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Exploration and pathogenicity test of Entomopathogenic fungus from brown plant hopper (*Nilaparvata lugens* Stal) Pest

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ABSTRACT

Brown planthopper (*Nilaparvata lugens* Stall.) is the main pest of rice in several countries in the East and Southeast Asia regions. Banyumas Regency is one of the endemic areas for brown planthopper in Indonesia Entomopathogenic fungi have the potential to control brown planthopper naturally. The study aims to obtain entomopathogenic fungi isolates that effectively control *Nilaparvata lugens*. The study was conducted with exploratory studies to obtain fungal isolates, followed by experimental research. Exploration was carried out in seven sub-districts, five villages were taken for each sub-district, and five rice fields were taken for each village. The results of the exploration found 66 fungi isolated from infected brown planthopper. Exploration isolates of each sub-district were then tested on brown planthopper using RCBD with three replications on a plastic house scale. The variables observed were mortality and time of death of brown planthopper. Data were analyzed by 5% F test if there were significant differences followed by 5% Duncan's Multiple Range Test. The test results found 8 of the best isolates, namely isolates J11 (*Aspergillus* sp.), J22 (*Paecilomyces* sp.), J34 (*Beauveria* sp.), J35 (*Beauveria* sp.), J41 (*Fusarium* sp.), J56 (*Fusarium*sp), J60 (*Paecilomyces* sp.) and J65 (*Curvularia* sp.). These fungus have the ability to kill brown planthopper ranges from 70–80% with a time of death ranges from 3.43 to 4.87 days.

Key words : *Exploration, Entomopathogenic fungi, Nilaparvata lugens, Pathogenicity*

Introduction

Brown planthopper (*Nilaparvata lugens* Stall.) is the main pest of rice in several countries in the East and Southeast Asia regions. Since 1968, brown planthopper (BPH) has severely damaged the rice industry in China and in other Asian countries (Hu *et al*., 2011; Hu *et al*., 2014). The frequency of BPH attacks has increased in Asian developing countries in 2005-2012. This is due to the killing of natural enemies due to the use of broad-spectrum insecticides (Phiyaphongkul *et al*., 2012; Ali *et al*., 2014).

In Indonesia, BPH is still the main pest that can potentially be a threat or inhibit the increase of national rice production. Banyumas Regency is one of the endemic areas of BPH in Indonesia. In the dry season (April-September) 2017, the intensity of attacks and the BPH population in Banyumas Regency were above the economic threshold. The intensity of BPH pest attacks ranged from 6.96 to 23.58%, while the population of BPH ranged from 0.84 to 27.36 individuals per clump. The high intensity of attacks and the population of BPH pests in Banyumas Regency is caused by continuous rice planting, planting is not synchronous, BPH resistance to insecticides made from imidacloprid,

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chlorpyrifos and fipronil as well as excessive insecticide applications (Minarni *et al*., 2018). BPH populations originating from Banyumas, Bantul, Bandung, Cianjur, and Serang which have a resistance level of 2-7 times against MIPC, also showed a decrease in AChE sensitivity to inhibition by MIPC of 2-4 times (Sutrisno, 2014)

In addition to the factors above climate changes are also considered as the main cause of the BPH pest explosion. Climate changes that are characterized by the occurrence of wet droughts cause differences between the rainy season and the dry season to be unclear. This can create cloudy weather, rising temperatures and high humidity. This condition is very suitable for the development of the BPH (Ali *et al*., 2014; Baehaki, 2011).

Entomopathogenic fungi play an important role as biological control agents for insect pest populations. Many species of entomopathogenic fungi have been known to kill BPH but have no effect on their natural enemies (Robert *et al*., 1992; Maketon *et al*., 2015). Entomopathogenic fungi can produce asexual spores (conidia) and resting spores (chlamydospora) that allow survival in the absence of new hosts. Both conidia and chlamydospora, both of which can cause natural epidemics or epizootics in vulnerable hosts (Shahid *et al.,* 2012; Gul *et al.,* 2014). The number of fungi spores will continue to increase and produce toxins that will kill insect pests. Entomopathogenic fungi also produce protease, chitinase and lipase enzymes to degrade insect cuticles. Then the fungus will come out of the insect cadaver. If temperature and humidity are appropriate, spores will spread to their environment. Some species of entomopathogenic fungi form resting spores when there is unfavorable environmental conditions. The resting spores will be able to infect the host if the environmental conditions are appropriate (Shahid *et al*., 2012; Gul *et al*., 2014; Safavi *et al*., 2010).

Exploration is the first step in implementing biological control to obtain fungal isolates that have a high killing power against BPH, which can then be developed and propagated as biological agents. Exploration activities were carried out by collecting BPH which was suspected of being infected with entomopathogenic fungi in six sub-districts in the Banyumas Regency area which was an endemic area for BPH. The six sub-districts are Sumpiuh, Karanglewas, Cilongok, North Purwokerto, East Purwokerto, Banyumas and Kebasen. The seven sub-districts have the same cultivation activities, namely planting rice continuously throughout the year, planting time is not simultaneous, excessive use of pesticides regardless of the population of pests and natural enemies and the average intensity of BPH attacks that were high in the previous planting season (Minarni *et al*., 2018). Furthermore, the fungus that infected the BPH was isolated and applied to healthy BPH on a laboratory scale.

It is expected that from this exploration and pathogenicity test, indigenous fungal isolates will be obtained which are effective for controlling BPH pests. The availability of virulent indigenous fungal isolates is a determining factor for the success of the biological control program, therefore the collection of indigenous strains and virulence determinations is the starting point for the implementation of biological pest management. Determination of the genetic diversity of fungal strains has also become something that is needed in biological control research, given that natural variability is the main source of new genotypes that have the potential as biopesticides.

Materials and Methods

The study consists of two stages. The first stage was exploratory research in the endemic areas of BPH pests in the Banyumas Regency and the second stage was the pathogenicity test of the entomopathogenic fungal isolates explored against the mortality of BPH.

Exploration research was carried out using survey methods. The research sample was taken using purposive random sampling method. Banyumas Regency is an endemic area for BPH. Seven out of 27 sub-districts showing the highest intensity of BPH pest attacks were chosen. Each sample sub-district was taken 5 villages and each village was taken 5 plots of rice fields. 10 clumps of rice were taken in each paddy field, and each clump was observed to get BPH infected with fungi. The infected BPH is characterized by the growth of hyphae on the surface of the brown planthopper's body. The infected BPHwas collected, then the entomopathogenic fungi that infect the BPH were isolated for the second stage of testing.

The second stage of the test was the pathogenicity test of the entomopathogenic fungi isolated from the exploration of the death of BPH. The factor tested was an exploratory fungal isolate found with

a spore density of 10^7/mL, as a control using imidacloprid insecticide 0.5 g/L with repeated 3 times.

Rearing mass of brown planthopper pests

Imago and BPH nymphs were collected from rice plantations in 7 endemic sub-districts of BPH attack in Banyumas Regency. Then the imago and nymphs were brought to the laboratory and kept in a 50 x 50 x 120 cm gauze cage. In the gauze cage, vegetative phase rice plants were planted in plastic pots with a diameter of 15 cm and 20 cm high for feed and nesting sites. Every day the instar 1 nymph formed was transferred into another gauze measuring 50 x 50 x 120 cm in which there was fresh feed. The BPH nymph used for the pathogenicity test was the second offspring (F_2) or later.

Isolating the entomopathogenic fungi found

Imago or BPH nymphs infected with surface fungi were sterilized with Alcohol 70% for three minutes. Then rinsed sterile water three times and dried it on sterile filter paper. BPH infected with the fungus was placed in a petri dish (9 cm in diameter) containing sterile moist tissue and incubated to stimulate the growth of fungi. The fungus that came out of the BPH body was taken with an inoculation needle, cultured on PDA (*Potato Dextrose Agar*) media and incubated for seven days at 23-25 ºC.

The pathogenicity test of fungi found in the laboratory

Pure 7-day-old fungi isolates were tested for their ability to infect them on the BPH by spraying method. This method was a modification commonly used for insecticides that work as contact poisons. The trick was to add 5 mL of NaCl 0.85% to pure culture from each isolate in a petridish. The petridish was shaken until the surface of the colony was completely submerged and the spores falled into the physiological solution. The physiological solution containing the spores was pipetted and transferred into a sterile test tube. Then shaken with the vortex until homogeneous. One drop of spore suspension was dropped into the Nebaeur haemocytometer to calculate the amount of spores

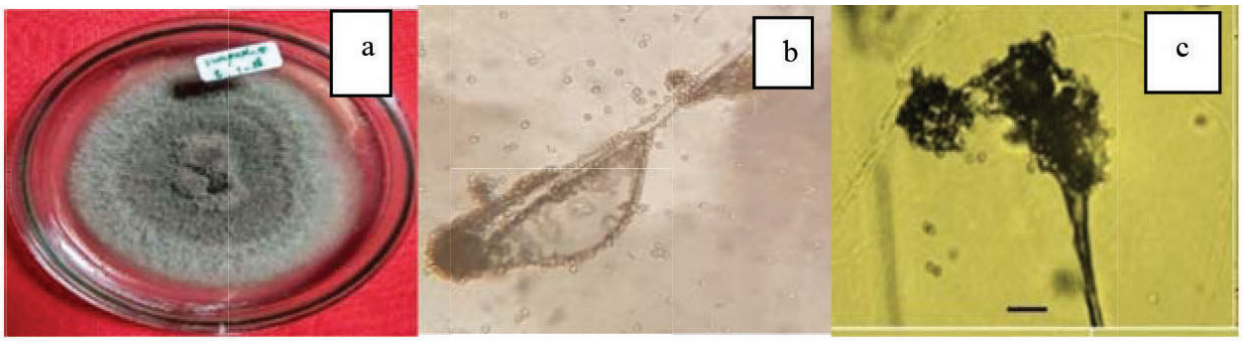
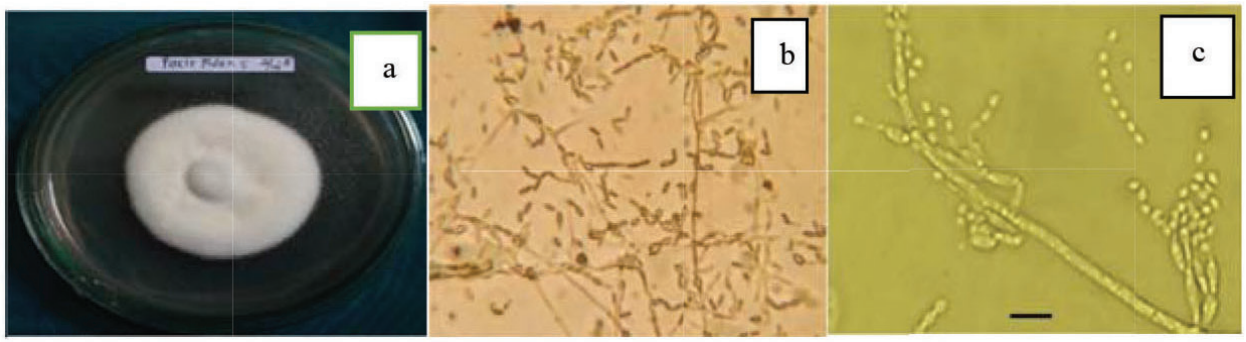


Fig. 1(a). Pure culture of Sumpiuh 11 (J11) isolates, (b) Conidia *Aspergillus* sp. (research documentation, 2018), (c) Conidia *Aspergillus* sp. (Dong *et al.*, 2016)



Fi.g 2a. (a) Pure culture of Pasir Kulon 5 (J22) isolates, (b) Conidia *Paecilomyces* sp. (Research documentation, 2018), (c) Conidia Dong *et al.*, 2016 and Nguyen *et al.*, 2016

with the help of a microscope and hand counter. The number of spores/mL was calculated based on the formula Kommedahl and Burnes, (1989).

Number of spore /mL = 50.000 x d (spores/mL)

Description: d = number of spores counted in five small boxes on the haemocytometer used.

Then, the density of the calculated spores was put into a 10 mL small sprayer bottle and sprayed evenly on the BPH test to be tested. The density of spores used was spore /mL and as a control sterile water and imidacloprid insecticide were used. Imago which has been applied with the suspension of the entomopathogenic fungus was then maintained in a plastic cylinder (8.5 cm in diameter and 40 cm in height) which was covered with gauze and in which there was a vegetative phase of rice plants. Every 24 hours the dead image was recorded. The fungi grown on the BPH imago were isolated, observations were made on the characteristics of these isolates and the results were compared with the initial isolates that had been obtained. Each treatment used 10 BPH imagos, and repeated 3 times for each isolate.

Data analysis

The data obtained were analyzed using the F test with a level of 5% to determine the effect of each treatment. If there is a difference, continue with 5% DMRT using IBM SPSS Statistics software.

Results

Exploration of the Entomopathogenic Fungi from Brown planthopper

Exploration activities were carried out in the endemic areas of BPH in Banyumas Regency, namely Sumpiuh, Karanglewas, Cilongok, North Purwokerto, East Purwokerto, Banyumas and Kebasen Districts. Exploration results in the sub-district found 66 entomopathogenic fungus from BPH infected with entomopathogenic fungi (Table 1).

Fig. 3. (a) Pure culture of Cipete 2 (J34) isolates, (b) Conidia*Beauveria* sp. (research documentation, 2018), (c) Conidia *Beauveria bassiana* Nuraida and Hasyim, 2009.

Table 1. The entomopathogenic fungi found in the brown planthopper endemic areas in Banyumas Regency

No	Sub- district	Village	Isolate Codes	Growth phase
1	Sumpiuh	Sumpiuh	J1,J2, J3, J4,J5, J6, J7, J8,J9, J10, J11, J12	vegetative
		Lebeng	J13, J14	vegetative
		Selanegara	J15,J16	vegetative
		Kebokuro	J17,J18	vegetative
2	Karanglewas	Pasir Kulon	J19, J20, J21,J22,J23	vegetative
		Karang Kemiri	J24	vegetative
3	Cilongok	Rancamaya	J25	vegetative
		Cilongok	J26	vegetative
		Panusupan	J27, J28, J29, J30, J31	vegetative
		Cipete	J32, J33, J34, J35, J36	vegetative
4	North Purwokerto	Karangwangkal	J37, J38, J39, J40, J41, J42, J43	vegetative
		Grendeng	J44, J45, J46, J47, J48, J49, J50	vegetative
5	East Purwokerto	Mersi	J51, J52, J5, J54, J55, J56, J57, J58	generative
6	Banyumas	Papringan	J59, J60, J61, J62, J63	generative
7	Kebasen	Kebasen	J64, J65, J66	vegetative

Pathogenicity test of entomopathogenic fungi isolates on mortality and time of death of brown planthopper

The exploration isolates of the entomopathogenic fungus were then tested for their ability to infect BPH. Tests are carried out in stages for each sub-district. The results of the DMRT test of the fungal isolates tested are presented in Tables 2 and 3.

Discussion

Based on Tables 2 and 3 of the 66 fungal isolates from the exploration and pathogenicity tests on a laboratory scale, eight entomopathogenic fungi isolates were able to kill more than 70 percent, ranging from 70 - 80 percent in 3.43 - 4.87 days after application (Table 3). The isolates are J11, J22, J34, J35, J41, J56, J60, and J65. The characteristics of each isolate are presented in Table 4. The morphological characteristics of each fungus are presented in Figures 1 to 8, which include the colors and shapes of colonies and conidia.

Brown planthopper infected by entomopathogenic fungi initially showed no symptoms, after three days the brown planthopper's activity decreased, and some even remained motion-

Table 2. Mortality of brown planthopper in the treatment of 66 isolates from the exploratory entomopathogenic fungi from Banyumas Regency

Fungi isolate	Mortality of BPH (%)		Fungi isolate	Mortality of BPH (%)	
J1	53.33	efghijkl	**J35**	**70.00**	**jklm**
J2	33.33	cdef	J36	53.33	efghijkl
J3	53.33	efghijkl	J37	30.00	bcde
J4	53.33	efghijkl	J38	56.67	fghijklm
J5	50.00	defghijk	J39	50.00	defghijk
J6	46.67	cdefghij	J40	66.67	ijklm
J7	46.67	cdefghij	**J41**	**70.00**	**jklm**
J8	40.00	cdefgh	J42	56.67	fghijklm
J9	46.67	cdefghij	J43	36.67	cdefg
J10	23.33	bc	J44	46.67	cdefghij
J11	**73.33**	**klmn**	J45	46.67	cdefghij
J12	56.67	fghijklm	J46	43.33	cdefghi
J13	60.00	ghijklm	J47	43.33	cdefghi
J14	50.00	defghijk	J48	43.33	cdefghi
J15	10.00	ab	J49	36.67	cdefg
J16	66.67	ijklm	J50	36.67	cdefg
J17	56.67	fghijklm	J51	33.33	cdef
J18	43.33	cdefghi	J52	40.00	cdefgh
J19	60.00	ghijklm	J53	50.00	defghijk
J20	50.00	defghijk	J54	46.67	cdefghij
J21	53.33	efghijkl	J55	53.33	efghijkl
J22	80.00	mn	J56	70.00	jklm
J23	66.67	ijklm	J57	43.33	cdefghi
J24	50.00	defghijk	J58	56.67	fghijklm
J25	53.33	efghijkl	J59	46.67	cdefghij
J26	60.00	ghijklm	**J60**	**76.67**	**lmn**
J27	50.00	defghijk	J61	56.67	fghijklm
J28	50.00	defghijk	J62	66.67	ijklm
J29	56.67	fghijklm	J63	26.67	bcd
J30	63.33	hijklm	J64	53.33	efghijkl
J31	63.33	hijklm	**J65**	**70.00**	**jklm**
J32	60.00	ghijklm	J66	66.67	ijklm
J33	66.67	ijklm	Aquadest	0.00	a
J34	**76.67**	**lmn**	Insecticide	96.67	n

Description: The numbers followed by the same letters show no significant differences based on the results of DMRT with accuracy of 95%

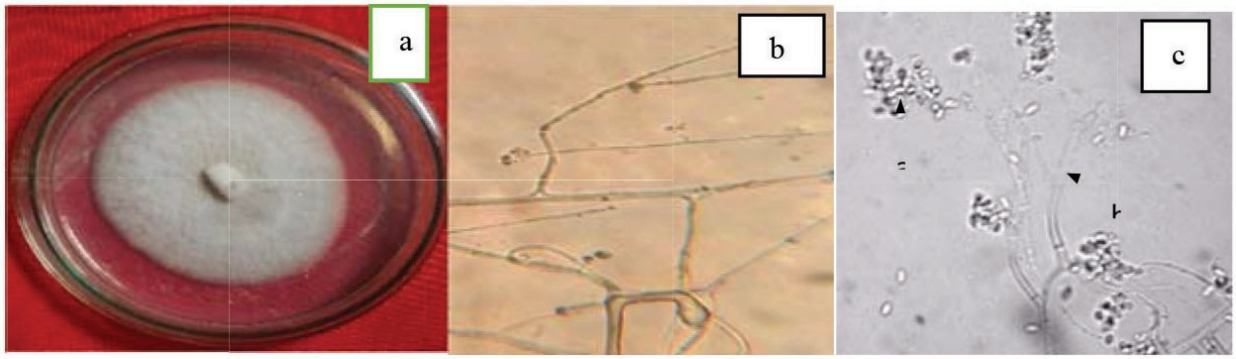


Fig. 4. (a) Pure culture of Cipete3 (J35) isolates, (b) Conidia *Beauveria* sp. (research documentation, 2018), (c) Microconidia *Beauveria bassiana*[37]

Table 3. The time of death of brown planthopper in the treatment of 66 isolates from the exploratory entomopathogenic fungi from Banyumas Regency

Fungi isolate	Time of death (days)		Fungi isolate	Time of death (days)	
J1	4.57	mnopqrstu	**J35**	**4.87**	**rstu**
J2	3.57	bcdef	J36	4.83	qrstu
J3	4.03	fghijkl	J37	3.42	bc
J4	4.47	lmnopqrst	J38	3.45	bcd
J5	4.37	ijklmnopq	J39	4.47	lmnopqrst
J6	3.42	bc	J40	4.94	tu
J7	3.90	cdefghi	**J41**	**4.17**	**ghijklmn**
J8	3.90	cdefghi	J42	4.32	hijklmno
J9	4.30	hijklmno	J43	3.93	defghij
J10	4.03	fghijkl	J44	4.92	stu
J11	**4.17**	**ghijklmn**	J45	4.98	u
J12	4.47	lmnopqrst	J46	4.15	ghijklm
J13	3.70	cdefg	J47	3.97	efghijk
J14	3.90	cdefghi	J48	3.78	cdefg
J15	3.77	cdefg	J49	4.36	ijklmnopq
J16	3.90	cdefghi	J50	4.33	hijklmnop
J17	3.17	b	J51	4.72	opqrstu
J18	3.20	b	J52	4.40	jklmnopqr
J19	3.90	cdefghi	J53	3.96	efghijk
J20	3.93	defghij	J54	4.30	hijklmno
J21	3.73	cdefg	J55	4.52	lmnopqrstu
J22	3.94	defghijk	J56	4.65	nopqrstu
J23	3.92	defghij	J57	4.33	hijklmnop
J24	3.95	efghijk	J58	5.50	v
J25	4.97	efghijk	J59	4.33	hijklmnop
J26	5.44	v	**J60**	**3.43**	**bc**
J27	4.67	opqrstu	J61	3.52	bcde
J28	4.82	pqrstu	J62	3.86	cdefgh
J29	4.43	klmnopqrs	J63	3.77	cdefg
J30	5.43	v	J64	4.55	mnopqrstu
J31	5.48	v	**J65**	**4.33**	**hijklmnop**
J32	4.65	nopqrstu	J66	4.55	mnopqrstu
J33	4.47	lmnopqrst	Aquadest	7.80	w
J34	**4.28**	**hijklmno**	Insecticide	1.19	a

Description: The numbers followed by the same letters show no significant differences based on the results of DMRT with accuracy of 95%

less and died. The average time of death ranged from 3.17 - 4.87 days after treatment (Table 2). The appearance of hyphae on the surface of the brown planthopper's infected body is around 5-7 days. This is in accordance with the opinion of Hou *et al*. (2018) which states that fungal hyphae *Metarhizium anisopliae* will appear on the body surface of *Rhynchoporus ferrugineus* at two or three days after death.

The death of BHP by entomopathogenic fungus is physically caused by the growth and development of fungi in the insect's body. This is in accordance with the results of a study by Hsia *et al*. (2014) and Li *et al*. (1998) which showed that the entomopathogenic fungus developed a certain structure of conidia as a tool to penetrate the cuticle called appresoria (sprout tube). After penetration, the sprout tube passes through the insect's cuticle and epidermis, the fungus multiplies into the insect's body cavity. Some Entomophthorales, multiplication by using protoplasts, while in some hyphomycete (eg, *M. anisopliae*), use blastospora as an initial proliferation. In addition, the death of BHP is allegedly caused by the presence of enzymes and toxins produced by the fungus.

The production of extracellular enzymes from

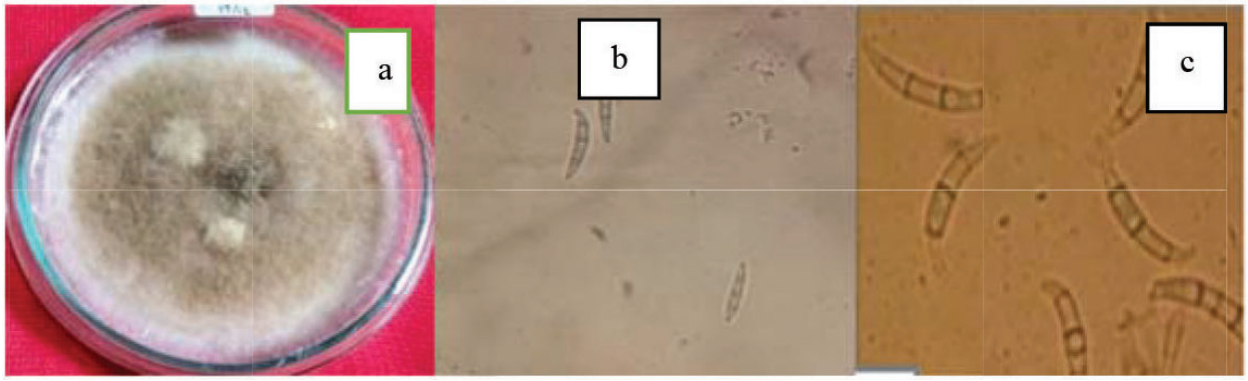


Fig. 5. a) Pure culture of Karangwangkal 5 (J41), (b) Conidia *Fusarium* sp. (Research documentation, 2018), (c) Conidia *Fusarium* sp.[38]

Table 4. Morphological characteristics of selected entomopathogenic fungi isolates

Isolate	Color and shape of the colony	Conidial form	Conidia color	Genus	References
J11	Blackish gray like cotton, uneven edges	Round	Hyaline	*Aspergillus* (Fig1.)	Kidd *et al*., 2016; Dong *et al*., 2016
J22 & J60	Round, flat, white which then turns to be creamy after old age	fusiform, sometimes cylindrical, smooth walled	Hyaline	*Paecilomyces* (Fig. 2 and 7)	Dong *et al*., 2016; Nguyen *et al*., 2017
J34 & J35	White and the edges are pale yellow and the base color is white. Round shape, widened growth	Oval slightly rounded, stick to the ends and sides of the conidiophores (branches), have long crossed hyphae, conidial growth clustered.	Hyaline	*Beauveria* (Fig.3 and 4)	Rosmini and Lasmini, 2010 Nuraida and Hasyim, 2009
J41 & J56	The yellowish white color then turns to light brown	Microconidia is round elongated, while curved macroconidia consists of 3-5 elongated septa with a narrowed or tapered tip	Hyaline	*Fusarium* (Fig. 5 and 6)	Rosmini and Lasmini, 2010 Bonde *et al.,* 2014 Tambingsila and Hidayat, 2014
J65	Black, uneven edges with a high center surface and edges slightly shrinking.	elliptical conidia has 2-3 bulkheads	Brown	*Curvularia* (Fig. 8)	Indria *et al.,* 2013

entomopathogenic fungi is one of a series of infections in insects. Important enzymes secreted by entomopathogenic fungi are chitinase, lipase and proteases. Some fungi that have been reported produce some of these enzymes. Fungi isolate *B. bassiana*, *Paecilomyces lilacinus* and *Metarhizium anisopliae* produce protease and lipase enzymes (Feng, 1998; Park *et al*., 2004; Nahar *et al*., 2004; Rustiguel *et al*., 2012). Chitinase enzymes are produced by fungi *Beauveria bassiana* (Sanivada and Mohan, 2014).

The enzyme chitinase, protease and lipase help

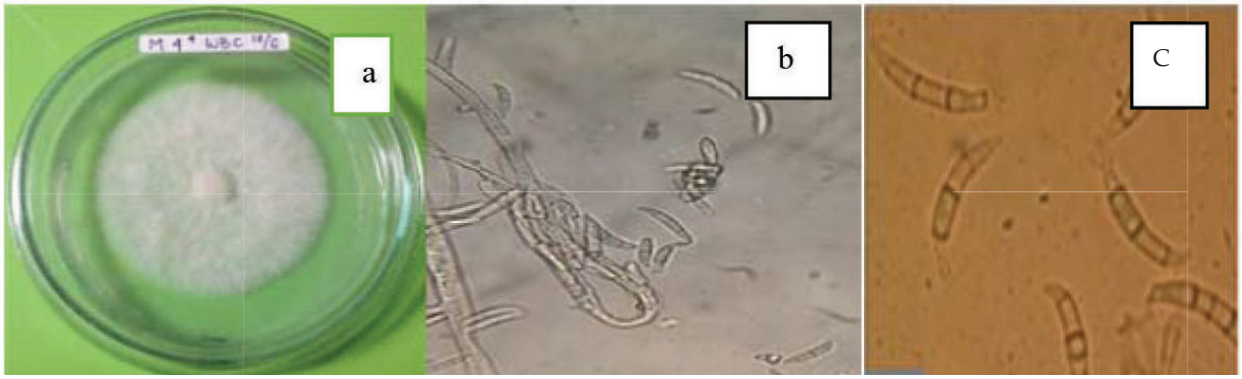


Fig. 6. a) Pure culture of Mersi6 (J56), (b) Conidia *Fusarium* sp. (Research documentation, 2018), (c) Conidia *Fusarium* sp.[38]

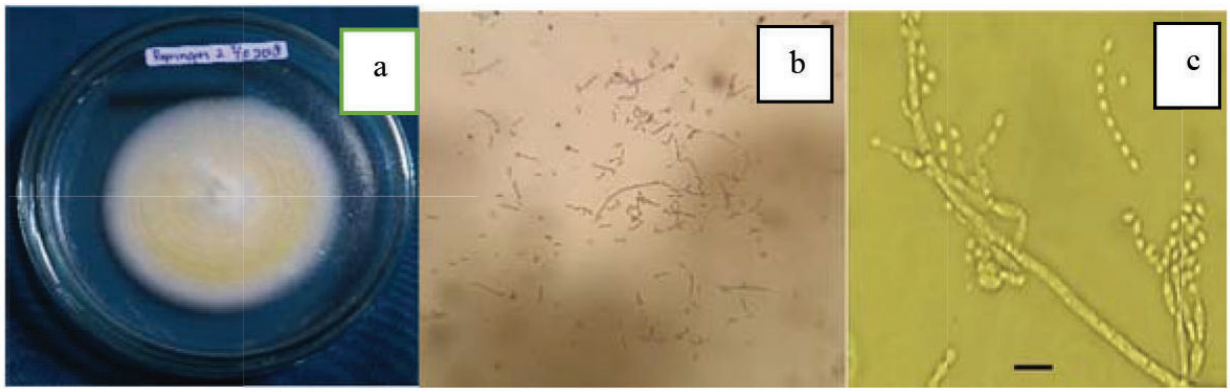


Fig. 7. (a) Pure cultures of Papringan 2 isolates (J60), (b) Microconidia *Paecilomyces* sp. (Research documentation, 2018), (c) Microconidia *Paecilomyces lilacinus* Dong *et al.,* 2016

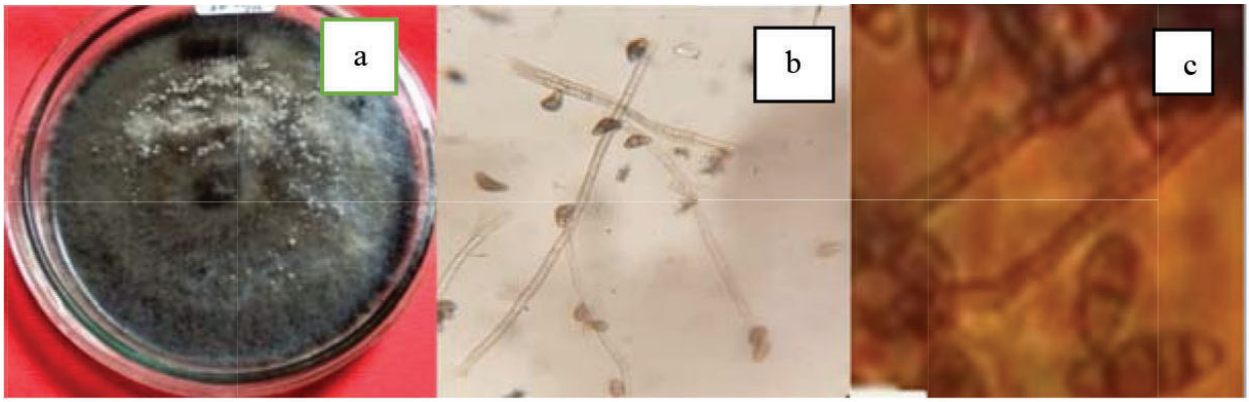


Fig. 8. (a) Pure culture of Kebasen 2 (J65) isolates, (b) Conidia *Curvularia* sp. (Research documentation, 2018), (c) Conidia *Curvularia* sp. Indria *et al.,* (2013).

degrade insect cuticles so that they are easily penetrated by fungi (Sanivada and Mohan, 2014). Besides enzymes, according to Hsia *et al.* (2014), entomopathogenic fungi also produce secondary metabolites that are toxic to insect pests that will increase the pathogenicity of the entomopathogenic fungus. Cuticles are used by insects as a method of other humoral and cellular defenses against fungal invasion. The toxins produced by some insect pathogenic fungi help them to improve pathogenesis and play their role as insecticides. While some other fungi produce antimicrobial metabolites. Destruxins in the *Metarhizium* fungi species are metabolites that can increase the pathogenicity of fungi. Beauvericins is produced by the *Beauveria* species. Some beauvericins are also isolated from *Isaria fumosoroseus* and some plants pathogenic species of *Fusarium*. Other products produced by *B. bassiana* are isarolides, bassianolides and beauverolides.

Genetic factors of entomopathogenic fungi also influence the pathogenicity of the fungus to brown planthopper. The results of entomopathogenic fungi exploration from BHP in Banyumas Regency have different levels of pathogenicity even though they come from the same type of pest, entomopathogenic fungi have large genetic variations between different isolates. The pathogenicity, virulence, enzymatic characteristics and DNA also vary between different isolates from different types of insects. The origin of the isolates affects the diversity of virulence of fungi against host insects, this is related to the type or race or strain of the fungus (Shahid *et al*., 2012; Gul *et al*., 2014; Wu *et al*., 2014). The genes of each fungal isolate are generally related to fungal cell wall synthesis and metabolic energy. The expression of fungal cell wall protein genes plays an important rolein the pathogenesis of fungi and enzymes dramatically regulating carbon metabolism by increasing energy use during fungal infections (Devi *et al*., 2005; Meyling *et al.,* 2009; Xia *et al*., 2013).

Conclusion

The test results found 8 of the best isolates, namely isolates J11 (*Aspergillus* sp.), J22 (*Paecilomyces* sp.), J34 (*Beauveria* sp.), J35 (*Beauveria* sp.), J41 (*Fusarium* sp.), J56 (*Fusarium* sp), J60 (*Paecilomyces* sp.) and J65 (*Curvularia* sp.). These fungus have the ability to kill BPH ranges from 70–80% with a time of death ranges from 3.43 to 4.87 days

Acknowledgment

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