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## THE USE OF ENDOPHYTIC FUNGI AS BIOPESTICIDE AGAINST DOWNY MILDEW *PERONOSCLEROSPORA* SPP. ON MAIZE

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### 20 ABSTRACT

Downy mildew is a major disease of maize caused by the fungus *Peronosclerospora* spp., widely distributed in all corn production centers in Indonesia. The disease can cause considerable losses even total losses have been reported occurring on susceptible varieties. The purpose of research was to determine the effectiveness of some isolates of endophytic fungi for the control of downy mildew on maize. The study consisted of four isolate treatments: *Aspergillus* spp., *Trichoderma* spp., *Beauveria* spp., *Gliocladium* spp., applied as seed treatment at planting and as flour formulation on rhizosphere 20 days after planting. Results showed that the average downy mildew disease intensity on the plots treated with *Beauveria* sp. was consistently lower than the control plots. The number of healthy plant was also consistently higher in the plots treated with *Beauveria* sp. than those in the untreated plots. Endophytism study results showed that *Aspergillus* spp. was not found in any part of the plant tissue. *Trichoderma* spp. and *Beauveria* spp. were found on 14% and 2% of the root pieces observed, respectively. Whereas *Gliocladium* spp., was reisolated from stem and leaf with the percentages of 16% and 92%, respectively. None of the tested isolates was detected in maize seed.

**Keywords:** Maize, downy mildew, fungal endophyte, biopesticide

### 7 INTRODUCTION

Downy mildew caused by a fungal obligate *Peronosclerospora* spp. is one of the most devastating diseases of maize (*Zea mays* L.). This disease can occur at any stage of maize development from seedling to harvest, though it primarily infects its host soon after seedling emergence until one month after planting (Wakman, 2004). Worldwide, the disease has been reported causing about 30% of economic loss (Jeffers et al., 2000). In several maize growing countries including Indonesia, yield losses can reach 50-100% for susceptible cultivars (Wakman, 2004). The only reliable control for decades until recent years is the use of chemical fungicides, especially metalaxyl. However, continuous use of certain fungicides in a long period of time can trigger resistance reaction of the target fungus to those fungicides and also pose detrimental impacts on the environment. It is therefore necessary to find alternative control measures, such as the use of biological agents to control the disease.

A new prospective area in agriculture is the use of microorganisms to promote plant growth and to protect the plant hosts from pests and diseases. One group of microorganism that can be used for this purpose is endophytic fungi (Pineda et al., 2000). Endophytic fungi are associated with various tissues and organs of terrestrial and some aquatic plants, whose infections are inconspicuous and the infected host tissue are at least transiently symptomless (Stone et al., 2000). Also, fungal endophytes live in intercellular space or inside cells of host plant causing no apparent damage (Saikkonen et al., 1998). However, endophytic fungi, which colonize and grow asymptotically within healthy plant tissues, may evolve from non-pathogenic fungi and become nonpathogenic (Carroll, 1988; Freeman & Rodriguez, 1993; Saikkonen et al., 1998; Kogel et al., 2006). Although, disease symptoms of host plant

caused by endophytes can be expressed under stress conditions (Clay & Schardl, 2002; Schulz & Boyle, 2005). During the long term co-evolution of endophyte and plant, equilibrium between these organisms has been established. Thus, the true endophyte will exist once equilibrium is achieved between fungal activity and the plant reaction and it is maintained over time (Giménez et al., 2007). Fungal endophytes benefit plant by promoting plant growth (Dai et al., 2008), improving resistance to multiple stresses (Lewis, 2004; Malinowski et al., 2004), and protecting from diseases and insects attacks (Wilkinson et al., 2000; Tanaka et al., 2005; Vega et al., 2008, Nur Amin et al., 2013 In Press).

In our previous study, four endophytic fungi (*Aspergillus* spp., *Trichoderma* spp., *Beauveria* spp., *Gliocladium* spp.) were found to be effective against leaf blight pathogen of maize *Helminthosporium maydis* (Nur Amin et al., 2011). The aims of the current investigation were to determine effects of those isolates on pathogen of downy mildew, *Peronosclerospora* spp., in field condition and the endophitism of the endophytes on several organs of maize plant (root, stem, leaf, and seed).

## MATERIAL AND METHODS

### Source of Fungal Endophytic Isolate

Four fungal endophyte isolates, e.g. *Aspergillus* spp., *Trichoderma* spp., *Beauveria* spp., *Gliocladium* spp., obtained from our laboratory collections, were used in this research. In our previous study, the isolates were effective against leaf blight of maize *Helminthosporium maydis* (Nur Amin et al., 2011).

### Preparation of Fungal Endophyte in Powder Form

The fungal endophytes as described in point 2.1 were propagated in rice medium containing chitin (1.0 gr). The rice medium that has been soaked for 3 hours was placed into a flask and autoclaved at 121 °C for 30 minutes. After that, five pieces of endophytic fungi (0.5 cm diam.) were inoculated into. Once the fungi started growing, the flasks were shaken to assure an even fungal growth. The grown fungi were then incubated at 30 °C for 48 hours. The rice medium along with the fungi was blended to produce a powder for further study.

### Preparation of Conidial Suspension

Maize plants with the symptoms of downy mildew infection, showing chlorotic stripes or overall yellowing on the first true and successive leaves, were collected in the field and brought back to our laboratory. The leaves were cut into pieces (about 5 cm long) and then placed into big glass jars containing about 1 cm deep water. At 8 o'clock pm, the leaves were taken from the base of the glass and wiped with a wet leaf tissue paper, then put into plastic zip-lock bags with the position of the upper leaf surface facing upwards, then kept outdoor to catch the cool air. The leaves were left outdoor until 04.00 am to allow *Peronosclerospora* spp. to sporulate. At 04.00 the plastic bag containing the leaf was taken and brought back into the laboratory. Then, the plastic bag was slowly unzipped; and leaves were taken out of the bag before they were rinsed with clean water. The rinsed water (conidial suspension of *Peronosclerospora* spp.) was collected in a plastic bowl, and then put into a plastic bottle with a perforated lid.

### Field Trial

Maize plants cv. Anoman, susceptible to *Peronosclerospora* spp. infection, was used as inoculum sources in this experiment. The source plants were planted in two rows (75 cm between rows and 25 cm within a row) around the experimental plots and between replicates one month before the treated plants were planted. Seven to 10 days after emergence, the

inoculums source plants were inoculated by spraying conidial suspension of *Peronosclerospora* spp. in the morning around 5:00 to 6:00 o'clock. Maize plants cv. Srikandi Putih-1 were used as treatment plants and planted one month after the inoculum source plants were planted or when the infection rates on the inoculums source plants around 80% - 90%.

The experiment consisted of five treatments, e.g. *Aspergillus* spp., *Trichoderma* spp., *Beauveria* spp., *Gliocladium* spp., and Control (no fungus isolate). The treatments were arranged in a randomized complete block design with four replications. Each replication consisted of one plot (4 rows wide and 5 m long) with a planting space of 75 cm between rows and 25 cm within a row. A single seed was sown in each planting hole made by using a sharp wooden poll. Each endophyte treatment was applied twice during the planting season. First application was carried out before planting as seed treatment. Seeds were soaked for 24 hours in a solution of endophytic fungal conidia whereas the control seeds were soaked in sterile distilled water. The second fungal endophyte application was performed 20 days after plant emergence. The fungi were applied as flour formulation mixed with soil in the plant rhizosphere. Field observations were performed on each treated plant 2, 3, 4, 5, and 6 weeks after the second endophyte application to determine the number of plants normally growing and the number of plants infected with downy mildew in each plot. Besides that, the capability of each endophyte to endophytically live in different plant organs (root, stem, leaf, and seed) was also determined. Root, stem, leaf, and seed samples were collected from each treatment plot. The parts were cut into small pieces and then briefly soaked into a bleach solution before they were individually put into a Petri dish containing PDA. Fungal colony growing in Petri dish was observed and identified under a compound microscope.

## RESULT AND DISCUSSION

### Results

The results showed that, from the first observation to the last observation, the percentage of severity damage on the maize crop with isolate *Beauveria* sp. treatment was the lower than the control. Statistically, however, this trial was not significant difference (Figure. 1). Overall, an upward trend could be obviously seen in all host-indophytic fungi against down mildew disease. At the beginning of the observation, the disease of down mildew in maize was only about 4% of damaging crop, increased slightly to about 30% in 4 WAI, afterwards, it rose significantly to reach a peak of about 90% of severity damage (particularly *Trichoderma* spp. and Control), and finally dropped slightly approximately 10% point, or reaching at about 80% of severity damage in the end of weeks. In terms of endophytism study, there varied among the isolates to exist within the crop layers. For instance, in the root tissues, the percentage of isolate coexisted were 14% of isolate *Trichoderma* spp. and 2% of isolate *Beauveria* spp. In addition, coexisting the isolate of *Gliocladium* spp. was detected 16% of inner the stem layers and 92% of within the leaf layers. However, during endophytism trials, in the seed tissues, no fungal endophytic isolate coexisted inner the seed layers. The fungal *Aspergillus* spp. was not found in all parts of the plant tissues.

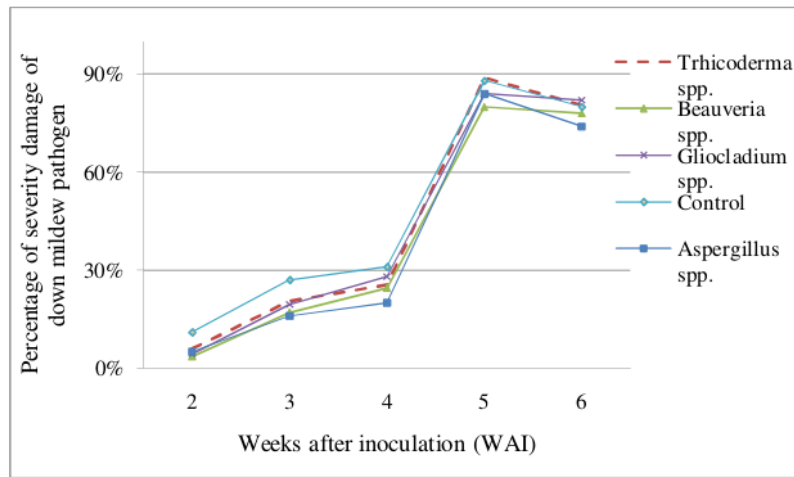


Figure 1. Percentage of severity damage of downy mildew pathogen

Figure 2 in contrast to Figure 1, The Line Chart reveals that the percentage of growth of host-endophytic fungi that infected by the down mildew pathogen dropped dramatically until the end of period of observation time. In short, there was a downward trend of the growth of host endophytic fungi in the period of time, from 2 WAI to 4 WAI, the growth of crops were a stable remain in all of treatments-endophytic fungi. However, the last 2WAI of period time, the growth of crops in all trials dropped gradually until reached at about 30%.

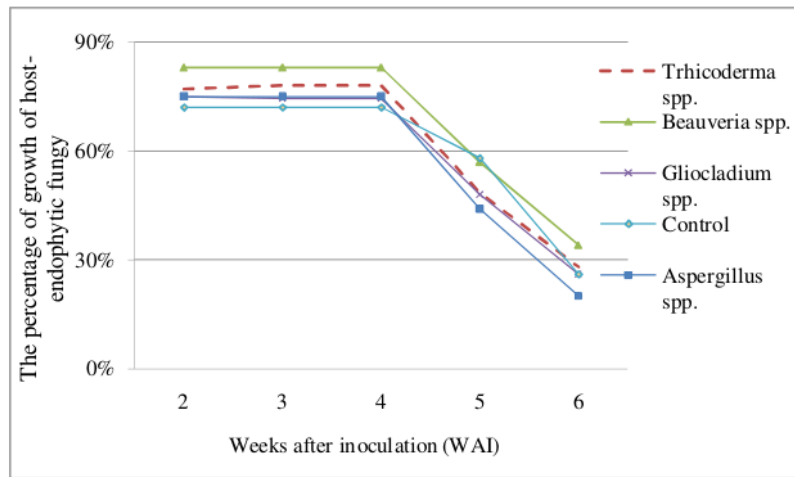


Figure 2. The Percentage of growth of host endophytic fungi

Figure 3. the Bar Chart shows that, the proportion endophytic fungi endured into the host layers, It can be seen that the endophytic fungi endured in different host-layers. the larger proportion of fungal *Gliocladium* spp. was the highest in host-leaves. However, the fewest existence of endophytism in the roots was *Beauveria* spp. and the higher percentage was *Trichoderma* spp. For instance, both in the leaves and stems layers, endophytic *Gliocladium* spp, was over 90% of total samples and 14% of samples respectively. In addition, Fungal *Trichoderma* spp., existed in the roots, was about 14%. There were not fungi that coexisted in the seed.

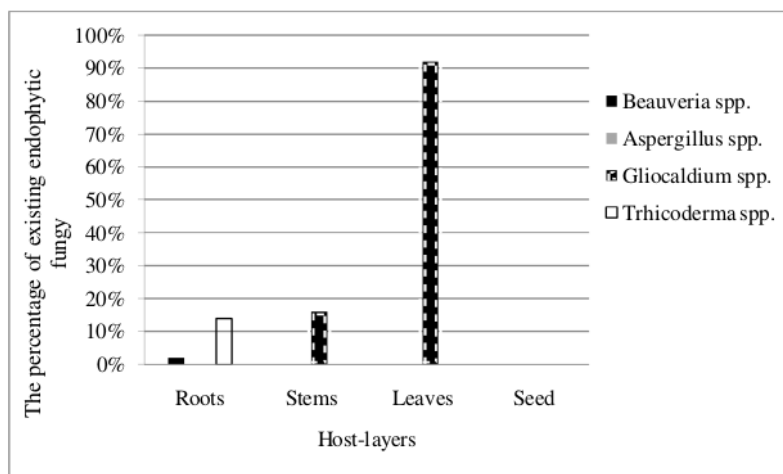


Figure 3. The Percentage of existing endophytic fungi

## DISCUSSION

The intensity damaged on the treatment isolates *Beauveria* sp. since the first observation to the last observation is always lower than the control, although did not show statistically significant difference (Figure 1). Some of researchers have described numerous modes of action of endophytic fungi in suppressing the growth of many plant pathogens. Gunatilaka (2006) reported that secondary metabolites and some of these compounds produced by fungal endophytes have antifungal and anti-bacterial properties, which strongly inhibit the growth of other microorganisms, including plant pathogens. A group of biocontrol strains can produce single or multiple kinds of antibiotics including terpenoids, alkaloids, aromatic compounds and polypeptides which have been proved that many plant pathogens are sensitive to. Five cadinane sesquiterpenes derivatives were isolated from *Phomopsis cassiae*, which is an endophytic fungus isolated from *Cassia spectabilis* and 3, 11, 12-trihydroxycadalene as one of those five derivatives was revealed as the most antifungal active compound against *Cladosporium sphaerospermum* and *Clad. sporioides* (Silva et al., 2006). Alkaloids also strongly suppress microbes. For example, altersetin, a new alkaloid isolated from endophytic *Alternaria* spp., showed antibacterial activity against several pathogenic grampositive bacteria (Hellwig et al., 2003). The other one fungal endophytes can also enhanced induction plant resistance through production of some PR protein (Vallad & Goodman, 2004; Tripathi et al., 2008).

Observations of the number of plants growing in the treatment of *Beauveria* sp. isolate always higher from the first observation to the last observation (Figure 2). Previous studies conducted by other workers demonstrated that plants infected with endophytes grew more quickly (Janarthine & Eganathan, 2012), probably due to production of gibberellins and indole acetic acid by the endophytes (Khan et al, 2012). Besides that, plants infected by endophytes became more tolerant to unsuitable soil conditions (Malinowski et al., 2004). The enhancement of plant growth may be influenced by compounds like phytohormones produced by fungal endophytes. *Colletotrichum* sp., an endophytic fungus in *A. annua* produces substances like indole acetic acid (IAA) to regulate plant processes (Lu et al., 2000).

In the study of endophitisms that the fungus *Aspergillus* spp was not found in all parts of the plant tissue, while *Trichoderma* spp. and *Beauveria* spp. were found in root tissue by 14%,

and 2%, respectively. Whereas on *Gliocladium* spp., found in the stems and leaves of maize plants with the rates of 16% and 92%, respectively (Figure. 3). None endophyte was detected in maize seed tissues. Nur Amin et al (2013 In Press) found the same result, where *Beauveria* spp. is not detected in the pod of cocoa after two week treatment with the fungus.

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