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Pesticides and Chemical Fertilizer are not Negatively Impact the Diversity of Entomopathogenic Fungi on Rice Plant in Malang Indonesia

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Abstract

The conventional system of rice plantation has been applied in Malang, Indonesia. Standard chemical fertilizer and pesticide application were used in this field. Soil samples in rhizosphere of rice plantation showed the existence of several entomopathogenic fungi including *Penicillium* sp, *Aspergillus* sp, *Trichoderma* sp and some unidentified fungi. The diversity value also demonstrated a medium diversity. We conclude that an application of pesticides and chemical fertilizer according to recommended practices, are not negatively affect the diversity of entomopathogenic fungi. *Aspergillus* sp and *Penicilium* sp can cause death against *Spodoptera litura*.

Keywords: Rhizosphere, Entomopathogen Fungi, Conventional System of Rice

INTRODUCTION[1]

Rice is one of staple food in Asia including Indonesia. Indonesia ranks 4th for rice production [1]. The rice field area in 2021 is estimated at 10.52 million hectares, a decrease of 141.95 thousand hectares or 1.33 percent compared to the rice field area in 2020 which was 10.66 million hectares. In 2021 is estimated at 55.27 million tons of Dried Milled Grain (GKG), an increase of 620.42 thousand tons or 1.14 percent compared to rice production in 2020 which was 54.65 million tons of GKG. In 2021, food consumption is estimated at 31.69 million tons, an increase of 351.71 thousand tons or 1.12 percent compared to rice production in 2020 which amounted to 31.33 million tons [2]. The amount of rice production is calculated based on the overall yield in the form of Dried Milled Grain (GKG). GKG is rice grain which has a maximum moisture content of 14 percent or has gone through the drying process.

The rice production can be concluded that the right land cultivation to make high production aimed at sufficient food. Its production is very much depending on the cultivation process and environmental condition as well. Various ways of applied cultivation aim to increase the production of rice crops. The use of fertilizers and pesticides cannot be separated from farmers' choice to increase their production and reduce pest populations. Pesticide used should be adjusted with the dose recommendation since an excessive use of pesticides can cause toxicity, lead to pest-resistance to pesticides and pesticide residue in agricultural sites including soil and irrigation water [3]; [4]. The environmental pollution caused by continues agricultural activity that rely on chemical fertilizers and synthetic pesticides. The application of conventional system can be found in several regions of Indonesia [5].

Soil is the most important habitat for entomopathogenic fungi, which can contribute significantly to the population control of insects in the ecosystem [6]. Microbial community decompose complex organic matters in the soil which then convert them as nutrients to be utilized by plants [7], thus the existence of microbial communities in soil play important role in nutrients availability. Other than that, soil and rhizosphere also function as habitat for various natural enemies of pests including entomopathogenic fungi which are widespread in agro-ecosystems of temperate and semi natural habitats [8]; [7]; [9]. Entomopathogenic fungi have been viewed as excellent biocontrol agents

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in the regulation of phytophagous insect and mite populations in agroecosystems [10]. The pest control efficacy of various entomopathogenic fungi species has been reported in *Hypocreales* (Ascomycota), but not limited to *Beauveria* [11].

The process of decomposition increased the abundance of entomopathogenic fungi, which are negatively impact the insect population such as Aphids on the above ground and soil fauna such as microfauna, bacterivorous, fungi plant feeding nematodes on the under ground [12]. Several species of fungi found in both under and above ground are potentially functioning as entomopathogenic fungi [9]; [13]. Several entomopathogenic fungi stimulate plant growth and inhibit bacterial pathogens in soil [8]. However, information regarding beneficial fungi in rhizosphere of rice plantation, especially entomopathogenic fungi are poorly understood.

In this paper, the diversity of entomopathogenic fungi in rhizosphere of rice plants in the conventional system in Malang, Indonesia were studied. Identification of entomopathogenic fungi as well as their virulence are also discussed.

MATERIAL AND METHOD

Study Area

The data collection was conducted in Malang, Indonesia. The rice cultivation practice were conducted according to standard recommendation application as described in Table 1.

Application of Pesticides

The systemic pesticides used in rice cultivation consists of 3% carbofuran. Application of pesticides used was according to those recommended by ministry of agriculture in Indonesia (Table 1). Carbofuran also has contact activity against pests. It is one of the most toxic pesticides still in use. It is marketed under the trade names Furadan. Carbofuran 3G applied after 1 month old rice [14].

Soil Sampling

In order to identify the diversity level of entomopathogenic fungi in rice rhizosphere, soil samples were collected at 3 months old rice in near the roots of rice plants using a trowel. Soil samples were taken at 5 points (25 gram soil) with diagonal system [15]. Rhizosphere soil section was taken with a depth of 30 cm [16]. Soil samples are a composite from 5 points of experiment area. The diversity index is calculated by the formula Shannon Wienner index [17]:

$$H' = -\Sigma \, pi \, In \, pi$$

Description:
H' = diversity index
pi = comparison of the number of individuals of one species with the number of individuals of the entire sample in the plot (n/N)

Isolation of Fungi

Isolation of the fungus was carried out by the dilution plate method [7]. For each land location, 25 g of soil samples were taken and dissolved with 225 ml of peptone and then in fortex, a dilution of 10^{-1} was obtained. Suspension of 10^{-1} dilution is taken 1 ml and dissolved with 9 ml of peptone then in fortex then obtained dilution 10-2. The dilution continues until the dilution level is 10-5. Before culturing in the Sabouraud Dextrose Agar Yeast Extract (SDAY) media, each dilution was carried out fortex to dissolve. Each level of dilution was taken 0.1 ml then cultured in SDAY media in petri dishes and then leveled. The dilution rate ranges from 10^{-5} to 10^{-1}, as it reduces the deposition of the tip in the micropipette. Then it was incubated for 3 days in an incubator at 35°C. The dilution activity was carried out in a Laminar Air Flow Cabinet (LAFC). After incubation, observations were made on each plate. If there is more than one fungal colony growing on one petri dish, then purification is carried out. Purification aims to separate each macroscopically different colony. The mushrooms were cultured again on SDAY media in tubes and incubated for 3 days in an incubator at 35°C. After the SDAY media in the tube was pure, then it was purified into SDAY media in a cup. Then it was incubated for 7 days in an incubator at 35°C. Purification activities are carried out in LAFC.

Observation Parameter

Macroscopic and Microscopic Observations

The purified and prepared isolates of entomopathogenic fungi were then observed macroscopically and microscopically and identified with the guidelines for fungal identification, namely [16]. Macroscopic observations were carried out on the 7th day after inoculation by observing the morphology

Table 1 Land use and rice plantation practice

Description	Land use and rice cultivation practice
Elevation	224 m asl
Geographical Location	S 07°46'12.1" and E 112°17'43.7"
Crop	Rice
Area	312 m^2
Variety	Ciherang
Fertilization	100 kg ha^{-1} za (1 times for each crop), 150 kg ha^{-1} urea (2 times for each crop: 50 kg ha^{-1} and 100 kg ha^{-1}), 300 kg/ha Phonska (2 times for each crop: 150 kg/ha), petroganik 300 kg/ha (1 times for each crop). Applications based on Ministry of Agriculture, 2014 about anorganic fertilizers usage recommendation
Pesticide	Carbofuran 3% at a dose of 20 kg ha^{-1}. Application based on standart brand of pesticide. 224 m asl need 0,5 kg of Carbofuran and 2 times for each crop.
Production (unhulled rice)	250 kg

Table 2 Index of Shannon Diversity

Index Value	Description
< 1	Low diversity, low distribution of the number of individuals per species
1-3	Medium diversity, the distribution of the number of individuals of each species is moderate
>3	High diversity, the spread of the number of individuals per species is high

of the fungal colonies which included colony color, colony surface texture (velvet, powder, cotton and cotton, wrinkled), colony distribution pattern in petri dishes (concentric and not concentric), colony diameter (cm).

Microscopic observations were carried out by observing the morphology of fungal colonies using a microscope which included hyphae shape, conidia shape, conidia distribution pattern, description of fungal parts. Observations were made using 1000x magnification. Observation of the presence or absence of septa on hyphae is done by observing the presence or absence of a septum (cross line). The septa on the hyphae can be seen tightly or rarely. Conidia can be round, oval, elliptical, oval or irregular in shape. Conidia distribution patterns such as clustered at the end of the conidiophores or clustered around the hyphae, spread, single, chained or not chained, as well as the form of conidia collections. Conidia collections are often seen in various shapes, such as round, radial (irregular), resembling the shape of a flower, and etc. Observations of conidiophores include the presence or absence of septa on conidiophores (insulated or not insulated), and the growth of conidiophores (branched or unbranched, long or short).

RESULTS AND DISCUSSION

Genera of Entomopathogenic Fungi Present in Rice Rhizosphere

Based on fungi identification in soil collected from rhizosphere around rice plants in Malang, eleven fungal isolates group were observed. Eleven groups of fungal isolates were identified and resulting three different genera of fungi including *Penicillium* sp., *Aspergillus* sp., and *Trichoderma* sp were collected. Macroscopic and microscopic morphological characteristics were analysed as presented in Figure 1-4 and table 2.

- ***Aspergillus* sp. (isolates b, c, e, f, h, k)**

Isolates b, c, e, f, h and k macroscopically showed yellowish to dark brown with a white edge (Figure 2a). Meanwhile microscopically showed conida (round-egg), fialid, Metula, vesicles, conidiophores, and hyphae (not insulated) (Figure 2b). The characteristics of this fungus colony also showed in table 2. For the colony *Aspergillus* sp. 1, its top-colored was black white edge and colonies reserve gray white edges, characteristic of surface colonies were powder ledges wrinkled. The colonies could grow to reach 9 cm and have oval conidia. The conidiophores were fialid and metula. The branching type was monoverticillate and hypha was not insulated. Meanwhile in *Aspergillus* sp.2, the top-colored was white, black powder and colonies reserve white, For *Aspergillus* sp. 3, the top-colored was dark brownish and colonies reserve dark brown whitish gray edges, characteristic of surface colonies were powder. The colonies could grow to reach 7,2 cm. For *Aspergillus* sp. 4 top-colored was white green

edge and colonies reserve yellow dark green edged, light brown, and characteristic of surface colonies were powder. The colonies could grow to reach 2,7 cm. For *Aspergillus* sp. 5, the top-colored was brownish white and colonies reserve white and characteristic of surface colonies were wrinkled velvet edges. For *Aspergillus* sp. 6, the top-colored was white edge yellow and colonies reserve white edge yellow and characteristic of surface colonies were cotton edge powder. These colonies could grow to reach 7,6 cm. characteristics of conidia, conidiofor, branching, fialid, metula and hypha for *Aspergillus* sp 2, 3, 4, 5, and 6 showed similar to those on *Aspergillus* sp 1. Morphological identification of *Aspergillus* species from corn grain in Penang Island, Peninsular Malaysia has characteristics black colony, biseriate conidial heads and small conidia (2,9 – 3,9 µm) [7].

- ***Penicillium* sp. (isolates a, d, g, l)**

Macroscopic result of isolates a, d, g, l showed that those isolates were included in genus *Penicillium* sp since the conidia were green and white edge (Figure 1a). This result was similar with the previous research that colony of *Penicillium* sp. has a green color, sometimes white, mostly have conidiophores. [11] also demonstrated that the *Penicillium* sp. found in land of Himalayas, India has the characteristic green color of top and bottom the white and the type of uniform spherical distribution.

In microscopic result, those isolates had konida (round-egg), fialid, conidiophores, hyphae (sectional) and metula (Figure 1b). A detail characteristics showed in table 2. For *Penicillium* sp. 1, its top-colored was old green edge white and colonies reserve white yellow, and surface colonies were velvet edges wrinkled. Diameter of *Penicillium* sp. 1 was 3,2 cm and had oval conidia. The colonies had conidiofor, fialid and metula. The branching type was monoverticillate and hypha was insulated. For *Penicillium* sp. 2, its top-colored was old green edge white and colonies reserve white yellow, and surface colony was velvet edges wrinkled. Diameter of *Penicillium* sp. 2 was 1,9 cm. For *Penicillium* sp. 3, its top-colored was green edge white and colonies reserve green edge white, and surface colonies were velvet. Diameter of *Penicillium* sp. 3 was 6,45 cm. For *Penicillium* sp. 4, its top-colored was green edge white and colonies reserve white yellow, the surface colony was velvet. Diameter of *Penicillium* sp. 4 was 1,15 cm. The characteristic of shape of conidia, conidiofor, branching, fialid, metula and hypha for *Penicillium* sp 2, 3, and 4 were similar to those on *Penicillium* sp 1.

According to [18], the characteristics of *Penicillium* sp. including red white color, with brush-like shape, having mycelium with septate shape and fruiting body shape is conidia in chain. According to [19], that single conidiophores (mononematus) or compound (synematous), consisting of a single rod share some phialid (simple / monoverticillata). All cells among Metula and potentially stem become a branch. Branching one level (biverticillata-symmetric), branching two levels (biverticillata asymmetric / terverticillata), three kinds or more tiers of branches (quaterverticillata). Phialid is a structure that sustains conidia, cylindrical basal section that narrows at neck, or lancoelate (more or less most part embedded in the tip of basal shoots). Conidia form long chains, divergent or column, globular, elliptical or fusiform, transparent or greenish, with walls smooth or bumpy. Thus, based on the result, it was confirmed that a, d, g and l isolates are *Penicillium* sp.

- ***Trichoderma* sp. (isolates i)**

A *Trichoderma* sp. was confirmed based on observation on isolate i. This isolate had yellow and green edges of yellow and white edge of conidia (Figure 3a). *Trichoderma* spp. from New Delhi has colour from whitish to greenish (either dark or light) during the growth period. After 7 days, culture plates were observed to be dark green in colour and all the isolates [20]. Microscopic data showed that genus *Trichoderma* has conida (round-elliptical), fialid, conidiophore, hyphae (not insulated) (Figure 3b). A more detail characteristic of this fungus was presented in table 2. In *Trichoderma* sp. 1, its top-colored was yellow green edge, yellow white. The colour of colonies reserve were gray white edges, characteristic of surface colonies were cotton edge powder. These colonies could grow to reach 9 cm and had ellipse conidia. The colonies had conidiofor, fialid and metula. The conidia were branched and the hyphae were not insulated. According [21] stated that Trichoderma spp. on organic potato plant rhizosphere Indonesia has a smooth-walled conidia, first a colony of hyaline, then becoming greenish white, and then dark green, especially on the show there are many conidia.

Conidiophores can be branched resembles a pyramid that is at bottom lateral branches are repeated, while getting to the end of branching are getting shorter. Phialid look slim and long, especially at the apex of the branches. conidia round to oval shaped a short spring.

- **Unidentified (isolates j)**

Isolates j is group of fungi that have not been identified because it only had conidiophore and conidia (Figure 4b) and is macroscopically white velvet and wrinkled (Figure 4a). Detailed information regarding this fungus showed in table 3. For the colonies of unidentified fungi, its top-coloured and its colonies reserve were white, characteristic of surface colony was velvet edges wrinkled. This colony could grow to reach 8,25 cm and had ellipse conidia. The conidia were branching and had no fialid, metula and hyphae. It was possible that this isolate was included in sterilia mycelia. According to [22], unidentified fungi have mycelium characteristics such as cotton, white or yellow or black, grow fast and some are slow. However further identification is needed.

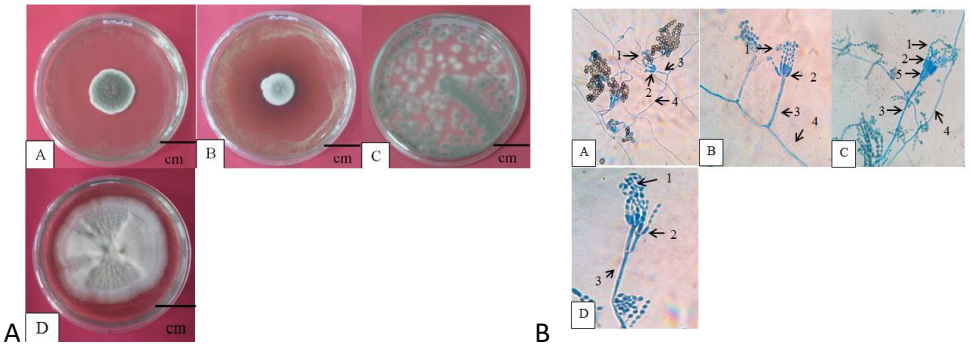

Figure 1 Macroscopic and microscopic (magnification 1000x) of *Penicillium* sp. A. *Penicillium* sp. 1; B. *Penicillium* sp. 2; C. *Penicillium* sp. 3; D. *Penicillium* sp. 4 ; E. *Penicillium* sp. 5

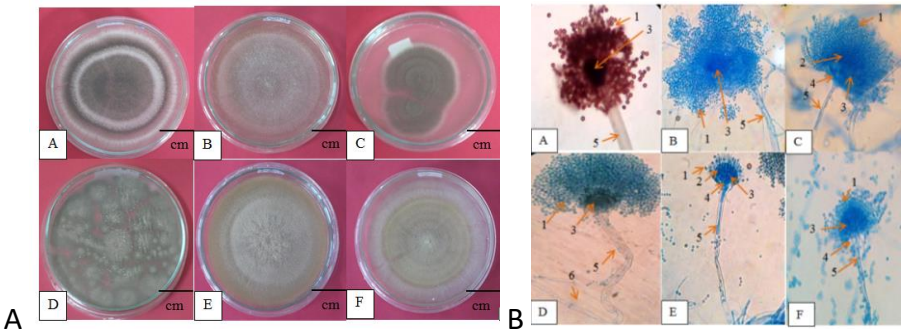

Figure 2 Macroscopic and microscopic (magnification 1000x) of *Aspergillus* sp. A. *Aspergillus* sp. 1; B. *Aspergillus* sp. 2; C. *Aspergillus* sp. 3; D. *Aspergillus* sp. 4; E. *Aspergillus* sp. 5; F. *Aspergillus* sp. 6

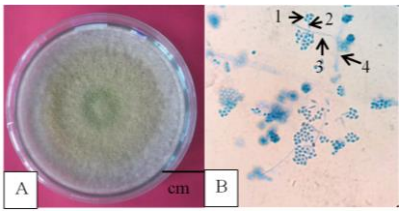

Figure 3 Macroscopic and microscopic (magnification 1000x) of *Trichoderma* sp.: A. Macroscopic; B. Microscopic (magnification 1000x) (1) Conidia; (2) Fialid; (3) Conidiophore; (4) Hyphae

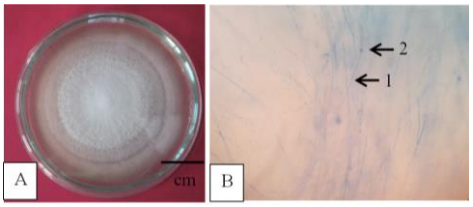

Figure 4 Macroscopic and microscopic (magnification 1000x) of Unidentified: A. Macroscopic of top view; B. Microscopic (magnification 1000x) (1) Conidiophore; (2) Conidia

Table 3. Morphology of macroscopic and microscopic isolate fungi

Observation	**IsolateA**	**Isolate B**	**Isolate C**	**Isolate D**	**Isolate E**	**Isolate F**	**Isolate G**	**Isolate H**	**Isolate I**	**Isolate J**	**Isolate K**	**Isolate L**
• **Colonies on Medium SDAY**												
- Colour	Old Green Edge White	Black White Edge	White, Black Powder	Gray White Edges	Dark Brown Old Chocolate	White Green Edge	Green Edge White	Brownish White	Yellow Green Edge, Yellow, White	White	White Edge Yellow	Green Edge White
- Colony Reserve	White Yellow	Gray White Edges	White	Black Edge Redness	Dark Brown Whitish Gray Edges	Yellow Dark Ggreen Edges, Light Brown	Green Edge White	White Yellow	Gray White Edges	White	White Edge Yellow	White Yellow
- Surface Colony	Velvet Edges Wrinkled	Powder Ledges Wrinkled	Powder	Velvet	Powder	Cotton Edge Powder	Velvet	Wrinkled Velvet Edges	Cotton Edge Powder	Velvet Edges Wrinkled	Cotton Edge Powder	Velvet
- Diameter (cm)	3,2	Full plate (9)	Full plate (9)	1,9	7,2	2,7	6,45	Full plate (9)	Full plate (9)	8,25	7,6	1,15
• **Conidia**	√	√	√	√	√	√	√	√	√	√	√	√
- Shape	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Oval	Ellipse	Ellipse	Oval	Oval
• **Conidiofor**	√	√	√	√	√	√	√	√	√	√	√	√
• **Branching**	Monoverticillate	Monoverticillate	Monoverticillate	Monoverticillate	Monoverticillate	Monoverticillate	Monoverticillate	Monoverticillate	Branched	Branched	Monoverticillate	Monoverticillate
• **Fialid**	√	√	√	√	√	√	√	√	√	-	√	√
• **Metula**	√	√	√	√	√	√	√	√	-	-	√	√
• **Hypha**	Insulated	Not-Insulated	Not-Insulated	Insulated	Not-Insulated	Not-Insulated	Insulated	Not-Insulated	Not-Insulated	-	Not-Insulated	Insulated
Genus	*Penicillium* sp. 1	*Aspergillus* sp. 1	*Aspergillus* sp. 2	*Penicillium* sp. 2	*Aspergillus* sp. 3	*Aspergillus* sp. 4	*Penicillium* sp. 3	*Aspergillus* sp.5	*Trichoderma* sp. 1	Not Identified	*Aspergillus* sp. 6	*Penicillium* sp. 4

The Entomopathogenic Fungi Shows Medium Diversity

In order to find out the diversity status of entomopathogenic fungi based on dilution plate analysis in rhizosphere area in rice paddy plantation using conventional system, calculation of fungal diversity index was conducted. The result showed a medium diversity values (1.13) as shown in Table 4. The calculation of evenness index showed a high category on *Aspergillus* sp and *Penicillium* sp which demonstrated value of 5.17 and 3.79, respectively. Whereas a low evenness index categories showed by *Trichoderma* sp. and unidentified isolates (0.00). A medium diversity index and high evenness index on *Aspergillus* sp and *Penicillium* sp might be is most dominant fungi in rice field. Aflatoxins are carcinogenic metabolites produced by several strains of *Aspergillus* sp and *Penicillium* sp group in food and feed. Aflatoxin contamination is one of the main factors affecting rice seed quality [20]. The medium index here still needs further testing by comparing other organic or conventional soils, the figures obtained from *Aspergillus* and *Penicillium* are based on Shannon Wiener's analysis.

Thus, no excessive usage of both chemicals applied in rice cultivation site. Similar to previous research, an application of nitrogen fertilizer on field, when applied according to recommended practices, are unlikely to negatively impact survival of B. bassiana in pecan orchards when the fungus is applied for Curculio caryae suppression during weevil emergence [21]. Urea had no significant affect on the fungus. Therefore, it seems that certain factors in manure that reduce survival of *Beauveria bassiana* are lost during decomposition [23].

However, a very low evenness index in *Trichoderma* sp. and unidentified isolates might be caused by there was no organic fertilizers such as compost or other organic manure which simultaneously applied in the rice field at all for several years. According to previous research in rhizosphere of *Chrysantemum* and other temperate crops showed that application of liquid organic fertilizers and manure compost can help to enhance the microbial diversity [22]; [23]; [24]. Evenness index values are influenced by the number of species present in the community. A type which has a high degree of stability has a greater opportunity to maintain the sustainability of its kind. To assess the stability of species in a community may use equity index value (E). The higher the E value, then more stable the diversity of species in the community, on the contrary, the lower the E value, then the less stable the diversity of species in the community. Based on this result we may suggest that in order to enhance entomopathogenic fungi diversity in rhizosphere of rice plant, application of organic compound such as organic fertilizers and manure composts are necessary.

Table 4 Value of index diversity (H ') and evenness index (E) fungi from rhizosphere of rice plants with conventional systems

Fungal Isolates	Total of Species	pi	ln (pi)	H’	E
Penicillium sp.	4	0,33	-1,10	0,37	3,79
Aspergillus sp.	6	0,50	-0,69	0,35	5,17
Trichoderma sp.	1	0,08	-2,48	0,21	0,00
Not Identified	1	0,08	-2,48	0,21	0,00
Total (∑)	12			1,13	

CONCLUSION

The diversity index value of entomopathogenic fungi was classified as medium category. It demonstrated that an application of pesticides and chemical fertilizer according to recommended practices, are not negatively impact the diversity of entomopathogenic fungi. However, application of organic fertilizers as well as manure compost is suggested thus it can improve the entomopathogenic diversity. Identified fungi found in rhizosphere of rice plant were *Penicillium* sp, *Aspergillus* sp, *Trichoderma* sp and some unidentified fungi. However, *Aspergillus* sp had the highest virulence among others. Soil acidity and the low soil fertility are barely impact the diversity of entomopathogenic fungi.

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