CMY018, CMY045, CMY113 and CMY057, were selected to examine their efficacy using the dual culture method; these isolates inhibited growth of the fungus by 62.86, 55.17, 54.28, 53.21 and 51.43% respectively (Figure 2).

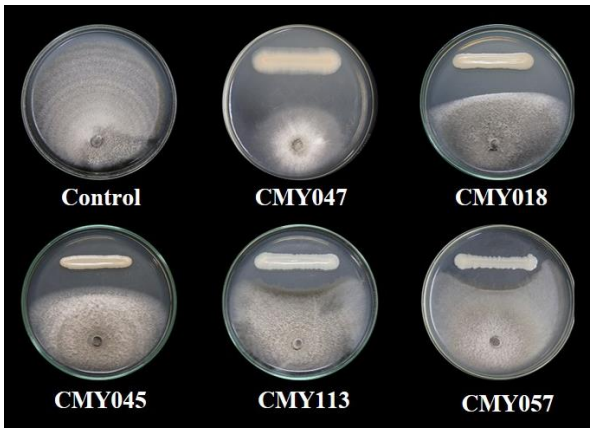

Figure 2. Efficacy of antagonistic yeast against *Pyricularia oryzae* causing rice blast disease by the dual culture technique on PDA.

Inhibitory effect of antagonistic yeast isolates on conidial germination of Pyricularia oryzae on PDA

All yeast isolates suppressed conidial germination at 3-6 h, but all except CMY047 did not significantly reduce conidial germination thereafter (Table 3, Figure 3). However, all the antagonistic yeasts reduced appressorium formation at 6-12h, and length of germ tube at 6-24h (Tables 4 and 5). The lowest appressorium formation at 12h (53.98 %) was produced by both CMY047 and CMY057. CMY045 and CMY018 also produced the lowest average germ tube lengths at 24 h, 54.88 and 55.32 µm, respectively.

Table 3. Effect of antagonistic yeasts on inhibition of conidial germination of *Pyricularia oryzae*.

Treatment	Conidial germination (%)		
	3h	6h	12h
Control	61.04 a	96.41 a	98.30 a
CMY045	49.06 b	78.62 d	96.50 ab
CMY113	40.54 c	80.72 cd	98.04 a
CMY057	50.64 b	84.14 b	97.42 ab
CMY018	50.84 b	79.50 d	96.50 ab
CMY047	49.24 b	82.88 bc	95.48 b
CV%	6.48	2.80	1.98
$LSD_{p=0.05}$	4.24	3.06	2.50

[1]The average of one hundred conidial observations for each treatment.
[2]Means followed by the same letter are not significantly different as determined by LSD, $P \leq 0.05$.

Table 4. Effect of antagonistic yeasts on appressorium formation of *Pyricularia oryzae***.**

Treatment	Appressorium formation (%)	
	6h	12h
Control	68.98 a	84.18 a
CMY045	55.99 d	68.29 b
CMY113	66.39 b	73.59 b
CMY057	27.98 f	53.98 c
CMY018	63.24 c	71.50 b
CMY047	45.18 e	53.98 c
CV%	3.39	6.32
$LSD_{p=0.05}$	2.41	5.59

[1]The average of one hundred conidial observations for each treatment.
[2]Means followed by the same letter are not significantly different as determined by LSD, $P \leq 0.05$.

Table 5. Effect of antagonistic yeasts on length of germ tubes of *Pyricularia oryzae*

Treatment	Length of germ tube (µm)			
	3h	6h	12h	24h
Control	11.60 a	24.81 a	43.91 a	149.20 a
CMY045	10.67 ab	17.70 d	34.67 c	54.88 d
CMY113	9.40 b	18.68 bcd	36.10 c	68.29 c
CMY057	10.45 ab	19.92 b	40.23 b	78.55 b
CMY018	9.69 b	18.28 cd	35.70 c	55.32 d
CMY047	10.19 ab	19.72 bc	38.64 b	60.63 d
CV%	11.56	5.70	3.99	6.35
$LSD_{p=0.05}$	1.56	1.47	2.1	6.45

[1]The average of one hundred conidial observations for each treatment.
[2]Means followed by the same letter are not significantly different as determined by LSD, $P \leq 0.05$.

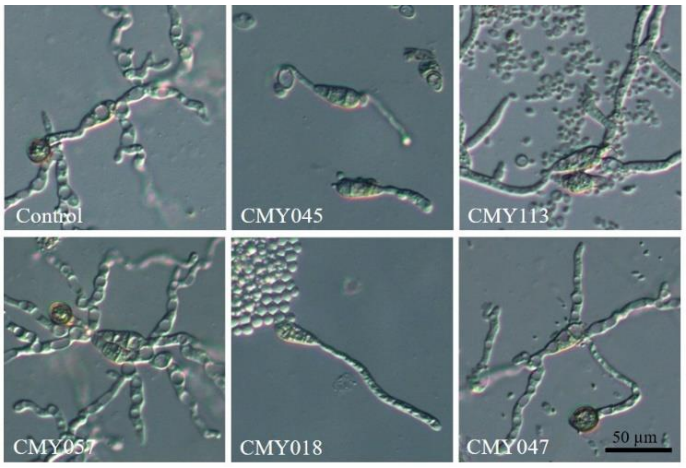

Figure 3. Effect of antagonistic yeasts on inhibition of conidial germination of *Pyricularia oryzae* on PDA at 24 h.

Appressorium penetration assays with onion epidermal cells

In appressorium penetration assays using onion epidermal cells treated with the antagonistic yeasts CMY057, CMY113 and CMY047, appressoria of *P. oryzae* formed in 24 h, but in tissue treated with CMY045 and CMY018 the fungus produced only long germ tubes. When compare with control treatment presence germ tube formed appressoria and produced invasive hyphae inside plant cells (Figure 4).

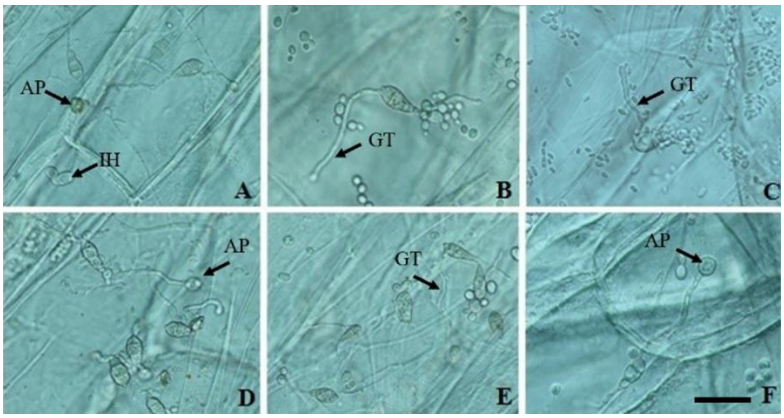

Figure 4. Appressorium penetration assays with onion epidermal cells**.**
Onion epidermal cells inoculated with antagonistic yeasts and examined at 48h
(A) Control (B) CMY045 (C) CMY113 (D) CMY057 (E) CMY018 (F) CMY047**;** AP, appressorium; GT, germtube; IH, infectious hypha. (Scale Bar=10 µm)

Infection assays with detached rice leaves

All isolates of the antagonistic yeasts significantly reduced blast incidence. The lowest disease incidences, 7.98 and 8.96%, were produced by CMY045 and CMY018, respectively (Table 6, Figure 5). Similar results were obtained in the appressorium penetration assays with onion epidermal cells (data not shown).

Table 6. Infection assays with detached rice leaves

Treatment	Disease incidence (%)
Control	32.47 a
CMY045	7.98 d
CMY113	22.08 b
CMY057	23.58 b
CMY018	8.96 cd
CMY047	10.66 c
CV%	11.62
$LSD_{p=0.05}$	2.67

[1]The average was calculated using data from15 replication for each treatment.
[2]Means followed by the same letter are not significantly different as determined by LSD, $P \leq 0.05$.

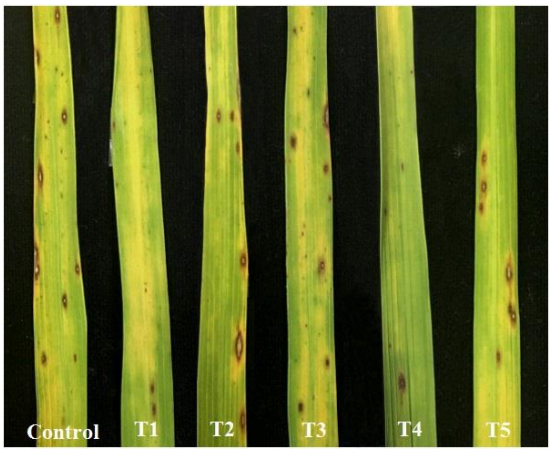

Figure 5. Infection assays with detached rice leaves sprayed with antagonistic yeasts to control rice blast disease. Typical leaves were photographed 7 d after inoculation. T1:CMY045, T2:CMY113, T3:CMY057, T4:CMY018 and T5:CMY04

Greenhouse experiment

All isolates significantly reduced rice blast disease incidence when compared with the inoculated control. After 15 d, isolates CMY045 and CMY018 showed the lowest disease incidence at 15.20 and 17.06%, respectively (Table 7, Figure 6). Similar results were obtained in laboratory experiments.

Table 7. Effect of antagonistic yeasts on rice blast disease incidence in a greenhouse experiment

Treatment	Disease incidence (%)
Sterile water	0.00 e
P. oryzae	80.14 a
CMY045	15.20 d
CMY113	27.07 c
CMY057	63.20 b
CMY018	17.06 d
CMY047	28.60 c
CV%	4.65
$LSD_{p=0.05}$	1.98

[1]The average was calculated using data from15 replications for each treatment.
[2]Means followed by the same letter are not significantly different as determined by LSD, $P \leq 0.05$.

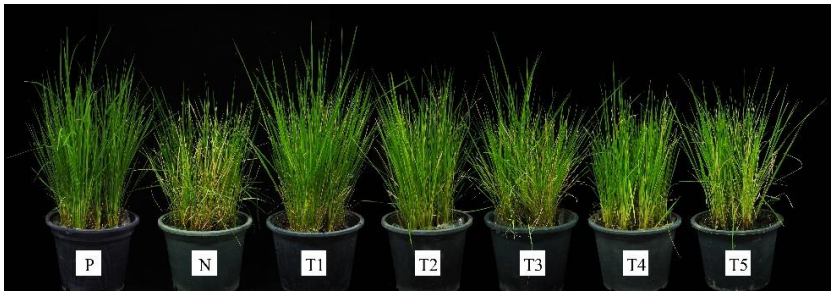

Figure 6. Effect of antagonistic yeast on disease incidence (%) of rice blast disease in greenhouse experiment, P: Sterile distilled water, N: Inoculated with *P. oryzae*, T1: CMY045, T2: CMY113, T3: CMY057, T4: CMY018 and T5: CMY047

Field experiment

All yeast isolates significantly reduced rice blast incidence when compared with the control treatment. Fifty five days after treatment, isolates CMY045 and CMY018 showed the lowest disease incidence at 18.18 and 19.54%, respectively. Likewise, at 70 d, CMY045 and CMY018 produced the lowest disease incidence at 15.74 and 18.12%, respectively (Table 8). These results were similar to those obtained in laboratory and greenhouse experiments.

Table 8. Percentage of rice blast disease incidence in feild experiment.

Treatment	Disease incidence (%)	
	55 days	**70 days**
Control	29.26 a	22.28 a
CMY045	18.18 d	15.74 d
CMY113	27.34 b	20.54 b
CMY057	28.14 ab	21.14 ab
CMY018	19.54 d	18.12 c
CMY047	22.50 c	19.28
CV%	4.67	4.72
$LSD_{p=0.05}$	1.47	1.20

[1]The average was calculated using data from 5 replication for each treatment.
[2]Means followed by the same letter are not significantly different as determined by LSD, $P \leq 0.05$

Discussion

The development of biological techniques using antagonistic microorganisms is an emerging field in crop protection to reduce the economic losses caused by plant pathogens in a biorational manner. The dual culture test of the antagonistic yeasts (CMY047, CMY018, CMY045, CMY113 and CMY057) grown *with P. oryzae*, indicated that the pronounced inhibition of fungal mycelial growth observed was caused by antibiosis, the production of extracellular antifungal substances by the yeasts. The modes of action reported for yeast antagonists of fungal pathogens are antibiosis, nutrient depletion around the sites of pathogen penetration, hyperparasitism with release of cell wall degrading enzymes such as glucanases, chitinases and stimulation of the plant's defense capacity (Harman *et al*., 2004; Bakker *et al*., 2007).

The various growth reductions of *P. oryzae* (mycelial growth, conidial germination, hyphal growth and appressorial growth and penetration) caused by the yeasts may also have been the result of competition for nutrients. It has

been reported that microbial antagonists take up nutrients more rapidly than pathogens, become established and inhibit spore germination of the pathogens at the infection site (Wisniewski *et al*., 1989; Droby and Chalutz, 1994; Droby *et al*., 1998).

Greenhouse and field experiments similarly demonstrated the potential utility of the yeasts in suppressing rice blast. Several antagonistic yeasts have previously been isolated from fruit and vegetables and efficaciously used as biocontrol agents. The phyllosphere yeast, *Rhodotorula glutinis* (strainY-44), isolated from leaves of tomato, has been reported to suppress gray mold (*Botrytis cinerea*) on both leaves and fruit of tomato (Kalogiannis *et al*., 2006). Another yeast, *Kloeckera apiculata* (strain 34-9), isolated from citrus roots, was effective in controlling *Penicillium italicum* and *B. cinerea* on citrus and grapes, respectively (Long *et al*., 2005).

However, there has been no report on the use of yeasts to control rice blast caused by *Pyricularia oryzae*. This study presents the first evidence that certain yeast strains, CMY045 and CMY018, can significantly reduce rice blast. The results suggest that these yeast strains have the potential to be biocontrol agents against *P. oryzae*.

Acknowledgments

This research was supported by Graduate School, Chiang Mai University, and is gratefully acknowledged.

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(Received 11 September 2017; accepted 29 November 2017)