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Fungal Epiphytes for Biological Control of Rice Blast Fungus (*Pyricularia oryzae*)

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Abstract

Fungal epiphytes are microbes residing on plant surfaces. However, their use has not been exploited in contrast to fungal endophytes in which a number of studies had been conducted in relation to plant protection. This study describes the potential of epiphytic fungi that can antagonize the rice blast fungus, *Pyricularia oryzae*. Ten epiphytic fungi isolated from rice leaves were used to challenge the rice blast fungus in vitro. Dual culture test of the fungi showed three modes of antagonism: (1) pathogen mycelia overgrown by fungal epiphytes mycelia [*Penicillium* sp. GM15 and *Fusarium* sp. LM1], (2) suppression by colonizing half of the plate [*Curvularia* sp. GM11, *Penicillium* sp. GT1, GM20, GM7, GT19, and GT27], and (3) through antibiosis as shown by zone of inhibition [*Aspergillus* sp. GT29 and *Penicillium* sp. GM6]. However, only *Penicillium* sp. GM15 and *Fusarium* sp. LM1 had parasitized the mycelia of *P. oryzae* after seven days. Extracellular activities of fungal epiphytes were screened. Eight of ten isolates produced endoglucanase while six of the isolates showed proteolytic activity on plate method. This study proved the potential of fungal epiphytes from rice leaves as biocontrol agents against *P. oryzae*.

Key words – antagonize – endoglucanase – mycoparasitized – proteolytic – zone of inhibition

Introduction

Rice is the staple food in the Philippines. In 2017, 19.28 million metric tons of rice were produced (PSA 2018). However, rice diseases initiated by pathogens are key constrictions in production in most rice-growing areas (Webster 1992). Among the fungal diseases, rice blast is the most serious disease worldwide (Padmanabhan 1974, Ou 1985, Orbach et al. 2000, Singh et al. 2000), and worldwide yield loss was estimated to be 30% of rice production, which could feed 60 million people (Nalley et al. 2016).

The discovery and use of synthetic chemicals has contributed greatly to the increase in food production by controlling insect pests and disease-causing microorganisms. However, their use during the last three decades has raised a number of ecological problems and posed threats to non-pathogenic organisms including humans, animals, and the environment. Therefore, the potential of various biological control methods has been investigated, with varying degrees of success.

Various components of the rice agroecosystem, including water in the paddies, soil, rice, seeds and its plant parts, have been found to be rich sources of antagonists against pathogens of rice such as *Pyricularia oryzae*, *Rhizoctonia solani* and *Fusarium moniliforme*. The phyllosphere, the above

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ground surface of the plants, is a habitat rich in bacteria, fungi, yeasts and algae, all epiphytic microorganisms. Epiphytes are a group of polyphyletic microorganisms found on the surface of leaves, stems or any aerial parts of plants. Fungal epiphytes belong mostly to phylum Ascomycota (Hongsanan et al. 2016). They play a wide range of roles as plant pathogens, natural antagonists of plant pathogens and plant growth promoters. The present study focused on screening potential antagonists from the phyllosphere of different rice varieties grown in different geographical locations in the Philippines, and determining their efficiency in controlling rice blast development under greenhouse conditions.

This study is one of the few works describing the potential of fungal epiphytes in suppressing rice blast disease caused by *Pyricularia oryzae*. In vitro screening of antagonistic fungal epiphytes against *P. oryzae*, mycoparasitism and detection of production of extracellular enzymes were done to identify potential antagonistic epiphytes.

Materials & methods

Collection of tissue samples and isolation of epiphytes

Isolation of epiphytes follows the procedure described by Kennedy & Ercolani (1978) with some modifications. Briefly, fresh and healthy leaves of rice plants were collected from rainfed areas. Five leaves of each plant sample were put separately in a sterile bag and taken back to the laboratory for isolation of epiphytic microorganisms. To isolate the epiphytic microflora, a leaf washing technique was used. A leaf sample was shaken for about 1 hour in 100 ml of sterile distilled water (SDW). The leaf wash (1 ml) was then spread onto malt extract agar (MEA) medium. Resultant colonies were selected, purified and used for further study.

Dual culture test (DCT)

Dual cultures of the antagonist and *P. oryzae* were conducted following the procedure described by Dhingra & Sinclair (1995). MEA culture plates were seeded with 5 mm diam. mycelial discs of each epiphyte and *P. oryzae*. Since the growth *P. oryzae* and the epiphytes are slow, mycelial discs were placed 3 cm apart. Culture plates without antagonist were included as control. Plates were incubated at room temperature. Mycelial growth was measured at 7 and 14 days. Percent growth inhibition was measured using the formula of Lee et al. (2014):

$$\%IRG = \frac{R1 - R2}{R1}\ x\ 100$$

where:
%IRG = Percent of inhibition of radial growth
R1 = Growth of pathogen alone
R2 = Growth of pathogen with epiphytes

Hyperparasitism

Agar blocks (10 mm^2) were cut from potato dextrose agar (PDA) with a sharp and flame-sterilized scalpel. These blocks were placed on opposite ends of a sterile glass slide. Mycelia of *P. oryzae* and epiphytic fungi (EF) that showed potential as biocontrol agents were placed on each of these agar blocks. A sterile cover slip was placed on top of each agar block. The slides were then placed inside sterile petri plates and incubated for 7 days with moistened sterile tissue paper. When visible growth of both the pathogen and the potential biocontrol agents overlapped, cover slips were removed and placed on separate glass slides with a drop of lactophenol cotton blue and sealed for examination under the microscope. Photomicrographs were taken using an Olympus CX23 microscope with attached camera XCAM1080PHB viewed in the application ToupTek ToupView version x64, 3.7.7892.

Extracellular endoglucanase activity

Endoglucanase activity of the potential control epiphytes was detected following the procedure used by Sazci et al. (1986). Five mm discs of the test organism were placed on PDA plates containing 1% (w/v) carboxymethyl cellulose (CMC) and incubated at 23 ^{o}C for 5 days. The plates were then flooded with an aqueous solution of 1% (w/v) Congo red and agitated at 50 rpm for 15 minutes. The plates were flooded with 1 N NaOH. The stained cultured plates were then agitated at 50 rpm for 15 minutes. Changes in the dye colour indicated enzymatic activity particularly inhibition in extracellular endoglucanase. Activities were detected as a visible halo around the colony. Hydrolytic activity was scored as for endoglucanase: (-) no visible zone of hydrolysis; (+) visible zone < 2 mm around the colony; (++) 2–4 mm zone.

Extracellular proteolytic activity

Protease activity was also tested using skim milk agar plates (skim milk powder 28 g, yeast extract 2.5 g, dextrose 1 g, casein 5 g, agar 15 g, water 1 L). Fungal plates were incubated for 3 days. Clear zones of hydrolysis indicated positive for proteolytic activity. Plates were scored as: (-) no visible zone of hydrolysis; (+) visible zone < 2 mm around the colony; (++) 2–4 mm zone.

Results

Dual culture test

In dual culture test, ten promising fungal isolates showed rapid growth of mycelia over or a zone of inhibition against *Pyricularia oryzae*. These fungi consisted of seven isolates of *Penicillium*, and one isolate of *Aspergillus*, *Curvularia* and *Fusarium*. Isolate GM15 covered the whole colony of rice blast fungus while LM1 overgrew half of the colony. Isolates GT29 and GM6 showed a zone of inhibition against *P. oryzae* while isolates GT1, GM11, GM20, GM7, GT19, and GT27 suppressed growth of *P. oryzae* by colonizing half of the plate (Fig. 1). Isolate LM1 gave the highest percent inhibition against *P. oryzae* after seven and fourteen days of dual culture test (DCT) (45.75% and 63.89%, respectively). Isolate GT27 exhibited the lowest inhibition percentage on the 7th day (20.75%) while isolates GT19, GT27, and GT29 showed the lowest inhibition percentage of 35.69% after 14 days (Table 1).

Hyperparasitism

Isolates *Fusarium* sp. LM1 and *Penicillium* sp. GM11 showed hyperparasitism against *P. oryzae* mycelia through coiling and penetration (Fig. 2A, B) of the *P. oryzae* hyphae.

Table 1 Antagonistic activity of potential epiphytic fungal isolates in dual culture test.

Isolate	Percent inhibition of growth	
	7 days*	14 days*
Penicillium sp. GT1	23.59^{bc}	41.67^{cd}
Curvularia sp. GM11	30.66^{b}	50.00^{bc}
Penicillium sp. GM15	26.87^{bc}	54.72^{ab}
Penicillium sp. GM20	27.80^{bc}	53.06^{ab}
Penicillium sp. GM6	25.71^{bc}	36.94^{d}
Penicillium sp. GM7	22.32^{c}	50.14^{bc}
Penicillium sp. GT19	27.12^{bc}	35.69^{d}
Penicillium sp. GT27	20.75^{c}	35.69^{d}
Aspergillus sp. GT29	23.58^{bc}	35.69^{d}
Fusarium sp. LM1	45.75^{a}	63.89^{a}

*Means with different letters are statistically different at 5% level of significance using Turkey's HSD.

Extracellular activities

Ability of epiphytic fungi to inhibit the growth of *Pyricularia oryzae* is correlated to their

ability to produce lytic enzymes. Thus, a plate assay was done to verify the extracellular activities of each isolates. A clear zone of hydrolysis around the colony indicates the presence of extracellular activities (Fig. 3). Of the ten fungal epiphytes, eight isolates produced extracellular endoglucanase and six epiphytes showed extracellular proteolytic activity (Table 2).

Fig. 1 – Antagonistic activity of epiphytic fungal isolates A GT1. B GM11. C GM15. D GM20. E GM6. F GM7. G GT19. H GT27. I GT29. J LM1 against rice blast fungus (*Pyricularia oryzae*) at 14 days of incubation in dual culture test. *Pyricularia oryzae* colony is to the right of each plate.

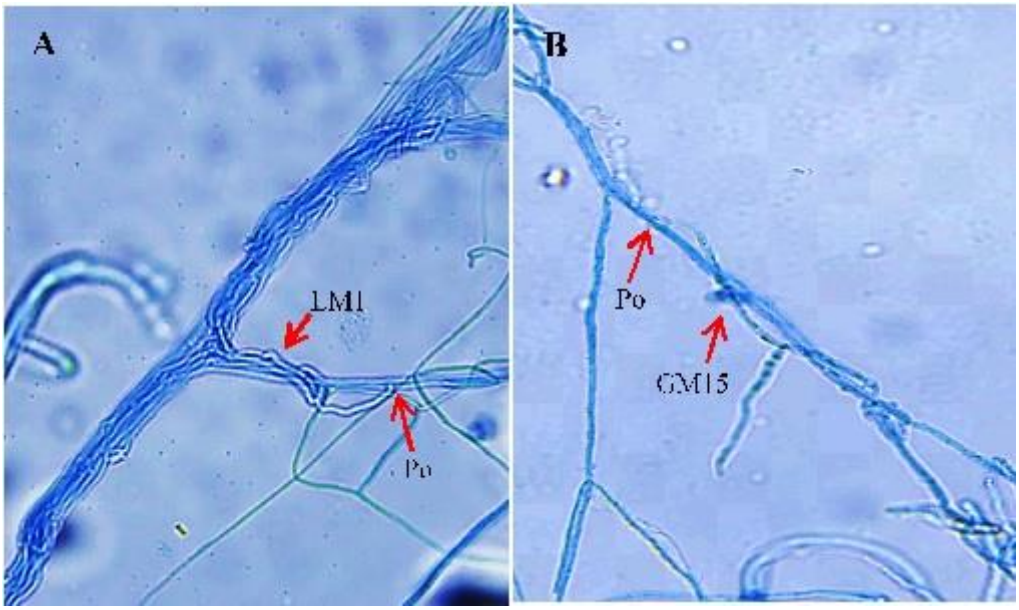

Fig. 2 – Hyperparasitism of *Pyricularia oryzae* (Po) mycelium by A *Fusarium* sp. LM1. B *Penicillium* sp. GM15 after 7 days on agar block set-up.

Table 2 Assay for activity of extracellular endoglucanase and protease for fungal epiphytic isolates.

Isolate	Endoglucanase[a]	Protease[b]
Penicillium sp. DT11	++	++
Curvularia sp. GM11	-	-
Penicillium sp. GM15	++	++
Penicillium sp. GM20	+	-
Penicillium sp. GM6	+	++

Table 2 Continued.

Isolate	Endoglucanase[a]	Protease[b]
Penicillium sp. GM7	++	-
Penicillium sp. GT19	+	++
Penicillium sp. GT27	-	++
Aspergillus sp. GT29	++	+
Fusarium sp. LM1	++	-

[a]Endoglucanase activity seen as zone of carboxymethyl cellulose hydrolysis: (-) no visible halo; (+) <2 mm halo; (++) ≥2 mm halo around the colony.
[b]Degree of proteolytic activity seen as visible halo around the colony on skim milk agar: (-) no visible halo; (+) <2 mm halo; (++) ≥2 mm halo around the colony

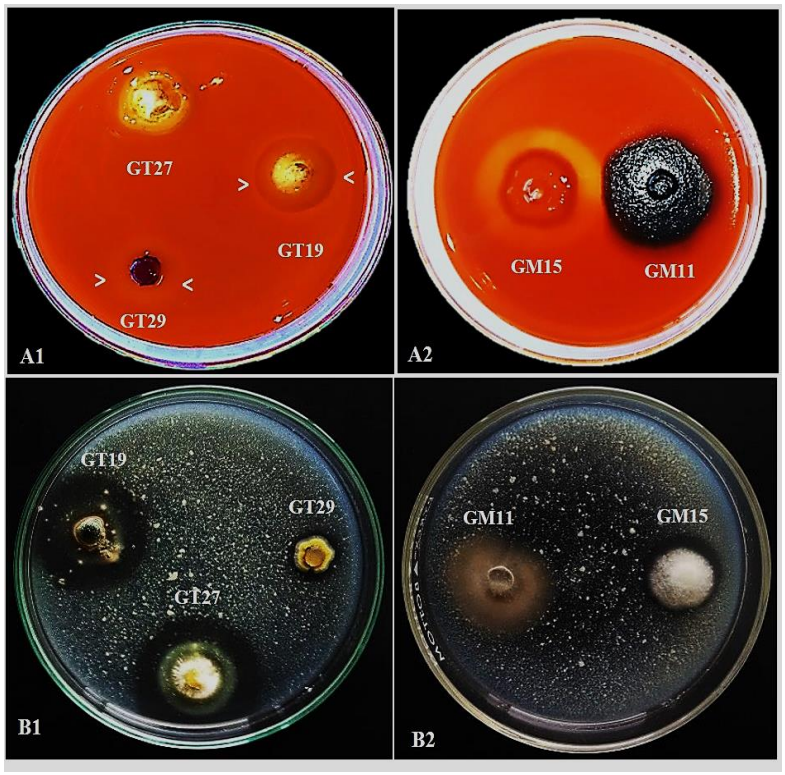

Fig. 3 – Representative plates showing the detection of extracellular activities of (A1 and A2) endoglucanase and (B1 and B2) protease as halo around the colonies of epiphytes.

Discussion

This study proved that fungal epiphytes of rice have potential for biological control against *Pyricularia oryzae*. Previous studies have reported that *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. have antagonistic potential against plant diseases (Lanceta 2010, Sadoral 2010, Naik et al. 2009, Tian et al. 2004). Moreno et al. (2005), Coca et al. (2004) found that *Aspergillus* sp. could control the rice blast fungus. *Hemicarpenteles paradoxus*, the sexual state of *Aspergillus paradoxus* (Sarbhoy & Elphick 1968), controlled anthracnose fruit rot of banana (Kanapathipillai & Jantan 1986). Although many *Fusarium* species cause diseases, Forsyth et al. (2006) reported that a strain of *Fusarium* (BRIP 29089) used against fusarium wilt in Lady Finger and Cavendish banana cultivars interestingly reduced the rate of infection. *Fusarium* strain Fo47 was also reported as a biocontrol agent against fusarium crown and root rot of tomato (Lemanceau & Alabouvette 1991). Yang et al. (2007) isolated *Penicllium oxalicum* strain PY-1 from soil and found that it produced antifungal substances with strong antagonism against plant pathogenic fungi. Sankhala (1968) reported the presence of gliotoxin in *Penicillium restrictum*, while Lumsden et al. (1996) reported that this gliotoxin had a role in fungal antagonism. Although compounds secreted by *Penicillium* spp. avoid

hyphal contact with fungal pathogens, work of Nicoletti et al. (2008) proved that some species of *Penicillium* overgrown the inhibition zone to pair with *R. solani* and *P. ultimum*. Avinash et al. (2015) extracted secondary metabolites of *Curvularia lunata* and showed the antimicrobial potential of *C. lunata* against bacterial pathogens. El Shafie & Webster (1979) observed mycoparasitism of *Curvularia* sp. on *Rhizopus arrhizus.*

In the present study, *Penicillium* sp. GM15 and *Fusarium* sp. LM1 were found to mycoparasitize the rice blast fungus. Donayre & Dalisay (2015) observed mycoparasitism of *Geotrichum* sp. on *R. solani* starting from hyphal contact followed by penetration, coiling and disruption of the pathogen's hyphae. Similarly, Chet et al. (1981) reported that *Trichoderma harzianum* antagonize a pathogens mycelium by dissolving the cell wall through secretions of extracellular enzymes in certain locations followed by hyphal penetration. Kubicek et al. (2001) found that degradation of fungal hyphae is due to the enzymatic hydrolysis and cellular activities of *Trichoderma*. Nicoletti and de Steffano (2012) described the mycoparasitisic activity of *Penicillium restrictum* to coil and penetrate the hypha of fungal pathogens through haustorium-like structures. Arora & Dwivedi (1980) observed mycoparasitism of *Fusarium* spp. on *R. solani* through penetration and coiling.

Extracellular enzymatic activity should be studied to determine the chemical that is released by the epiphytes in antagonizing the rice blast pathogen when applied under field condition. For this study, endoglucanase and protease were determined. Theis & Stahl (2004) reported that β-1,3-glucanase shows antifungal activity when combined with chitinase in vitro. Likewise, Selitrennikoff (2001) discussed that the hydrolysis of structural β-1,3-glucan, present in fungal cell wall, is the one responsible for the antifungal effect of β-glucanase. Moy et al. (2002) stated that degradation of fungal cell wall is due to the role of β-1,6-glucanase. Fuchs et al. (1997) inoculated strain Fo47 of *Fusarium* sp. in tomato which increases the chitinase, β-1, 3-glucanase, and β-1, 4-glycosides activity in plants. Studies of Pozo et al. (2004) suggested that secretion of protease by *Trichoderma* increases the efficiency of controlling *Rhizoctonia solani*. Gopinath et al. (2005) screened fungi for proteolytic activity. Among the screened fungi, only *Aspergillus versicolor* had shown a very strong activity of protease using gelatin medium flooded with ammonium sulphate. Several *Penicilium* spp. and *Fusarium* spp. were found to have extracellular protease on gelatin agar (Gopinath et al. 2005). Moscoso & Rosato (1987) reported that highest values of protease were obtained by diploid and heterokaryon strains of *Aspergillus nidulans* in solid medium. Strong extracellular enzymatic activity exhibited by *Epichloe* isolates was observed by Niones & Takemoto (2014). However, even other *Epichloe* isolates with no inhibitory activity exhibited extracellular endoglucanase and protease activities.

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