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THE POTENTIAL OF ENDOPHYTIC FUNGI AS BIOCONTROL AND PHOSPATE SOLUBILIZATION AGENT IN *Capsicum anuum*

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ABSTRACT

The productivity of chili (*Capsicum annuum*) in Indonesia is currently very low. Some factors that influenced it including the presence of pathogenic microorganisms which lead to the low availability of phosphate in the soil. This condition become a limiting factor for plant growth and production. Endophytic fungi can be used as antagonistic agents in inhibiting pathogenic fungi and to increase the efficiency of phosphate solubilization known as phospholytic fungi. This study aimed to find antagonistic agents from endophytic fungi that can suppress the growth of pathogenic fungi and test the ability of endophytic fungi to dissolve phosphate. Fungi isolates used were BB-Biogen collection isolates, consisting of 42 endophytic fungi isolates, and 3 pathogenic fungi isolates (*Fusarium* sp., *Colletotrichum acutatum*, *Phytophthora capsici*) on chili plants (*C. annuum*). The antagonism test was carried out using the dual culture method in the Potato Dextrose Agar (PDA) medium for 5 days incubation at temperature (± 28 ° C). The parameters measured were based on the formation of inhibition zones and the calculation of the percentage of growth inhibition of fungi isolates. The test results obtained 7 representative fungi isolates (RIVA4, RIVA5, MIVD2, *Aspergillus niger*, *Cladosporium sp.*, *Cladosporium oxysporum*, and *Chaetomium globosum*). Based on the calculation of the percentage of growth inhibition, fungi isolates with RIVA5 code have a higher potential in inhibiting the growth of all three pathogenic fungi. Calculation of the percentage of endophytic fungi inhibition of RIVA5 were 70.3% (*Fusarium* sp.), 63.3% (*C. acutatum*), and 60% (*P. capsici*). Phosphate test was carried out by the cork borer method in pikovskaya medium for 4 days incubation at 27-28 ° C. The parameters measured were based on the formation of clear zones around the colony. There were 4 endophytic fungi isolates (MIVA4, MIVF7, *Aspergillus sydowii*, and *A. niger*) formed a clear zone around the colony, which indicates the presence of phospholytic activity. Based on the calculation of the phospholytic index, *A. niger* isolates have a high phosphate solubility index value of 5.

Keywords: *Capsicum anuum*, endophytic fungi, pathogenic fungi, phospholytic fungi

INTRODUCTION

Chili plants (*Capsicum annum*) is one of the most popular vegetable commodities in Indonesian and has a high economic value (**Mariyono *et al.*, 2015**). Chili is one of the main commodities for Indonesian farmers because it can be planted in various fields and known to have high adaptability (**Ali, 2006**). Chili productivity in Indonesia is still very low, due to many factors such as pathogenic microorganisms which can reduce the quality and quantity of production (**Than *et al.*, 2008; Kim *et al.*, 1999**).

Indonesian farmers generally still uses a lot of synthetic pesticides because of the ease of obtaining and effectiveness. Even though many research results show that the excessive use of synthetic pesticides results in environment problems and endanger to human health (**Nantawanit *et al.*, 2010**). A fairly safe and environmentally friendly control is needed as an alternative control, one of which is by using endophytic microbes. Endophytic microbes are microbes found in plant tissue systems such as leaves, flowers, twigs, or plant roots. Endophytic microbes grow and get nutrient from their host plants. Endophytic microbes can be in the form of bacteria or fungi, but in the last decade, fungi gained more attention to be explored (**Putra *et al.*, 2015**). Endophytic fungi can produce bioactive compounds such as antibiotic, enzyme, plant growth promoting substances **(Sun *et al.*, 2008; Bezeerra *et al.*, 2015, and Hwang *et al.*, 2011)**. As antibiotic producer, endophytic fungi have a potency to inhibit the growth of pathogenic fungi and are expected to be able to effectively become biocontrol agents for pathogenic microorganisms (**Gao *et al.*, 2010**). Pathogenic microorganisms can be a limiting factor that can reduce the quality and quantity of production in plants, in addition to these factors, the form of P availability in the soil is also a limiting factor for growth and production in plants. The increase in phosphate causes the phosphate fertilizer given is inefficient, which as the result, phosphate needs to be given in high quantities (**Brindaban *et al.*, 2020**).

An alternative that can be used to improve the efficiency of phosphate fertilization is to utilize microorganisms that can dissolve phosphate. Phosphate solvent microorganisms are microorganisms that can extract phosphate from an insoluble form into a form available to plants through the secretion of organic acids produced to release P from the sorption complex (**Khan, *et al.*, 2009**). This study aims to find the antagonistic agent of endophytic fungi against pathogenic fungi as well as their ability to dissolve phosphate.

MATERIAL AND METHODS

Sample Collection

Endophytic fungi isolates and pathogenic fungi were obtained from Biogen Culture Collection. About 18 isolates of endophytic fungi were obtained from *Rhodomyrthus tomentosa*, and 15 isolates from *Melastoma malabathricum*, 9 isolates from the *Alpinia malaccensis*. while as many as 3 isolates of pathogenic fungi (*Fusarium* sp., *Colletotrichum acutatum*, *Phytophthora capsici*) were taken from *Capsicum annuum*.

Morphology Observation of Fungal Endophyte

The fungi were firstly grouped on the bases of their colony appearance on PDA such as colony shape, color, elevation, texture, mycelia type, edges, density, and diameter. Fungal colonies with similar characteristics were grouped into the same morphotypes.

Antagonism test of fungi

Antagonism test of fungi was carried out by the dual culture method based on **Naik *et al.*, (2009**). Pathogenic and endophytic fungi were inoculated on PDA medium in a Petri dish (diameter 9 cm) with a distance of 3 cm. Incubation was carried out at room temperature (± 28 °C) for 5 days. Percentage inhibition of endophytic fungi against pathogen was calculated using the formula:

$$H = \frac{J1 - J2}{J1} \times 100\%$$

H= Percentage of inhibition
J1 = the radius of the pathogenic fungi colony towards the edge of the Petri dish
J2= the radius of the pathogenic fungi colony that is headed toward the fungus

Phosphat sollubilization activity test

Endophytic fungal isolates were inoculated using sterile straws and transferred into Pikovskaya medium in a petri dish. The media was incubated at room temperature (± 28 °C) for 7 days. Indications of phosphate dissolution by fungi can be characterized by the formation of clear zones around colonies. The activity of fungi in phosphate degradation is expressed by the value of the Phospholytic Index measured using the following formula:

$$\text{Phospholytic Index} = \frac{\text{diameter of clear zone (cm)}}{\text{diameter of fungi colony (cm)}}$$

RESULTS AND DISCUSSION

A total of 41 endophytic fungi were characterized based on their morphology on PDA medium. Each endophytic colony shows a unique and varied appearance of colonies. Most of endophytic fungi have circullar shape, flat elevation, hypha cottony texture, aerial myselium, and average colony size after 11 weeks by 6.8 cm (Fig. 1; Table 1).

Table 1 Culture characteristics of the Endophytic Fungi on PDA

Isolate code	Shape	Colour		Elevation	Texture	Mycelium	Edge	Size (cm) of colony after 11 days
		Above	Reverse					
RIVA1	circullar	brown	cream to yellow	raised	Cottony	aerial	entire	7.2
RIVA2	circullar	dark green	cream to yellow	raised	Cottony	aerial	entire	7.2
RIVA3	irregular	white	Cream	raised	fluffy	aerial	dentate	8
RIVA4	circullar	white	Cream	Flat	cottony	aerial	filamentous	8.5
RIVA5	circullar	white	Cream	Flat	velvety	aerial	filamentous	9.5
RIVD1	irregular	white,black	cream to yellow	convex	rocky	immersed	dentate	9
RIVD2	circullar	white	Cream	raised	rocky	aerial	entire	3
RIVD3	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	7.4
RIVD5	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	2.5
RIVD6	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	2.5
RIVD7	irregular	black,white	Cream	convex	rocky	immersed	dentate	2.8
RIVD8	circullar	white	Cream	raised	cottony	aerial	entire	2.7
RIVD9	irregular	black,white	cream to yellow	raised	rocky	immersed	dentate	9
RIVD10	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	2.6
RIVD11	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	3.2
RIVD14	circullar	white	Cream	raised	cottony	aerial	entire	1.5
RIVD15	circullar	white	Cream	raised	cottony	aerial	entire	8.4
RIVD16	irregular	black,white	cream to yellow	convex	rocky	immersed	dentate	9
MIVF3	irregular	grey	Grey	Flat	fluffy	aerial	undulate	2
MIVF4	circullar	white	Cream	raised	cottony	aerial	undulate	8.3
MIVF5	circullar	white	Cream	convex	fluffy	aerial	filamentous	8.3
MIVF6	circullar	white	Cream	convex	cottony	aerial	dentate	8.2
MIVF7	circullar	white	Cream	raised	cottony	aerial	dentate	8.5
MIVA1	circullar	white	Cream	raised	orcky	immersed	entire	2.5
MIVA2	irregular	white	Cream	Flat	cottony	aerial	dentate	9
MIVA3	circullar	white	Cream	raised	cottony	aerial	entire	9
MIVA4	circullar	white,cream	cream to yellow	raised	cottony	aerial	entire	9
MIVB1	irregular	white	Cream	raised	cottony	aerial	dentate	6.5
MIVB2	circullar	grey	Grey	Flat	fluffy	aerial	filamentous	7
MIVD1	irregular	white	Cream	Flat	fluffy	aerial	entire	7
MIVD2	circullar	white	Cream	Flat	cottony	aerial	entire	9
MIVF1	circullar	white	Cream	Flat	cottony	aerial	filamentous	9
MIVF2	irregular	white	Cream	Flat	cottony	aerial	entire	9
Colletotrichum boninense	circullar	black, White	cream to yellow	Flat	fluffy	aerial	entire	7.7
Aspergillus sydowii	circullar	black	Yellow	Flat	cottony	aerial	entire	7.5
Cladosporium oxysporum	circullar	black	Yellow	Flat	cottony	aerial	entire	8.5
Aspergillus niger	circullar	black	Yellow	Flat	cottony	aerial	entire	8.8
Cladosporium sp.	circullar	black	Yellow	Flat	cottony	aerial	entire	8.3
Guignardia mangiferae	circullar	black	Yellow	Flat	cottony	aerial	entire	8.7
Chaetomium globosum	circullar	grey, cream	cream to yellow	raised	cottony	aerial	entire	8.7
Diaporthe anacardii	circullar	white	cream to yellow	raised	cottony	aerial	entire	7.6

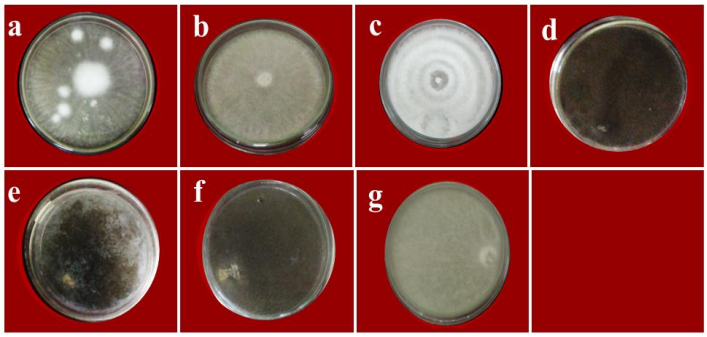

Figure 1 Colony of endophytic fungi isolates a) RIVA4, b) RIVA5, c) MIVD2, d) *Aspergillus niger*, e) *Cladosporium* sp., f) *CladosporiumOxysporum*, g) *Chaetomium globosum* on PDA medium for 7 days.

Antagonistic testing was carried out by the dual culture method, in which endophytic fungi isolates and pathogenic fungi isolates were grown together in a petri dish (**Naik *et al*., 2009**). It aims to create a mechanism of competition that occurs between the two. When pathogenic fungi isolated are inoculated in a medium that already contains endophytic fungi, the growth, and development of endophytic fungi are inhibited due to reduced space and nutrients.

The results confirmed that 7 isolates have a higher antagonistic ability than others in inhibiting the growth of pathogenic fungi. The parameters observed in antagonistic testing are the presence of inhibitory zones between pathogenic and endophytic fungi, and the reduction of pathogenic fungi mycelium. Based on the data obtained, it is known that the most potential endophytic fungi isolates in inhibiting the 3 pathogenic mold isolates are RIVA5 isolates with inhibition values of 70.3% (*Fusarium*), 63.3% (*Colletotrichum acutatum*), 60% (*Phytophthora capsici*) (Table 2).

Table2 Value of growth inhibition of pathogenic fungi by potential endophytic fungi

No	Isolate code/ name	Growth inhibition (%) of pathogenic fungi isolates		
		Fusarium sp.	*Colletotrichum acutatum*	*Phytophthora capsici*
1	RIVA4	16	61	-6
2.	RIVA5	70,3	63,3	60
3.	MIVD2	7,4	45,4	37
4.	*Aspergillus niger*	40	20	0
5.	*Cladosporium* sp.	40	50	0
6.	*CladosporiumOxysporum*	66	31,52	0
7.	*Chaetomium globosum*	52	56	0

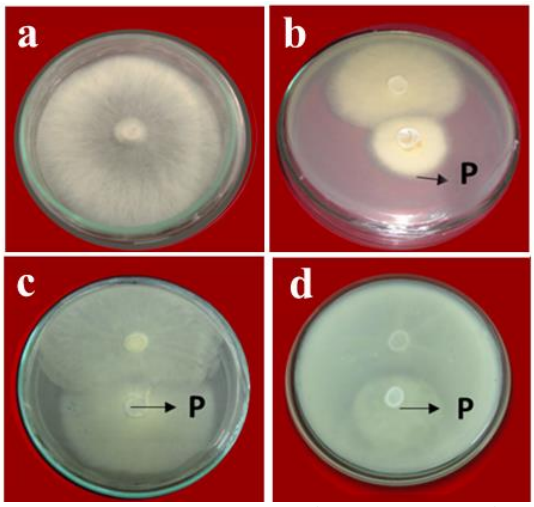

Figure 2 Antagonistic test results a) control of RIVA5 endophyticfungi isolates, antagonist test of RIVA5 endophytic fungi isolates against b) *Fusarium*, c) *Colletotrichum acutatum*, d) *Phyopthora capsici* on PDA medium aged 5 days at room temperature ± 28°C. *P= Pathogen).

Control fungi isolates without antagonistic treatment and fungi isolates with dual culture antagonist treatment had differences in colony growth (Fig. 2). Treatment fungi isolates showed reduced mycelium, non-sporulating, and had smaller diameters while control isolates without antagonistic treatment showed normal my growth of mycelium, which was not reduced, and sporulated. That is because there is an antagonist interaction between endophytic fungi isolates and pathogenic fungi isolates in antagonistic testing.

Endophytic fungi isolates in dual culture treatment have antagonistic ability to inhibit the growth of pathogenic fungi colonies. This is in line with **Talapatra *et al*., (2017)** which stated that the inhibition of the growth of mycelium colonies of pathogenic fungi is due to the antagonistic nature of endophytic fungi. Antagonistic interaction is a form of defense that includes self-defense, territory, and nutrition.

The reduction of hyphal or mycelium width that occurs in pathogenic fungi is suspected due to the antagonistic nature of endophytic fungi. Pathogenic fungi lack nutrients to grow when they are grown with endophytic fungi in the same medium so that the mycelium that is formed becomes less and there is no sporulation. The inhibition zone formed is due to the antagonism characteristic of endophytic fungi isolates. The inhibition zone is a clear zone that indicates inhibition of fungi growth due to the secretion of metabolite compounds by endophytic fungi isolates. The presence of antagonistic mechanisms in endophytic fungi against pathogenic fungi is a form of antibiosis. Secondary metabolites are metabolite compounds that function as inhibitors of growth of pathogenic fungi. These compounds are not essential for growth and are produced at certain times. Secondary metabolites are a form of self-defense from adverse environmental conditions. Secondary metabolites are in the form of pelysis enzymes, volatile compounds, sidospores, or other toxic compounds. compared to bacteria and plants, fungi are among the most productive producers of secondary metabolites (**Keller *et al*., 2005**).

Differences in the ability of antagonism between fungus can be caused by many things, including the speed of spore formation, the number of antibiotic compounds produced and the differences in specific enzymes produced. Some antagonistic mechanisms are space and nutritional competition, production of antifungal compounds, and lytic enzymes (glucanase, chitinase, and protease) (**Chet and Chernin, 2002**). The mechanism of space and nutrient competition occurs when endophytic fungi try to obtain limited space and nutrients when grown together with pathogens so that the growth activity of pathogenic fungi colonies is disrupted due to lack of nutrients and space to grow (**Janisiewicz & Korsen, 2002; Sharma *et al*., 2009**). Lytic enzymes cause degradation of protein components making up the fungi cell walls, resulting in inhibition of cell wall growth in mold mycelium (**Chet and Chernin, 2002**; **Nunes 2012**).

Regarding to the phosphate test, it was found that endophytic fungi isolates which can dissolve phosphate, including 4 fungi isolates, namely MIVA4, MIVF7, *Aspergillus sydowii*, and *A. niger*. *A. niger* has the highest phospholytic ability among other fungi isolates, with a phospholytic index value of 5.0 (Table 3).

The media used for phosphate testing is psychovaya media, which turbid into white because it contains insoluble P such as calcium phosphate. After 48-72 hours of incubation, the potential for microorganisms to grow on tricalcium phosphate agar will indicate the presence of a clear zone (Fig. 3), while other microorganisms do not exhibit this characteristic.

The presence of clear zones seen around the colony is an indicator that the fungi can dissolve phosphate in the media. Hydrolysis activity qualitatively illustrates the ability of mold isolates to remodel phosphate by comparing the size of the clear zone around the colony with the size of the colony's diameter.

The mechanism of biological phosphate dissolution occurs because these microorganisms produce enzymes such as the enzyme phosphatase and phytase enzyme. The activity of the enzyme phosphatase produced by these fungi is known through the phospholytic index.

Table 3 Value of Phospholytic Index

No.	Isolate code/ name	Diameter of clear zone (cm)	Diameter of fungi colony (cm)	Phospholytic Index
1	MIVA4	3.2	2.9	1.1
3.	MIVF7	1.6	0.9	1.7
4.	*Aspergillussydowii*	2.13	0.87	2.4
5.	*Aspergillusniger*	2.15	0.43	5.0

The observations showed a high Phosphate dissolving index and had a fast-growing ability obtained from *Aspergillus niger* isolates. *Aspergillus* are known to be everywhere and grow on almost all substrates. *Aspergillus* is a dominant group of phosphate solvent fungi found in acid soils in Indonesia. This *Aspergillus* genus has high potential in dissolving Phosphate bound to become Phosphate available in the soil.

The difference in the value of the phospholytic index in each isolate shows the difference in the activity of the enzyme phosphatase in hydrolyzing phosphate. Isolates with high index values indicate high extracellular phosphatase activity, and vice versa in isolates with low index values, phosphatase activity is also low extracellular.

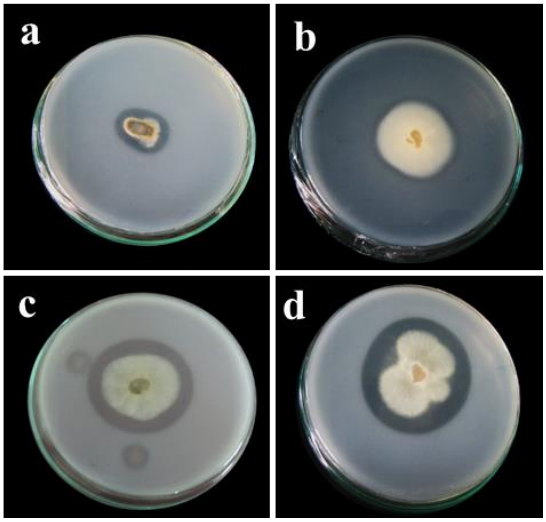

Figure 3 Phospholytic fungi isolates at 4 days after inoculation a) MIVA4, b) MIVF7, c) *A. sydowii*, d) *A. niger*

The amount of phospholytic enzyme activity produced by endophytic fungi is shown by the increasing width of the clear zone. The result of the phosphate breakdown is only indicated by the presence of a clear zone which indicates that phosphate has been overhauled into peptide compounds and amino acids which are dissolved in the medium.

The wide clear zone around the colony explains the ability of fungi to qualitatively dissolve Phosphate varies depending on the genetic nature of each microbe in producing organic acids that play a role in determining the ability of Phosphate dissolution (**Mittal *et al.*, 2008**). The superior phosphate solvent microbes will produce the largest diameter of the clear zone and are faster than other colonies.

CONCLUSION

Based on the results RIVA5 has the highest potential in inhibiting the growth of all three pathogenic fungi. Calculation of the percentage of hyphal or mycelium width inhibition of RIVA5 were 70.3% (*Fusarium* sp.), 63.3% (*C. acutatum*), and 60% (*P. capsici*). In the phosphate test, it is known that *A. niger* endophytic fungi isolates have the highest ability among other fungi isolates in hydrolyzing phosphate, with phosphate solubility index value of 5.

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