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Isolation and Identification of Endophytic Fungi and Its Antagonistic Potential against *Curvularia* sp. in Maize

ANDI IQBAL[1]
MS in Department of Plant Pests and Diseases
Faculty of Agriculture
Hasanuddin University Makassar, Indonesia
NUR AMIN
Department of Plant Pests and Diseases
Faculty of Agriculture
Hasanuddin University Makassar, Indonesia
ANDI NASRUDDIN
Department of Plant Pests and Diseases
Faculty of Agriculture
Hasanuddin University Makassar, Indonesia

Abstract:

Curvularia leaf spot disease on maize caused pathogenic fungi Curvularia sp. The disease is a minor disease in Indonesia but the plant attacked could reach 40-90%. Commonly symptoms in small brownish yellow spots caused leaves dry. When the diseases in higher level, caused damage in stem and cobs. The purpose of the research is to isolate, identify and observe in vitro condition the antagonistic activities of endophytic fungi against Curvularia sp. caused leaf spot disease in maize. The research was conducted in two place of maize plantation in Gowa and Maros district, South Sulawesi. The stage of research is : (1) isolation of endophytic fungi from healthy maize used plant part (root, stem and leaves) in BBPP Batangkaluku, Gowa district; (2) isolation of pathogenic fungi taken from maize attacked Curvularia sp. on the leaves. The sample was carry out from experimental area in Maros district; (3) pathogenicity test of

[1] Corresponding author: iqbalandi91@gmail.com

endophytic fungi and (4) dual culture test for potential endophytic fungi to be antagonist against Curvularia sp. The results showed endophytic fungi from healthy maize in Gowa district divided into four genera including : Fusarium sp. found in leaves and stems, Trichoderma sp. in stems and roots, Rhizoctonia sp. in leaves and Gliocladium sp. in root. Endophytic fungi has potential as antagonists after a dual culture test are Trichoderma sp. found in the stem having a PIGR (Percentage Inhibition of Radial Growth) value of 60%, Trichoderma sp. in the root about 66% and Gliocladium sp. has the highest PIGR value of 69%. Fusarium sp. and Rhizoctonia sp. has PIGR values about 10%, 23%, 23%, and 17%, respectively. The endophytic fungi considered as potential antagonists against Curvularia sp. at maize.

Keywords: endophytic fungi, leaf spot disease, *Curvularia* sp., *Trichoderma* sp., maize

1. INTRODUCTION

Maize (*Zea mays* L.) is famous food crop besides rice, wheat, potatoes and cassava. In Indonesia, especially in the regions of East Java (Madura) and Nusa Tenggara, maize as the important crops for staple food. Maize have many benefits such as human food, cattle feed, fiber and fuel [6, 18].

The one of important diseases attacked maize plantation is *Curvularia* leaf spot. Current status of *Curvularia* sp. in Indonesia especially in South Sulawesi, there are still classified as a minor disease. In contrast, *Curvularia* sp. caused high intensity of maize disease reaching 40 - 90%. The conidia easy spread through the wind, splashing rain and human. *Curvularia* sp. has many alternative hosts including cereal and weedy plants. The conidia able survive in the waste of maize harvest [2, 22].

The maize leaf spot disease caused *Curvularia* sp. has symptoms of small brownish-yellow spots measuring between 1 to 2 mm, attacking from the first leaf and faster moving to the another leaves. The spots causing dry leaves and die. Beside attacked leaves, the leaf spot of *Curvularia* sp. can attack stem and cobs [2]. The specific pathogen cause maize leaf spot is *Curvularia lunata* (Walker)

Boedijn. *C. lunata* belongs to the Family : Pleosporales, Genus : *Cochliobolus*, Species : *Cochliobolus lunatus* (=*C. lunata*) [2]. The fungi transmit a diseases as soil-borne and seed-borne pathogens. The fungi produce conidiophores in the form of angled brown with conidia curved in the host tissue and culture medium. The morphological form of the *C. lunata* colony commonly brown, gray, or black. The conidia contain 3-5 cells, the middle cell is enlarged, dark, and curved. The hypha is branching and cycling while the conidiophores are not branching and cycling. The size of the converse ranges from 29 × 8-10µm [5, 11, 24].

Agriculture development is now leading to eco-friendly agriculture where chemical pesticides are reduced. The biological control activities focused maintain of endophytic fungi in suppressing plant diseases. Endophytes are organisms that live in plant tissues that are parasitic or non-parasitic [1]. Another opinion, endophytes fungi are microorganisms that live in plant tissue without causing symptoms of disease [19]. The disease symptoms from host plant also caused endophytes under stress conditions. Endophytic fungi giving benefit to plants by increasing plant growth [9], increasing plant resistance to various types of stress [15], protecting plants from disease and insect pests [23].

Many endophytic fungi produce secondary metabolites and some contain anti-fungal and anti-bacteria which are very strong in inhibiting other microorganisms including plant pathogens [16]. The biocontrol group can produce one or several antibiotics including terpenoids, alkaloids, aromatic compounds and polypeptides which have proven plant pathogens are sensitive to these compounds. Five sesquiterpenes cadinane derivatives were isolated from *Phompis cassiae*, which is an endophytic fungus isolated from *Cassia spectabilis*. One of these is known as *3, 11, 12-trihydroxycadalene* as one of the most active antifungal compounds against *Cladosporium sphaerospermum* and *C. sporioides* [20]. The purpose of the research is to isolate, identify and observe in vitro condition the antagonistic activities of endophytic fungi against *Curvularia* sp. caused leaf spot disease in maize.

2. MATERIAL AND METHODS

2.1. Isolation and Identification of Endophytic Fungi

Isolation of endophytic fungi from maize plantation conducted at BBPP Batangkaluku, Gowa district, South Sulawesi. Maize plant parts (leaves, stems and roots) are washed with running water, cutting into small pieces with a diameter 0.5 cm. Maize roots were sterilized by NaOCl (2.5%), stems and leaves used NaOCl (1%) for 5 minutes, then rinse with distilled water five times, respectively. The sterilized pieces of maize part transferred to 25% Potato Dextrose Agar (PDA) medium in several petridishes and stored in the incubator at 28°C and observed every day. The next step, the growth fungi from maize plant part was purified and stored using a PDA into a refrigerator. The fungi was observed in macroscopically and microscopically with digital microscope [4, 5, 24].

2.2. Isolation of Pathogenic Fungi

Maize leaves infected with *Curvularia* sp. taken from Maros district, South Sulawesi. Leaf samples cutting into small pieces with diameter 1 cm. Leave pieces taken between healthy part of the leaf and the site of fungi infection. The pieces of maize leaves sterilized by immersing in 1% sodium hypocloride (NaOCl) suspension for 5 minutes then rinsed with distilled water five times and dried in several minutes. The small pieces put into a petridish containing PDA and stored in incubator at 28°C for 3 days. The purified process by transferring culture to a new PDA medium [3].

2.3. Pathogenicity Test of Endophytic Fungi

The process of pathogenicity test conducted by planting cucumber seeds into a petridish with wet filter paper media. Each isolate of endophytic and pathogenic fungi was diluted to a concentration of 10^{-6}. The cucumber seeds were soaked in each isolate for half an hour. The control treatment was soaked cucumber seed with distilled water. Each petridish was planted with 10 cucumber seeds and repeated three times for each isolate of endophytic fungi, pathogens and control. After two weeks observation of the percentage length of leaves, crown and root. Samples were observed three sprouts per cup [17]. The data from observation were processed using Oneway of ANOVA [1).

2.4. Dual Culture Test

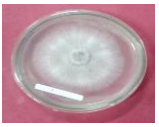
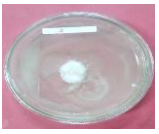
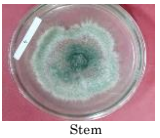
Endophytic fungi and *Curvularia* sp. were taken with cork borer and micro needle. Both fungi, endophytes and pathogens were grown together at a distance of 3 cm in the same petridish and incubated at 27°C. Then measured the diameter both of the colony for seven days. Control was made with the same steps, but only pathogens are grown. Each treatment was repeated three times. The effect of antagonism of endophytic fungi was calculated using PIGR (*Percentage Inhibition of Radial Growth*) [2, 3, 12].

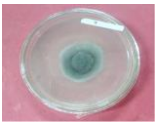
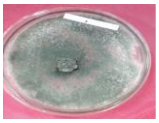
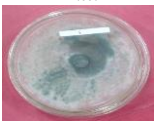
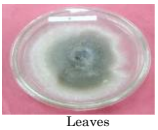
3. RESULTS AND DISCUSSION

3.1. Isolation and Identification of Endophytic Fungi

There are seven types of endophytic fungi isolated from the root, stem and leaves of healthy maize are planted in the BBPP Batangkaluku, Gowa District. All of endophytic fungi have been purified on PDA medium. Based identification under a microscope, isolate 1 and isolate 2 are *Fusarium* sp., isolate 3 and isolat 5 namely *Trichoderma* sp.; isolate 4 and isolate 7 are *Rhizoctonia* sp. while isolate 6 is *Gliocladium* sp. (Table 1).

Table 1. Characteristic of Endophytic Fungi based Isolation from Healthy Maize

Isolate Code	Macroscopic		Hyphae		Forming of conidia/spore	Genus	Source of Inoculum
	Upper Surface	Lower Surface	Color	Septum condition			
Isolate 1	White, the color similar to outer surface, smooth texture.	White	Hyaline	Septum	Ellips	*Fusarium*	 Leaves and stem
Isolate 2	White, smooth and solid texture.	White	Hyaline	Septum	Ellips, conidia has septum, slighty curved	*Fusarium*	 Stem
Isolate 3	Dark green, white in the surface outer, solid and smooth texture.	Yelllowish white	Hyaline	Septum	Round	*Trichoderma*	 Stem

Isolate 4	Gray with darker in the middle, white in the outer surface, solid texture.	White	Hyaline	Septum	Small round	*Rhizoctonia*	 Leaves
Isolate 5	Dark green, white in the outer surface, smooth texture	Yellowish white	Hialin	Septum	Round	*Trichoderma*	 Root
Isolate 6	Green, white in the outer surface, small part separate one to another, solid texture.	Putih	Hialin	Septum	Round	*Gliocladium*	 Root
Isolate 7	Dark gray, white in the outer surface, solid and smooth texture.	White	Hyaline	Septum	Small round	*Rhizoctonia*	 Leaves

In every cropping system, there are contain many microorganisms (fungi, bacteria, viruses, nematodes etc.) that grow on the surface of plants including plant tissues. In this study, seven types of endophytic fungi were found in the roots, stems and leaves of healthy maize plant. According to observation result, the endophytic fungi have great variety of colors, shapes and textures. After process identified consisted of four genera based on the characteristics morphology of colony and conidia/spores including: *Fusarium* sp. (leaves and stems), *Trichoderma* sp. (stems and roots), *Rhizoctonia* sp. (leaves) and *Gliocladium* sp.(root).

In the other hand, root of healthy pulut maize isolated and resulted 63 endophytic fungi isolates that grew in PDA medium. There were six genera including *Trichoderma, Fusarium, Aspergillus, Penicillium, Acremonium*, and *Botryodiplodia* isolated from pulut maize [3]. Isolation of maize roots in Maros (South Sulawesi) and Tumpang (East Java) areas was found endophytic fungi *Gliocladium* sp., *Trichoderma* sp. and *Rhizoctonia* sp. causing decay of maize fronds isolated from symptomatic maize plants in the same location [22].

3.2. Isolation of Pathogenic Fungi

Maize plants in the experimental areas from Maros district infected with *Curvularia* leaf spot showed symptoms of small yellow or brown spots measuring between 1 mm - 2 mm, taken and taken in the laboratory to be observed under a microscope. *Curvularia* sp. colonies growth on PDA medium are shaped like feathers or black-colored cotton, whereas under the microscope as brown conidiophores and conidia divided by 3-5 parts (Figure 2).

Figure 2. Colony and conidia from *Curvularia* sp.

3.3. Pathogenicity Test of Endophytic Fungi

After two weeks of treatments, observation focused on the growth of cucumber seeds were treated with seven isolates of divided into : endophytic fungi, pathogens and distilled water/control. The results showed after statistical tests using the Oneway ANOVA, all of the treatment non significant effect on the growth of length of leaves, stems, and roots of cucumber seeds (Table 2).

Table 2. Impact of cucumber seed viability after soaked in different isolates

Isolates	Number	Average	SD	Sig
Fusarium sp.	3	2.9533	2.46293	
Fusarium sp.	3	2.4267	1.64974	
Trichoderma sp.	3	1.8367	.81586	
Rhizoctonia sp.	3	2.1000	1.66874	
Trichoderma sp.	3	2.4367	1.44507	0.979
Gliocladium sp.	3	2.7367	2.19108	
Rhizoctonia sp.	3	1.4767	1.76398	
Control (distilled water)	3	2.9267	2.37744	
Curvularia sp.	3	1.9300	1.61332	
Total	**27**	**2.3137**	**1.60918**	

Sig < 0.05 relationship between isolates of endophytic fungi and growth of cucumber seeds

In another study found that cucumber more susceptible than radish when in vitro pathogenicity testing of the virulence level of *Rhizoctonia* sp. isolates [13]. The term endophytes refers more to microbes that are beneficial to the host plant [10, 19]. Pathogens that infect plants also include endophytic microbes when viewed from the theory of evolution. Endophytes originate from minor pathogens and then interact with their hosts in mutualism. Secondary metabolites produced by endophytic fungi can change the balance of defense interactions or mutually beneficial interactions because sometimes endophytic fungi can turn into pathogens [10, 16].

3.4. Dual Culture Test

The observation of the dual culture test conducted a week after planting fungi on PDA medium. The observations in the value of PIGR (*Percentage Inhibition of Radial Growth*) indicate that *Trichoderma* sp. and *Gliocladium* sp. showed higher results than other endophytic fungi isolates in suppressing the growth of *Curvularia* sp. on maize.

The results of dual culture test showed there are three endophytic fungi in higher PIGR values : *Trichoderma* sp. from stem (60%), *Trichoderma* sp. from root (66%) and *Gliocladium* sp. (69%). Endophytic fungi in lower PIGR value such as *Fusarium* sp. (10% and 23%) and *Rhizoctonia* sp. (23% and 17%) are considered unable to suppress development of the pathogenic fungi *Curvularia* sp. Based on the results, not all of endophytic fungi can suppress pathogenic fungi, because differences in the speed of growth and unable competition for space and nutrition. According to [8, 12, 14], normally endophytic and pathogenic fungi in nature always competition with another fungi in the scope of space and nutrition. In the other side, the capability inhibiting the growth of other pathogenic fungi will improve plant healthy.

The direct interaction of endophytes and pathogens showed in hyperparasitism, competition or antibiosis [7]. *Trichoderma* sp. living in stem and root suppress *Curvularia* sp. growth through nutritional competition and space. This is indicate rapid growth of *Trichoderma* sp. covering petridishes., then inhibit *Curvularia* sp. Based testing the activity of protease, lipase and chitinase enzymes, all of the results were negative. The ability of inhibit pathogenic fungi is possible because *Trichoderma* sp. produces antibiotic compounds.

Trichoderma sp. produces gliotoksin and viridian as antibiotics against pathogens [22]. Gliocladium sp. also suppresses the growth of *Curvularia* sp. through space and nutrition competition. The endophytic fungi growth faster and dominant covered growth of *Curvularia* sp. Besides being competitive, it is suspected that *Gliocladium* sp. produces antibiotic compounds inhibit pathogens. *Gliocladium* sp. produces antifungal for inhibit pathogens such as gliovirin and viridan.

4. CONCLUSION

There are four genera of endophytic fungi found in healthy maize plantation in BBPP Batangkaluku Gowa district include : *Fusarium* sp., *Trichoderma* sp., *Rhizoctonia* sp. and *Gliocladium* sp. The results of the dual culture, there are three endophytic fungi has high PIGR values and potential to suppress growth of pathogenic fungi cause *Curvularia* leaf spot : *Trichoderma* sp. found in the stems (60%), *Trichoderma* sp. found in the roots (66%) and *Gliocladium* sp. (69%), respectively.

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