Exploration and Antagonistic Test of Endophytic Fungi from Soybean (*Glycine max* L. Merr) With Different Resistance to *Sclerotium rolfsii*

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Abstract

The research aimed to determine the diversity of endophytic fungi in soybean crop with different resistance against *Sclerotium rolfsii* and find out their potential antagonist in controlling *S.rolfsii* by *in vitro* and *in vivo*. Materials used in this study were soybean with a variety of Wilis (susceptible variety) and Sinabung (resistant variety). This research was conducted at the Microbiology Laboratory of Central Laboratory of Life Science (LSIH), Brawijya University and in the trial plantation of Malang Research Institute for Food Crops, Lawang, Malang subdistrict in September 2015 until May 2016. Type of experimental design used was Complete Randomized Design with 16 treatments and three times repetitions at *in vitro* experiment with 16 treatments and four times repetitions at *in vivo* experiment. Observation on *in vitro* test is covering to colony morphology of fungal pathogens on PDA medium. On the test of *in vivo*, it was observed a disease occurrence and effectiveness rate of endophytic fungi. There are 15 species of endophytic fungi produced from isolation, namely *Trichoderma* sp., *Aspergillus* sp.2, *Aspergillus* sp.3, *Acremonium* sp.1, *Acremonium* sp.2, *Acremonium* sp.3, *Acremonium* sp.4, *Fusarium* sp.1, *Fusarium* sp.2, *Cephalosporium* sp, *Microsporum* sp.*, Penicillium sp., and* unidentified fungi called W_2 and W_4. The highest inhibitory of endophytic fungi against *S. rolfsii* by *Aspergillus* sp.2 is 89.18% (*in vitro*) and 61.21% (*in vivo*), while *Trichoderma* sp. 91.88% (*in vitro*) and 63.29% (*in vivo*). Diversity index value of Wilis variety is higher than Sinabung, i.e. 1.878 and 1.606 respectively. While dominance index value of Sinabung variety is 0.2035 and Wilis is 0.1528.

Keywords: Endophytic fungi, diversity, *S. rolfsii*.

INTRODUCTION

Endophytic is microorganisms that live inside plant tissues without causing a symptom of disease in the host plant. There is a mutualistic interaction between endophytic microbes and host plant, each benefiting from the interaction [1]. The mutual interaction benefits the endophytic fungi through provision supply of energy, nutrients, shelter as well as protection from environmental stress. On the other hand, fungal endophytes indirectly benefit plant growth by producing special substances mainly secondary metabolites and enzymes, which are responsible for the adaptation of plants to abiotic stresses such as light, drought and biotic stresses, e.g. herbivore, insect and nematode attack or invading pathogens [2]. The research aimed to determine the diversity of endophytic fungi in soybean with different resistance to *Sclerotium rolfsii* and find out their potential antagonist in controlling *S. rolfsii* by in vitro and in vivo.

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MATERIALS AND METHODS

This study used exploratory and experimental method. Exploratory method was used to find out endophytic fungi from Wilis and Sinabung variety. Experiment performed was antagonistic test of endophytic fungus, i.e. isolated *S. rolfsii* through direct opposition method. This research was undertaken in Microbiology Laboratory of Central Laboratory of Life Science (LSIH), Brawijya University and field trials placed in Malang Research Institute for Food Crops, Bedali Malang in September 2015 until May 2016.

Materials used in this study were soybean with a variety of Wilis and Sinabung. It is a collection of Indonesian Legumes and Tuber Crops Research Institute (ILETRI) Malang, isolates of *S.rolfsii*, PDA, aquades steril, alcohol 7%, NaOCl 5%.

Isolation and Identification of *S. rolfsii*

Sclerotium rolfsii was isolated from the root of soybean which indicates to be withered, the stem turn reddish brown, and there is mycelium in the form of hyphae at the surrounding aboveground. Symptomatic root was cut, sterilized using NaOCl 5%, rinsed with sterile distilled water, and dried using tissue. It was then grown in

ISSN. 2087-2852
E-ISSN. 2338-1655

the petri dish containing PDA medium until the hyphae grow. Fungi colonies which grow in accordance with macroscopic morphology (shape and color) of *S. rolfsii* subsequently identified using identification guides of Barnett and Hunter.

Isolation and Identification of Endophytic Fungi

Endophytic fungi were isolated from the health plant's root tissue. First the plant's root was rinsed in tap water to remove the dust and debris then cut into small pieces by a sterilized blade under aseptic conditions. Each sample's surface was sterilized by 70% ethanol for 1 minute and after that immersed the plant parts in sodium hypochloride (NaOCl) solution for 1 minute. It was meant to be sterile from the outside fungi so that the growing fungi are expected from the inside of the plant tissue. The samples were rinsed in sterile distilled water for 1 minute and then allowed to surface dry on filter paper.

After proper drying, 4 pieces of plant parts were inoculated in PDA plate and incubated at 28^0C for 5 to 7 days. Pure colonies were transferred on PDA. The fungal strains in the pure culture were preserved on potato dextrose agar (PDA) [3]. In the last rinse of distilled water, it was then taken 1 ml of distilled water and being poured into PDA medium as a control. Fungi that grow and have a colony considered to be different based on the macroscopic morphology (color and form) will be performed purification. Furthermore, isolates of fungi were identified by macroscopic and microscopes using Barnett and Hunter's identification guides.

Antagonistic Test of Endophytic Fungi with Direct Opposition Method

The isolates of *S. rolfsii* and endophytic fungi were put together on a petri dish containing PDA medium within 3 centimeters length and incubated in the room temperature of 28-30°C during a week. For a treatment of control, pieces of isolate were put on the petri dish without endophytic fungi. The treatment was repeated for 3 times. The observed variable was colony radius that grows to the direction of endophytic fungi. Formulation of growth inhibition (I) of pathogenic colony of endophytic fungi by Sharfuddin and Mohanka [4]:

$$I = [(r_1-r_2)/r_1] \times 100\%$$

Description:
I= growth inhibition of *S. rolfsii* colony (%)
r_1= colony radius of *S. rolfsii* grows in the control (cm)
r_2= colony radius of *S. rolfsii* grows to the direction of endophytic fungi (cm)

Effectiveness of Endophytic Fungi in the Greenhouse (*In vivo*)

The testing aims to find out the potential of endophytic fungi isolates in curbing a disease occurrence caused by *S. rolfsii*. It was done at the nursery phase through seed soaking method. This treatment utilizes 15 isolates of endophytic fungi from exploration and control treatment, i.e. soybean with pathogen but without endophytic fungi. Soybean was planted on the growth medium of sterilized soil and compost under the comparison of 2:1. Soybean seeds were sterilized using alcohol 70%, soaked in the suspension of endophyte fungi isolates with concentration of 10^6 conidia.mL^{-1} for ± 12 hours. At the control treatment, seeds submersion were performed using sterile distilled water. Soybean which have been 2 weeks after planting given pathogenic treatment by dripping a pathogen suspension of *S. rolfsii* by 1 ml with density of 10^6 conidium.mL^{-1} in every planting hole. A variable observed is disease occurrence and effectiveness level with formulation [4]:

$$1\ \text{Disease Occurrence} = n/N \times 100\%$$

$$\text{Effectiveness level} = DO_c - DO \times 100\%$$

Description:
n= the number of infected plant
N= the number of observed plant
DO_c=Disease occurrence in control
DO=Disease occurrence

Index of Diversity and Dominance

Diversity index (H') was used to calculate endophytic fungi diversity. This calculation aims to determine the level of the diversity of endophytic fungi at the different resistance against *S. rolfsii*. Formulation of diversity index [5]:

$$H' = -\sum P_i \ln P_i$$

Description:
H' = Diversity Index of Shannon Wiener
n_i= the number of species i
N= the total number of individual
$P_i = n_i/N$

Dominance index was used to find out a dominance of endophytic fungi at a community. The calculation aims to determine whether there is dominance or not of particular endophytic fungi. Formulation of dominance index [6]:

$$C = \sum (P_i)^2$$

Description:
$P_i = n_i.N^{-1}$
n_i= the number of species i
N= the total number of individual

ISSN. 2087-2852
E-ISSN. 2338-1655

DATA ANALYSIS

The data obtained from observation was analyzed using F-test at the level of 5%. It was then continued with HSD (Honest Significant Different) test at the same level.

RESULTS AND DICUSSION

Isolates of Endophytic Fungi from Soybean

There are 15 fungi resulted from the isolates of endophytic fungi of soybean. Sinabung variety resulting in 7 species of endophytic fungi, they are: *Trichoderma* sp., *Aspergillus* sp.1, *Aspergillus* sp.2, *Penicillium* sp., *Acremonium* sp.4, *Fusarium* sp.2, and *Cephalosporium*. In the other side, Wilis variety produces 8 species of endophytic fungi, i.e *Aspergillus* sp.3, *Fusarium* sp.1 *Acremonium* sp.1, *Acremonium* sp.2, *Acremonium* sp.3, *Microsporum* sp., W_2 and W_4 (unidentified fungi).

Antagonistic Test of Endophytic Fungi and *S. rolfsii* by Using Direct Opposition Method

The result of antagonism test of endophytic fungi against *S. rolfsii* is provided in the Table 1.

Table 1. Endophytic Fungi Inhibition against *S.rolfsii*

Type of Endophytic Fungi (Treatment)	Growth Inhibition Average (%)
Aspergillus sp. 2	91.88 a
Trichoderma sp.	89.18 ab
Aspergillus sp. 1	83.77 abc
Aspergillus sp. 3	72.07 abcd
Acremonium sp. 2	71.16 bcd
Acremonium sp. 1	68.46 cd
Fusarium sp. 2	63.05 de
Cephalosporium sp.	60.35 de
Fusarium sp. 1	60.35 de
Acremonium sp. 4	59.45 de
Penicillium sp.	53.14 de
Acremonium sp. 3	52.24 de
Microsporum sp.	45.94 e
W_2	45.04 e
W_4	45.04 e
Control (without endophytic)	0.00 f

At the control treatment (without endophytic), growth inhibition (I) of colony is 0.00%. The highest growth inhibition of control treatment is in *Aspergillus* sp.2 (91.88%) and *Trichoderma* sp. (89.18%). While the lowest growth inhibition of control treatments are in W_2 and W_4 (unidentified) with inhibition of 45.04%.

Mechanism of the inhibition among tested isolates of endophytic fungi is different. Competition between pathogen and endophytic fungi is the most common mechanism. *Trichoderma* sp., *Aspergillus* sp.1, and *Aspergillus* sp.2, have the highest competition in seizing space and nutrition. This is shown by mycelium growth of endophytic fungi which is dominant and suppressing pathogenic growth (Fig. 1).

Another mechanism happened is mechanism of antibiosis which is characterized by clear zone around endophytic fungi and pathogen. Mechanism of antibiosis, antagonistic degrading enzyme has to be directly contact to the pathogen [7]. Endophytic fungi have a directly mechanism to suppression disease of plants, that is, through antibiotic production and secretion of lytic enzymes [8]. In this study, the mechanism of antibiosis shown by the isolates of *Penicillium* sp. which forms a clear zone so that the hyphae of *S. rolfsii* not able to penetrate the colonies of *Penicillium* sp. (Fig. 1). *Penicillium* species can secrete bioactive function as antibiosis, such as penicillin and riboksin [9].

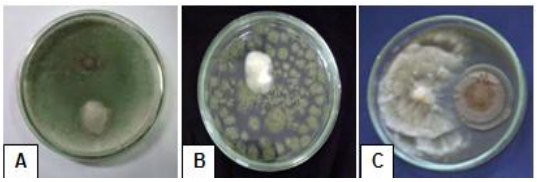

Figure 1. Antagonistic test of endophytic fungi against *S.rolfsii*
A: Competition of *Trichoderma* sp.
B: Competition of *Aspergillus* sp.
C: Mechanism of Antibiosis by *Penicillium* sp.

Meanwhile, the isolates of endophytic fungi which is not having mechanism of competition and antibiosis content at inhibition testing of *S.rolfsii,* supposed to have another activity in controlling diseases, such as the ability to induce plant resistance and also increase plant fitness. Resistance induction of endophytic fungi positions plants not as target pathogens directly, but through physiological and metabolic changes that allow the plant to further streamline their resistance to disease [10].

The Effectiveness of Endophytic Fungi at Greenhouse (*In vivo*)

The result of test effectiveness at greenhouse is provided in the Table 2. The lowest disease occurrence is on *Trichoderma* sp. treatment (21.25%) which significantly different from control (61.25%), while the highest disease occurrence is on *Microsporum* sp. (58.75%), W_4 (58.75%) and W_2 (56.25%) are not significantly different from control. It shows that not all of endophytic fungi which successfully isolated have potential in suppressing the dumping off progression. The highest effectiveness of endophytic

ISSN. 2087-2852
E-ISSN. 2338-1655

fungi in suppressing *S. rolfsii* disease is in the treatment of *Trichoderma* sp. (63.29%), *Aspergillus* sp.2 (61.21%), *Acremonium* sp.2 (61.21%). On the other, the lowest effectiveness level is in *Microsporum* sp. (4.16%), W_4 (4.16%), and W_2 (8.01%).

Table 2. The Rate of *S.rolfsii* Attacks against Endophytic Fungi Treatment

Type of Endophytic Fungi (Treatment)	Disease Occurrence (%)	The Level of Effectiveness (%)
Control	61.25 a	0.00 g
Microsporum sp.	58.75 ab	4.16 fg
W_4	58.75 ab	4.16 fg
W_2	56.25 abc	8.01 efg
Acremonium sp. 1	46.25 bcd	22.43 defg
Fusarium sp. 1	45.00 bcd	24.35 defg
Acremonium sp. 3	43.75 cd	28.52 def
Cephalosporium sp.	41.25 de	32.84 cde
Acremonium sp. 4	41.25 de	32.84 cde
Fusarium sp. 2	33.75 def	36.85 bcd
Penicillium sp.	32.5 def	40.54 abcd
Aspergillus sp. 3	28.75 ef	56.89 abc
Aspergillus sp. 1	26.25 f	57.04 abc
Acremonium sp. 2	23.75 f	61.21 ab
Aspergillus sp. 2	23.75 f	61.21 ab
Trichoderma sp.	21.25 f	63.29 a

Protection mechanism of endophytic fungi can be competition, antagonism, and microparasite and induction resistance. To inhibition the disease occurrence of other endophytic fungi the system of plant resistance has to be activated. Mechanism of plant resistance towards pathogen is commonly a combination between two resistance systems, namely structural and biochemical resistance [11].

Endophytic fungi also cause the induction of secondary metabolites that can inhibit other fungi which live on the same host. Biocontrol agents can weaken or kill the pathogen of plants through a resistance to be parasite directly, its ability in the competition of space and nutrients, production of enzyme to fight the pathogenic cell components, production of plant metabolism in stimulating the germination of spores of pathogens and production of antibiotics [11].

The presence of a combination among several biological agents can be independent, synergistic, or antagonistic. Among the endophytic fungi that colonize plant tissues and inoculated pathogens can cause a wide range of possibilities, such as they do not affect each other, compete with each other, synergistic in causing the symptoms of the disease or suppress the disease occurrence [12].

Index of Diversity and Dominance

Diversity Index of Wilis Variety (susceptible) is 1.878 while Sinabung (resistance) is 1.606. Those two varieties have moderate diversity. However, Sinabung variety (resistance) has lower diversity index value than Wilis (susceptible). This study proves that the diversity of endophytic fungi on resistance variety is not always higher than susceptible. It causes since not all endophytic fungi that are successfully isolated able to suppress the pathogen of *S.rolfsii*. It guesses since endophytic fungi are not containing anti-fungus and resulted secondary metabolite has another function. The factors affecting the ineffectiveness of biological agents to inhibit the growth of pathogens is antibiotics produced by endophytic fungi are less effective against pathogens; among others antibiotic concentrations are low and decomposed by other microorganisms [13].

Dominance index value (C) of susceptible variety (Wilis) is 0.1528, while the resistance (Sinabung) is 0.2035. Dominance index value of those two varieties are including in the low category. For that reason, there are no dominant endophytic fungi on those two varieties.

CONCLUSION

Not all endophytic fungi which were successfully isolated are able to suppress *S. rolfsii*. *Trichoderma* sp. (63.29%) and *Aspergillus* sp.2 (61.21%) have the best capability in suppressing *S. rolfsii* disease, both at laboratory or greenhouse.

REFERENCES

[1] Wilson, D. 1995. Endophyte the evolution of a term, and clarification of its use and definition. *Oikos.* 73. 274-276.

[2] Barz, W., S. Daniel, W. Hinderer, U. Jaques, H. Kessmann, J. Koster, K. Tiemann, 1988. In: Pais, M., F. Mavituna, J. Novais (Eds). Plant Cell Biotechnology. Springer (NATO ASI series). Berlin, Heidelberg, New York. 211-213.

[3] Muhibuddin, A., L. Addina, A.L. Abadi, A. Ahmad. 2011. Biodiversity of soil fungi on Integrated Pest Management Farming System. *Agrivita.* 33(22). 111-118.

[4] Sharfuddin, C and Mohanka, R. 2012. In vitro Antagonism of Indigenous Trichoderma Isolates Against Phytopathogen Causing Wilt of Lentil. *Int. J. Life Sci. Pharm. Res.* 2(3). L195-L202.

ISSN. 2087-2852
E-ISSN. 2338-1655

[5] Ludwig, J.A., J.F. Reynold. 1988. Statistical ecology: Primer on methods and computing. John Wiley and Sons Inc. Canada.
[6] Odum, E.P. 1994. Dasar-dasar Ekologi, 3rd Ed. Samingar, T. (Transl). Gadjah Mada University Press. Yogyakarta.
[7] Hallmann, J. 2001. Plant interaction with endophytic bacteria. In: Jeger. M.J., N.J. Spence (Eds). Biotic Interaction in Plant-Pathogen Associations. CABI. Wallingford (US). 87-119.
[8] Arnold, A.E., L.C. Mejia, D. Kyllo, E.I. Rojas, Z. Maynard, N. Robbins, E.A. Herre. 2003. Fungal endophytes limit pathogen damage in a tropical tree. *PNAS Journal.* 100. 15649–15654.
[9] Djarir, M. 1993. Mikotoksin pangan. Kanisius. Yokyakarta.
[10] Slusarenko, A.J., R.S.S. Frases, L.C. van Loon. 2000. Mechanism of resistance to plant desease. Kluwer Academic Publisher. Den Haag (NL).
[11] Agrios, N.G. 2005. Plant pathology 5th Ed. Department of Plant Pathology. University of Florida. United States of America.
[12] Petrini, O., T.N. Sieber, L. Toti, O. Virent. 1992. Ecology, metabolite production, and substrate utilization in endophytic fungi. *Nat. Toxins.* 1. 185–186.
[13] Kasutjianingati. 2004. Pembiakan mikro berbagai genotipe pisang (*Musa* spp.) dan potensi bakteri endofitik terhadap layu fusarium (*Fusarium oxysporum* f. sp. cubense). Master Thesis. Graduate Program, Bogor Agricultural University. Bogor.

ISSN. 2087-2852
E-ISSN. 2338-1655