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Endophytic fungi play important role in rice protection against brown planthopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae)

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Abstract. Endophytic fungi has both beneficial and detrimental effect on plants. Knowledge on the role of endophytic fungi of rice in relation to associated insect pests is limited. The research aimed to investigate the diversity of endophytic fungi of rice sheath and to examine its importance in rice protection against brown plant hopper (BPH), *Nilaparvata lugens*. The fungi was isolated from 2 regencies, Cianjur and Subang -West Java, with 2 cultivars in each locations, Ciherang and Pandanwangi. Cianjur samples were differentiated in 3 level of BPH infestation *i.e.* zero, low and high. Some predominant fungi were further tested their activity on population growth and nymph-adult survival of BPH in a greenhouse experiment. Number of isolated fungal species, diversity index and total colonization of endophytic fungi of rice from Cianjur was higher than Subang. Diversity index and total colonization of endophytic fungi of cv. Pandanwangi was higher than of Ciherang. Three predominant fungi in free-BPH rice: *Nigrospora* sp.1, *Nigrospora* sp.2 and *Nigrospora* sp.3, enhanced rice resistance against BPH, indicated by decreased of nymph-adults survival, non-preference phenomenon and decreased of population growth.

1. Introduction

Nowadays, theoretical and applied biologists pay great attention to endophytic microorganisms, including endophytic fungi and its interaction with host plants. The ecological role of endophytic fungi is favoring growth, development and survival of host plant. Some endophytic fungi have inhibitory effect on phytophagous insects of various taxonomic groups. Natural colonization of an endophytic fungus *Acremonium coenophialum* in tall fescue *Festuca arundinacea*) deterred the feeding of *Rhopalosiphum padi* and *Schizaphis graminum* [1]. In addition, it was reported the significant inhibition of population growth of mealy bug *Phenacoccus solani* and barley aphid, *Sipha maydis* Passerini, on fungal endophyte-infected tall and meadow fescues [2].

Endophytic fungi of rice varies among varieties, location and plant parts in China [3]. However, up to now, knowledge on endophytic fungi of rice in tropical ecosystem is limited. The objectives of the research were to study the diversity of endophytic fungi of rice in 2 rice cultivars and 2 regencies in West Java, Indonesia and to examine their importance in rice protection against brown planthopper.



2. Methods

2.1. Isolation and identification endophytic fungi

Isolation of fungal-endophytes was carried out by modified technique of Petrini [4]. Samples of rice sheaths were obtained from rice at early vegetative stage, with low infestation level of brown planthopper / BPH (1-5 insects/hill), from farmer's field at Warungkondang-Cianjur (350 m asl) and Ciasem-Subang (20 m asl) – both are regencies of West Java Province-Indonesia. Samples collection period was in July-December 2008. Location in each regency was divided in 4 villages, from where 40 samples of cv. Ciherang and 40 samples of cv. Pandanwangi were taken out. Especially for Cianjur location and cv. Ciherang, in which variability of infestation level of BPH existed, separate sampling was done on 3 BPH infestation category; zero, low (1-5 insects/hill) and high (>5 insects/hill). Samples (cut of sheath with 5 cm length, with 1 sheath per hill) were packed in plastic bag and placed in cool box then transported to the laboratory in Bogor.

The collected sheaths were cut into 5 mm x 5 mm size, disinfected 2 times with 70 % ethanol and 1% sodium hypo chloride, each for 3 min, then rinsed by sterilized water and excessive water tapped by towel paper and plated on medium potato dextrose agar (PDA) pH 5.5. Isolated fungi were then purified by re-culturing on PDA. Identification was conducted based on morphology up to genus level using identification books [5-8].

Recorded variables were relative isolation frequency, number of fungi species, diversity index and total colonization. Relative isolation frequency of each endophytic fungus was determined by the percentage of samples containing certain endophyte. Shannon index was applied for diversity index and calculated as $H' = -\sum \ln(i) \cdot (p_i)$ [9], in which H' = Shannon diversity index, $\ln(i)$ = natural log of proportion endophyte i_{th} , p_i = proportion of endophyte- i_{th} . Total endophyte colonization was determined by percentage of samples with endophytes without regard the species, and express in percent. The collected endophytic fungi then stored on test tubes containing PDA and store at 15 °C. Propagation was carried out by reculturing this isolate on PDA. The 14-d old colony of endophyte was used for inoculation.

2.2. Artificial inoculation of endophytic fungi.

Existing seed fungi was eliminated by hot water treatment at 52 °C for 20 min. Then, conidia of 14-d old culture of selected endophytic fungi were obtained by soaking with sterilized water, then filtered and adjusted to 10^4 conidia/mL. Endophytic fungi were inoculated on rice plants twice, seed treatment and conidia spraying. For seed treatment, rice seeds cv. Ciherang was dipped by 10^4 conidia/mL suspension for 6 hr, then grown with sterilized soil in pot tray. Meanwhile, for conidia spraying, 50 mL conidial suspension containing 10^4 conidia/mL was sprayed by hand sprayer to each plant 10 d after transplanting.

2.3. Effect of endophytic fungi on the population growth of BPH

Ten nymphs of *N. lugens* (1st instar) were infested into rice potted plant then covered by cheese cloth cages to avoid BPH migration and the attack of it's natural enemies. This experiment was conducted in a complete randomized design with ten replications. Treatment consisted of endophytic fungi i.e. *Nigrospora* sp. 1, *Nigrospora* sp. 2, *Nigrospora* sp. 3, and *Fusarium semitectum*. The fungi represent predominant fungi of rice plants associated low/zero field BPH population (*Nigrospora* sp. 1, *Nigrospora* sp. 2, *Nigrospora* sp. 3,) and high field BPH population (*Fusarium semitectum*). Number of live BPH was measured weekly started from the 1st wk and stopped at 7th wk after BPH inoculation.

2.4. Effect of endophytic fungi on survival of BPH nymphs

Ten nymphs of *N. lugens* (1st instar) were infested on 4-wk old rice plants. Treatments and experimental design used was similar to 2.3. methods. The number of alive BPH was observed every day from 1-12 d after BPH inoculation.

2.5. Preference test

Methods of Johnson *et al.* [10] was applied for preference test. Potted rice plants treated by endophytes and untreated, placed 75 cm equidistant to a center. Then, 20 macroptera of BPH in an opened tube (diam. 2.5 cm, height 5 cm) was placed in the center. Number of alighted BPH on each plant was measured on 24 and 48 hr after the infestation. The preference experiment was arranged in completely randomized design with 5 replications.

Relative frequency of re-isolation (%) was calculated. Another variables such as fungal population growth and survival was statistically analyzed using analysis of variance (ANOVA) by SPSS continued with Duncan multiple range test (DMRT).

3. Results and discussion

At total 15 rice endophytic fungi species has been isolated from Cianjur and Subang. *Nigrospora* and *Fusarium semitectum* were the most dominant endophytic fungi with frequency of isolation more than 10%. Another fungi such as *Acremonium*, *Cephalosporium*, *Penicillium*, *Phyllosticta*, *Curvularia*, *Chaetomium*, and *Alternaria padwickii* had lower colonization rate, with isolation frequency less than 5%. The species domination of these isolated endophytic fungi was significantly different than which reported in China [11] and India [12]. In China, dominating endophytic fungi in rice were *Penicillium*, *Fusarium*, *Aspergillus*, *Helminthosporium*, *Pyricularia*, *Paecilomyces* and yeasts. Moreover, dominating endophytic fungi in Bhadra River India, were *Chaetomium globosum*, *Penicillium chrysogenum*, *Fusarium oxysporum* and *Cladosporium cladosporioides* [12]. This significant difference might be caused by different rice varieties planted, altitude and latitude, soil condition, climate, and cultivation practices in China, India, and Indonesia. These factors are strongly affecting the dominance and diversity of endophytic fungi [13, 14]. In addition, different plant organs also obtains different diversity of isolated endophytic fungi. Endophytic fungi in China and India were isolated from leaves, compared to sheath in this research.

Without considering the varieties difference, species number and diversity index of endophytic fungi from Cianjur was higher than of Subang. The fungi *Chaetomium*, *Pestalotia*, *Penicillium* were only isolated from Cianjur's samples. There was no difference of species number between cultivars. However, cv. Pandanwangi cultivars are colonized by more diverse endophytic fungi (Table 1). The 2 hr environmental factors affecting the fungal diversity between Cianjur and Subang are the altitude and rainfall. Warungkondang, Cianjur is located at 350 m asl with annual rainfall at 2843 mm, while Ciasem Subang is located at 20 m asl with lower annual rainfall at 1926 mm. Cultivar Pandanwangi supported higher diversity in term of diversity index and samples with endophytic fungi. Pandanwangi is a local rice cultivar, while Ciherang is a modern varieties – a cross with genetic parental partially from outside Indonesia [15]. Generally, primitive rice varieties have higher diversity and colonization rate of endophytic fungi than modern varieties [16].

The level of BPH population did not highly associated to the species number and diversity index, but it had a positive association with species composition and the total of endophytic fungi colonization. Endophytic fungal community on rice plants having zero and low BPH level were dominated by *Nigrospora* sp. 1, *Nigrospora* sp. 2, *Nigrospora* sp.3, and *Nigrospora* sp. 4. On the contrary, rice plants with high level of BPH were dominated by *F. semitectum* and *Cephalosporium* sp.. Another key finding stated on Table 2 is with the presence of endophytic fungi was decreased if there is BPH increasing, i.e. 65%, 45% and 40% for zero, low, and high population of BPH, respectively.

Artificial inoculation of selected endophytes on rice conferred resistance against BPH. The increasing of resistance is depicted by decreasing of population growth (Fig.1) with the highest suppression provided by *Nigrospora* sp. 2, and the lowest was by *F. semitectum*. Endophytic fungi dominating in BPH-free plants were *Nigrospora* sp. 1, *Nigrospora* sp. 2 and *Nigrospora* sp. 3 suppressed population growth of BPH with average population reduction was 21.87%, 32.30%, and 27.00% respectively. On the contrary, *F. semitectum* which was dominant on attacked plants showed minor effect on the population growth (Fig. 1).

Table 1. Diversity and abundance of sheath endophytic fungi of rice in 2 Subang and Cianjur.

Species of endophytic fungi	No. Sample ^a			
	Subang		Cianjur	
	cv. Ciherang	cv. Pandan wangi	cv. Ciherang	cv. Pandan wangi
<i>Acremonium</i> sp.	-	2 (5)	2 (5)	2 (5)
<i>Alternaria padwickii</i>	-	2 (5)	2 (5)	2 (5)
<i>Cephalosporium</i> sp.	2 (5)	-	2 (5)	-
<i>Chaetomium</i> sp.	-	-	-	2 (5)
<i>Curvularia lunata</i>	2 (5)	2 (5)	2 (5)	-
<i>Fusarium semitectum.</i>	6 (15)	4(10)	2 (5)	2 (5)
<i>Nigrospora</i> sp. 1	2 (5)	4 (10)	6 (15)	4(10)
<i>Nigrospora</i> sp.2	6 (15)	12 (30)	6 (15)	6 (15)
<i>Nigrospora</i> sp. 3	4 (10)	12 (30)	2 (5)	6 (15)
<i>Nigrospora</i> sp.4	-	2 (5)	-	-
<i>Penicillium</i> sp. 1	-	-	4(10)	4 (10)
<i>Pestalotia</i> sp.	-	-	-	1 (5)
<i>Phyllosticta</i> sp.	2 (5)	-	2 (5)	1 (5)
Sterile white 1	2 (5)	2 (5)	-	-
Sterile white 2	-	-	2 (5)	1(5)
Species number	8	9	11	11
Species number each locations		11		13
Shannon Index	1.69	1.93	1.99	2.31
Sample with endophyte (No.)	16	22	22	26
Sample with endophyte (%)	40	55	55	65

^a Number in parentheses represent percentage (%), n=40.

Table 2. Diversity and abundance of sheath endophytic fungi in various population level of BPH.

Endophytic fungi	BPH population level ^a		
	Free BPH	Low (1-5 /hill)	High (>5 /hill)
<i>Acremonium</i> sp.	0	0	2 (5)
<i>Alternaria padwickii</i>	0	2 (5)	0
<i>Cephalosporium</i> sp.	0	0	12 (30)
<i>Chaetomium</i> sp.	0	2 (5)	0
<i>Fusarium semitectum.</i>	0	0	12 (30)
<i>Nigrospora</i> sp. 1	8 (20)	4 (10)	6 (15)
<i>Nigrospora</i> sp.2	20 (50)	8 (20)	6 (15)
<i>Nigrospora</i> sp. 3	8 (20)	14 (35)	0
<i>Nigrospora</i> sp.4	0	2 (5)	0
<i>Phyllosticta</i> sp.	4 (10)	4 (10)	0
Sterile white 2	0	2 (5)	14 (35)
Species number	4	8	6
Shannon Index	1.22	2.21	2.04
Sample with endophyte (No.)	26	18	16
Sample with endophyte (%)	65	45	40

^a Number in parentheses represent percentage (%), n=40.

Artificial inoculation by selected endophytic fungi, on population growth of BPH gave an evidence that pre-dominant endophytic fungi in low level of BPH infestation, such as *Nigrospora* sp.1, *Nigrospora* sp.2, and *Nigrospora* sp.3 increase rice resistance against BPH, indicated by the decreasing of nymphs survival. This is a first report that diversity of endophytic fungi of rice sheath associate to BPH abundance in the field. Moreover, the effect of endophytic fungus *Nigrospora* in conferring resistance of rice against bph is also proven. All of endophytic fungi treatments also have adverse effect on nymph survival (Fig.2). *Nigrospora* sp.2 and *Nigrospora* sp.1 provided strong suppression of nymph survival rate at 50% and 40% respectively. Moreover, *Nigrospora* sp.3 and *F. semitectum*, has lower inhibitory effect, suppression level of 35.7% and 32%, respectively.

Table 3. Effect of endophyte inoculation on preference of macropterous BPH.

Endophyte treatments	Number of BPH /plants ^a	
	24 hr	48 hr
<i>Nigrospora</i> sp. 1	3.00 ± 0.24 a	3.00 ± 0.22 a
<i>Nigrospora</i> sp. 2	3.33 ± 0.15 a	3.33 ± 0.36 a
<i>Nigrospora</i> sp. 3	2.66 ± 0.76 a	2.66 ± 0.64 a
<i>Fusarium semitectum</i>	5.00 ± 0.22 ab	5.00 ± 0.17 ab
Uninoculated	6.00 ± 0.37 b	6.00 ± 0.24 b

^a Number followed by different symbol in the same column is significantly different according DMRT test with P<0.05.

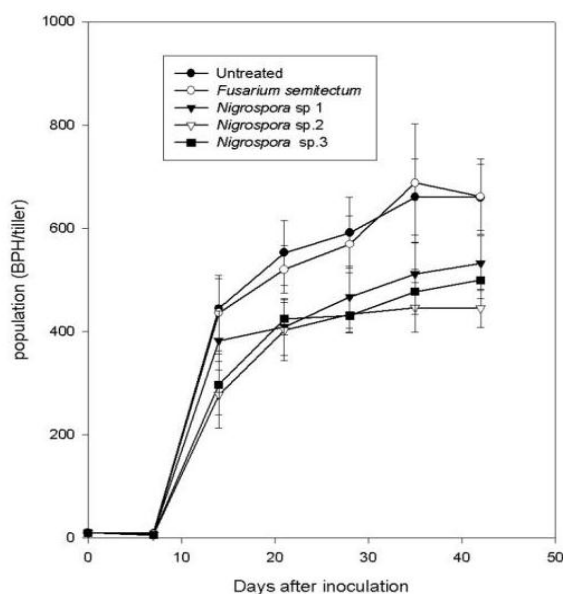


Figure 1. Population growth of brown planthopper on endophyte-treated rice.

The main mechanism of increase of plant resistance against insects due to endophytic fungi is antibiosis [1]. Decrease of BPH nymph's survival due to endophytes treatments as resulted in this research was in line with the theory. Moreover, endophytic fungi mediating host plant resistance against herbivory insects is strongly related to toxin production [14]. Toxin produced by endophytic fungi are alkaloids, terpenoid, steroid, quinone, and flavonoid, phenylpropanoids and lignans, peptides, phenol, phenolic acids, and aliphatic compounds [17]. Endophytic fungi *Phyllosticta* sp. and

Hormonema dematioides in balsam fir produce toxic compounds, mainly heptelidic acid and rugulosine which has strong insecticidal effect against spruce budworm [18]. The type of toxin or insecticidal properties causing non preference and nymph's survival of BPH, produced by *Nigrospora* is not known yet. It was reported that *Nigrospora sphaerica* produced phomalactone [19], which has insecticidal activities [20].

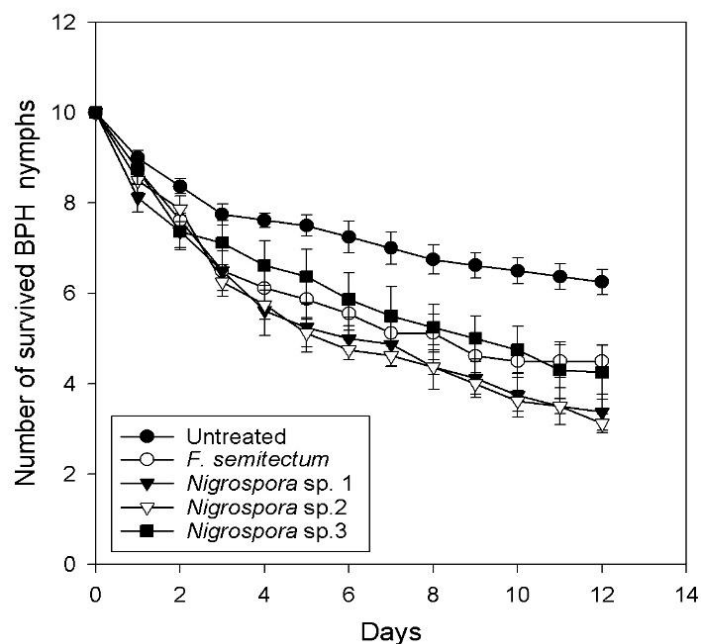


Figure 2. Nymph survival of brown planthopper in endophyte-treated rice.

Non-preference mechanism play also role in inducing plant resistance against insect pests due to endophytes. This mechanism involve in endophyte-mediated resistance of rice against BPH (Table 3). Table 3 shows that winged-BPH adult preferred to choose plants with no endophytic fungi colonization. Host non preference to herbivory insects mediated by fungal endophytes, was reported in aphids *Ropalosiphum maydis*, *Schizaphis graminum* [1], *Phenacoccus solani*, and *Sipha maydis* [2]. Non preference or change in host selection by phytophagous insects on endophyte infected plants was caused by the alteration of host plant production of volatile substances such as reported in system of *Acremonium*-tomato-*Helicoverpa armigera* [21, 22].

By comparing the exploratory and experimental data, it can be said that there is relation between the dominance of endophytic fungi and rice resistance against BPH. Fungus has no different dominance between plants either with or without BPH infestation. *Fusarium semitectum* has minor effect on the suppression of BPH. On the contrary, endophytic fungi with high dominance in BPH-free plant, have significant suppressing effect on BPH population.

Three endophytic fungi i.e. *Nigrospora* sp.1, *Nigrospora* sp.2 and *Nigrospora* sp.3 had considerable inhibitory effect on BPH, makes them greatly potential to be developed as biocontrol agent against BPH. The 3 potential endophytic fungi had no potency as pathogens, even promoted plant growth, increasing of emergence rate, seedling height and seedlings root length (data not shown). Endophytic *Nigrospora* sp. was also reported increase resistance of cauliflower against *Spodoptera litura* [23]. In addition, by having enough colonization rates ($\geq 30\%$ of reisolation frequency), the use of *Nigrospora* endophytes fungi as biocontrol agent will ensure better success rate in application.

The importance of endophytic fungi in protecting rice against BPH - a most devastating pest of rice in many Asian countries may underlay the big paradigm change in rice insect pest management.

Further investigation on environments, plant factors and agronomical practices related to endophyte colonization and BPH build-up is required. Other direction of research relating to development of endophytic fungi as biocontrol agents of BPH is also very challenging. Two groups of research related rice endophytic fungi may contribute to knowledge enrichment on complex ecological process related to pest population regulation and formulate better strategy for BPH management.

4. Conclusion

Number of isolated fungal species, diversity index and total colonization of endophytic fungi of rice sheath from Cianjur was higher than Subang. Diversity index and total colonization of endophytic fungi of rice cv. Pandanwangi was higher than of cv. Ciherang. Three predominant fungi in free-BPH rice: *Nigrospora* sp.1, *Nigrospora* sp.2 and *Nigrospora* sp.3, enhanced rice resistance against BPH, indicated by decreased of nymph-adults survival, non-preference phenomenon and decreased of population growth.

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