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Identification of Vesicular Arbuscular Mycorrhiza (VAM) From Soil and Its Potency in Reducing Disease Development (*Phytophthora* sp.) on 5 Citrus Rootstock

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Abstract

Phytophthora spp. is one of the fungal pathogens that kills plants on several kinds of the citrus rootstock. In other countries, it is reported that disease pathogens were reduced in roots containing Vesicular Arbuscular Mycorrhiza (VAM interaction). However, in Indonesia, there is less information about the effect of VAM on the roots of citrus plants against root disease caused by Phytophthora sp. This study aimed to identify VAM in citrus roots and study the potential of VAM in controlling root rot of Phytophthora sp. on five types of the citrus rootstock. The research was carried out at the Phytopathology Laboratory, Indonesian Citrus, and Subtropical Fruits Research Institute (ICSFRI). Phytophthora spp. and VAM samples originated from several citrus centers endemic to Phytophthora were collected. VAM was isolated from the rhizosphere area of citrus plants, while Phytophthora sp. was isolated from infected plant roots. The fungus isolates were isolated, purified, then identified through references. The test of the potential of VAM in increasing resistance of root diseases caused by Phytophthora sp. was performed at the screen house in ICSFRI. The results of the study showed that VAM was identified in 39 gardens in 6 districts from samples collected in 49 yards in 10 regions of citrus centers. The dominant VAM genus is Glomus sp. with the highest density of spores was originated from Ponorogo area. The results of the identification of Phytophthora morphologically showed a diversity of Phytophthora, namely P. parasitica, P. palmivora, and P. citrophthora. The test of the potential of VAM in increasing plant resistance to Phytophthora results showed that Kanci, JC, RL, and Volkameriana varieties inoculated with Phytophthora sp. and Glomus sp. have higher plant height than healthy plants..

1. INTRODUCTION

One of citrus disease that causes plant death and a serious loss is root rot and stem end rot (Phytophthora sp.). This disease was reported to cause 50% loss of lateral roots (Erwin & Ribeiro, 1996). In addition, fungi can cause damping off at high temperatures and nursery beds in humid conditions (Erwin & Ribeiro, 1996). The average loss of production due to Phytophthora, Diplodia and Huanglungbin in citrus in Indonesia in 2001-2003 was 60,960 tons or Rp. 236,926,500,000 (Anonymous, 2008).

Systemic fungicides commonly used by farmers to control root disease are feared to cause environmental balance disorders (Sudewa et al., 2008). The pesticide can kill non-target organisms, increase the resistance of target organisms, its residues can absorb and accumulate in the fruit, seep into the soil, and flow of water that can kill

Aquatic organisms, and is dangerous for farmers. Based on the awareness of the dangers of unwise use of pesticides, currently agricultural practices are directed at organic farming, one of which is to use beneficial and environmentally friendly microorganisms.

VAM (*Vesicular Arbuscular Mycorrhizal*) almost can be found in all ecosystems, including acid soils (Kartika, 2006) and alkalis. According to Smith & Read (1997), VAM can be associated with almost 90% of plant species. However, the population level and composition of VAM types are very diverse and are influenced by plant characteristics and environmental factors such as temperature, soil pH, soil moisture, phosphorus and nitrogen content, and heavy metal concentrations (Daniels & Trappe, 1980 in Suamba et al., 2014). The use of VAM as a biofertilizer has recently begun to gain attention, not only because of its ability to increase absorption of water and nutrients from the soil, it generates growth hormones and also acts as an inhibitor of pathogen-borne pathogens (Hartoyo et al., 2011).

VAM can reduce disease attacks caused by Phytophthora sp., by increasing plant resistance, for example in tomato commodities (Cordier et al., 1996; Laetitia, et al., 2008), strawberries (Murphy et al., 2000). On citrus with sweet orange rootstock containing mycorrhiza (Glomus intraradices), P. parasitica decreased its development. Protection mechanisms that occur can be antibiotics, synthesis of fungistatic substances by roots, formation of physical effects of mantles caused by mycorrhizal fungi (Duchesne, 1996), using excess carbohydrates in roots, protecting rhizosphere microbial populations along roots, utilizing metabolic results symbiotic cortex cells (Fakuara, 1988), increasing nodulation, nitrogen accumulation by Rhizobium sp. in legumes (Bagyaraj et al., 1979).

The aim of this study was to determine the VAM and Phyphopthora of citrus and determine the potential of VAM isolates which were dominant in the roots of 5 citrus rootstock species of Japanese Citroen, Rough Lemon, Volkameriana, AA 23, and Kanci.

2. MATERIALS AND METHODS

The study was conducted on the Phytopathology Laboratory and Screenhouse of Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI), and citrus centers in East Java. The stages of the activity included the collection and identification of VAM and pathogenic phythopthora fungi in citrus roots originated from 18 sub-districts in 5 districts of citrus that are Blitar, Tulungagung, Ponorogo, Jember and Banyuwangi, of East Java province. The plant material used for testing were 5 varieties of citrus rootstock : *Japanese Citroen* (JC), *Rough Lemon* (RL), Volkameriana, AA23, and Kanci grown from seeds. Transplanting was carried out after plants were ± 2 months old in pots sized 30 cm in diameter and 35 cm high; each replication of them was 10 pots. The media used were a mixture of soil and manure with ratio of 1:1.

Plant maintenance was carried out optimally. Corn plants (*Zea mays*) were planted in plastic polybags to maintain and multiply VAM from the field collection. The variables observed in this study were the morphological characters of VAM spores which included the shape and color of spores, character, and morphology of *Phythopthora* spp. and VAM infection at the root.

VAM Identification from Several Citrus Centers in East Java

Samples were taken from the rhizosphere area and the mass of the roots of healthy plants at a depth of 10, 20, and 30 cm. Each soil sample was taken approximately 200 grams, taking distance 10-50 cm from the base of the stem, and placed in a plastic bag. Taking root samples was done by cutting the roots of the young roots. Isolation and identification of VAM were done by weighing a sample of 100 gr, then put in a 1000 ml beaker glass and add water to a volume of 1 litre. The soil was stirred for ± 10 minutes until it was homogeneous and the soil aggregate was broken down by hand so that the spores were free from the soil. The suspension was left for ± 1 minute until large particles settled. The supernatant liquid was poured into a multilevel filter with a hole diameter of 270 µm, 150 µm, 100 µm, and 45 µm. (this procedure was repeated 2-3 times). Each filter was rinsed with tap water to ensure that all small particles were carried away. Filter residues sized of 270 µm, 150 µm, 100 µm, and 50 µm were inserted into the test tube containing sterile water and centrifuged for 5 minutes at a speed of 2000 rpm, the supernatant and the remaining roots were removed, then the pellets were taken and resuspended in 50% sucrose then centrifuged for 1 minute at 2000 rpm. The supernatant was washed on a 40-50 µm sieve to remove sucrose before filtration with vacuum. After filtration with vacuum, the spores obtained were placed on petridish and observed under a microscope with a magnification of 100 x. The microscopic features of the spores found were then matched with the identification guidelines used by INVAM to determine the VAM genus found.

***Phythopthora* sp. Pathogen Identification from Several Citrus Centers**

Isolates of Phytophthora sp were collected from roots of infected plants and isolation of pathogens using bait techniques (Erwin & Ribeiro, 1996) with lemons and apples to isolate fungi from soil. Roots and infected stem-end samples from citrus orchards in East Java namely Banyuwangi, Jember, Ponorogo, Blitar and Tulungagung districts, a total of 24 isolates on plates containing V8 media. These isolates were incubated at room temperature in the dark for a week. Colonies showing the morphological characteristics of Phytophthora were observed under a microscope. These isolates were planted at 24°C in 10 ml water culture for microscopic identification under a binocular microscope.

Test of the Potential of VAM in Reducing the Attack of Root Disease Phytophthora sp

The rhizosphere area of 5 citrus varieties, namely JC, RL, Volkameriana, AA23, and Kanci (4 months old), inoculated with 5 grams of *Glomus* sp., Together with plants transplanting of large polybag using sterile media. After two months, the plants were inoculated with 100 ml of *Phytophthora* sp. by soil drenching around the roots. Each treatment was repeated 10 times. As a comparison, an observation on plants that were not treated by VAM and inoculated with *Phytphtora* sp was conducted. Parameters observed were (1) strength of root infection of VAM spores by calculating the percentage of infection in plant roots (vesicles), (2) visual symptoms of phytophthora disease, (3) vegetative growth of plants (plant height, number of leaves). Calculation of the percentage of VAM infection in plant roots was done by taking 2 g of random root samples, ± 1 cm, and staining with trypan blue according to the Kormanik & Mc Graw (1982) method. Observations were

carried out under a microscope with 250 x magnification in 3 fields of view of the microscope. Indikator of VAM infection characterized by spores, vesicles, or VAM hyphae in the root tissue. VAM infection in roots were calculated based on the number of vesicles as follow:

0 = There are no vesicles
1 = There are vesicles 1-100
2 = There are vesicles 101-200
3 = There are vesicles >200

Calculation of symptoms of the disease at the base of the stem/root and leaf loss observed, calculated based on the formula as follows:

$$I(\%) = \frac{n.v}{N.Z} \times 100\%$$

I = Disease intensity
n = number of plants observed for each attack score
v = damage scale value of each attack score
Z = damage scale value from highest attack score
N = number of plants observed

Damage score (v) is determined by the base of the stem as follows:

	Category of stem end and rootrot attack	leaves attack Category
0	healthy	healthy
1	≤ 10 % Stem-end rot and root rot	≤ 10 % Leaves turn yellow / fall
2	≤ 10 % Stem-end rot and root rot (wet wounds / fungus spore)	≤ 10 % x ≤ 25 % Leaves turn yellow / fall
3	≤ 10 % x ≤ 25 % Stem-end rot and root rot (wet wounds / fungus spore)	≤ 25 % x ≤35 % Leaves turn yellow / fall
4	≤ 25 % x ≤35 % Stem-end rot and root rot (wet wounds / fungus spore)	≤ 35 % x ≤ 50 % Leaves turn yellow / fall
5	≤ 35 % x ≤ 50 % Stem-end rot and root rot (wet wounds / fungus spore)	> 50 % Leaves turn yellow / fall

3. RESULTS

Identification of VAM from Several Citrus Centers in East Java

It was found that there were differences in the shape, color, and size of the spores on the VAM identification results carried out in the ICSFRI laboratory and Gadjah Mada University Laboratory in Yogyakarta. VAM was found from citrus centers area with alluvial soil types, in 5 sub-districts in Blitar, 2 from 3 sub-districts in Tulungagung, and Ponorogo, 4 sub-districts in Jember and Banyuwangi, East Java. It were identified as genus *Glomus,* while samples from Ngunut Tulungagung and Pulung sub-districts Ponorogo, the genus *Gigaspora* was found (Figure 1 and Table 1). *Glomus* sp is the most and dominates the findings in 5 East Java districts. This shows that *Glomus* sp. has a fairly high level of adaptation to the environment both in acidic soil conditions.

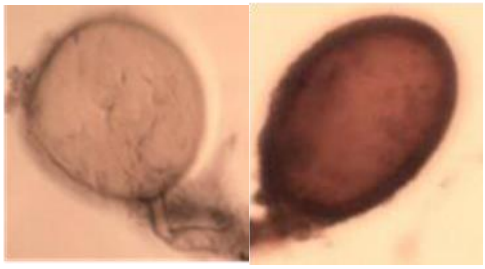

Figure 1. a) Spore of *Glomus* sp, b) Spore of *Gigaspora* sp. (results of identification at UGM laboratory)

Table 1. Identification of VAM isolates from several East Java citrus centers.

No	District	Sub-district	Characteristics of spores (shape, surface color, texture)	Identification Results
1	Blitar	Binangun	globose, Black orange, white gray	*Glomus* sp
2		Kesamben	Globose, Black orange, white gray	*Glomus* sp
3		Lodoyo	globose, Black orange, white gray	*Glomus* sp
4		Panggungrejo	globose, Black orange, white gray	*Glomus* sp
5	Tulungagung	Sumber Gempol	globose, white gray, rough	*Glomus* sp
6		Ngunut	ellipsoid, white gray, rough	*Gigaspora* sp
7		Ngantru	globose, white gray, rough	*Glomus* sp
8	Ponorogo	Jenangan,	Globose, white gray, smoth - rough	*Glomus* sp

9		Ngebel	Globose, white gray, smoth - rough	*Glomus* sp
10		Pulung	ellipsoid, white gray, rough- smooth	*Gigaspora* sp
11	*Jember*	Sumber Baru	Globose, white gray, smooth	*Glomus* sp
12		Semboro	Globose, white gray, smooth	*Glomus* sp
13		Umbulsari	Globose, white gray, smooth	*Glomus* sp
14		Sumberbaru	Globose, white gray, smooth	*Glomus* sp
15	Banyuwangi	Tambakrejo	white to gray, smooth	*Glomus* sp
16		Purwoharjo	white to gray, smooth	*Glomus* sp
17		Cluring	white to gray, smooth	*Glomus* sp
18		Tegaldlimo	white to gray, smooth	*Glomus* sp

Alluvial soils were known to be rich in nutrients, including P, whereas VAM usually found in soil types that have drought stress and limited P availability. The results of NPK analysis on land originating from 5 districts showed that NPK content was quite good except in Tulungagung. This condition is thought to affect the type of VAM which were found to be small (Table 2).

Table 2. Nutrient content (N, P.K)

No.	Sample origin from	Nutrient content		
		N. Total %	P. Bray 1 mg kg^{-1}	K me/100g
1	Banyuwangi	0.14	16.01	0.75
2	Jember	0.19	22.88	0.81
3	Ponorogo	0.09	8.39	2.13
4	Blitar	0.09	25.91	0.68
5	Tulungagung	0.12	0.07	1.36

Identification of Pathogens *Phythopthora* sp. from Several Citrus Centers

Three species of pathogenic fungi Phytopthora was found based on morphological observations (shape, size of sporangium, shape and diameter of colonies), according to the identification key by Stamps (1990). Isolates from Banyuwangi, Jember, Blitar, and Tlekung which have the form of sporangium papilate, ovoid, such as pears can be categorized as species *P. parasitica.* Isolate from Tulungagung and Kraton, Pasuruan has forms of non papilate sporangium, globuse and rossaceous-cottony colonies and based on their size are included in the category of *P. palmivora.* While isolates from Ponorogo have a form of non papillate sporangium, globuse and the form of colonies of cottony are in the category of *P. citrophthora* (Table 3 and Figure 2).

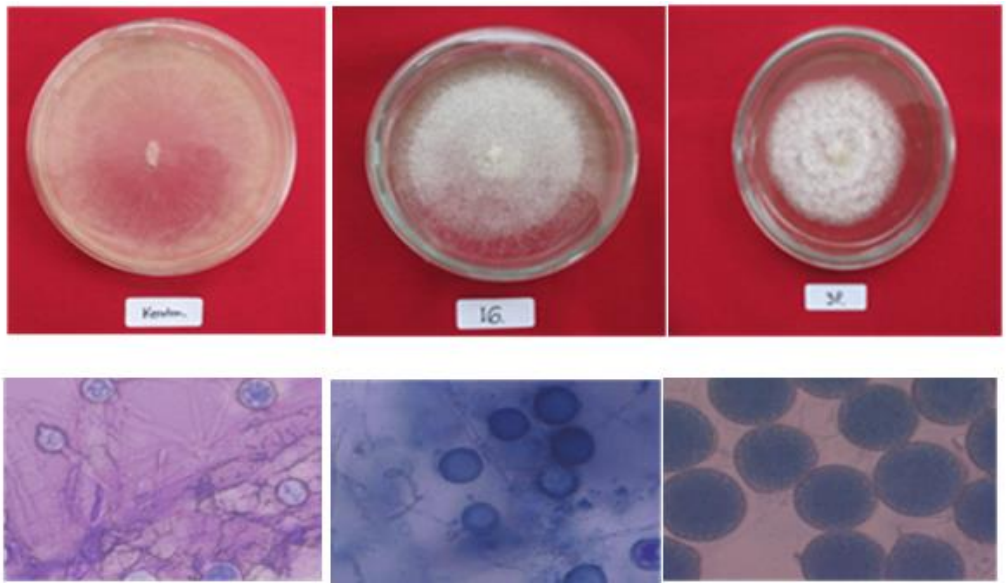

Figure 2. Colony and sporangium/oospora from left to right: *P. parasitica; P. palmivora; P. citrophthor*

Table 3. Phytophthora Morphological Identification

Sample origin from	Form of Sporagium	Size of Sporagium (µm)	Form of colony	Diameter of colony (cm)	Morphological Identification Results
Banyuwangi	Papilate, Ovoid	21.47-32,6 x 21.48-29,88	Cottony Stelate	6.32	*P. parasitica*
Jember	Papilate, Ovoid	14.74 -30 x 13.98-30,09	Cottony	6.55	*P. parasitica*
Ponorogo	papilate Globuse	19.28-33,10 x 17-30	Cottony	6.85	*P. parasitica*
Blitar	Papillate, ovoid	17.02-28,3 x 14.46-28,9	Cottony	4.9	*P. parasitica*
Tulungagung	Semi Papilate elepsoid	14.44-30,22 x 13.86	Rossaceous-cottony	6.35	*P. palmivora*
Tlekung	Papilate Ovoid	38.25 -43,56 x 33.22-50,00	Cottony Stelate	6.35	*P. citrophthora*
Kraton, Pasuruan	Semi Papilate ellepsoid	19.32-40 x 17.28-35,5	Rossaceous-cottony	6.37	*P. palmivora*

Test of the Potential of VAM in Reducing the Attack of Root Disease *Phytophthora* sp

VAM infection, density of VAM spores and the percentage of colonization in root tissue of JC roots are the highest, compared to other citrus species. After that, followed by RL, Volkameriana, A23 and Kanci with the least spore density and percentage of VAM colonization in the roots (Table 4). The potential of VAM in increasing resistance to attacks of root diseases *Phytophthora* sp. can be seen from the percentage of symptoms of attack on the root that were inoculated with VAM in all smaller citrus species (10.00-16.66%) compared to those with no VAM inoculated (25.00-33.33%), the percentage of disease severity in the roots appear to be higher in all citrus varieties with no VAM inoculated, although the difference is not too large.

VAM inoculation has not shown differences in the severity of attacks on

leaves, growth in plant height and number of leaves in all varieties (Table 5). It was found that there was an increase in resistance to Phythopthora sp. on all varieties tested, seen from the percentage of disease events and the severity of disease in the roots. The highest increase in resilience was found in JC and RL varieties compared to other varieties tested (Table 5)

Symptoms of the disease are not clearly visible on the stem above the ground, but seen in the leaves that are marked with yellowing leaves such as nutrient N deficiency, but with a small percentage in all treatments (8.33-21.67%), while damage in the root observations are found between 0.56 -1,44%, with the highest in Kanci varieties (Table 5). The small attack of Phythopthora occurs due to the histological defense process of plants that have been invested with VAM, and colonization has occurred in the roots (Table 5) so that the plant becomes more resistant

Table 4. VAM of *Glomus* sp. infection in roots of 5 types of citrus 90 days after application

No	Citrus varieties	Spore density (spore/100 g soil)	Percentage of VAM colony VAM dalazation in roots (%)
1	JC	800a	91,60a
2	RL	446b	83,05a
3	Volkameriana	313b	87,00a
4	A23	86,5c	70,83b
5	Kanci	81,1c	79,16b

Table 5. Symptoms of phytophthora disease and vegetative growth of 5 citrus varieties

No	Citrus varieties	% diseases incidence	% phytophthora severity		Vegetative growth	
			root	leaf	Plant height (cm)	Number of leaves (cm)
Plants are inoculated with VAM (+) and Phythopthora sp (+)						
1	JC	10,00 a	0.56a	8.33 a	8.83a	9.25a
2	RL	10,00 a	0.56a	16.67b	8.67a	8.33a
3	Volkameriana	16,66 b	0.78a	16.67b	5.58a	5.17a
4	A23	16,66 b	0,89a	10,33a	6.08a	7.75a
5	Kanci	16,66 b	1.44b	21.67b	7.08a	8.00a
Plants non inoculated with VAM (+) and inoculated with Phythopthora sp (+)						
1	JC	25,00 a	1,44 a	8.33 a	8.83a	9.25a
2	RL	33,33b	1,44 a	16.67b	8.67a	8.33a
3	Volkameriana	33,33b	1,44 a	16.67b	5.58a	5.17a
4	A23	33,33b	2,56 b	10,33a	6.08a	7.75a
5	Kanci	33,33b	2.56 b	21.67b	7.08a	8.00a

4. DISCUSSION

Soil samples taken from these 18 sub-districts are partly river alluvial with texture of sandy clay, clay clay and sandy clay so that it is more suitable for the development of Glomus genus mycorrhiza, because Glomus spores range from 20-200 µm, smaller than Gigaspora genus measuring 120 -130 µm (Brundrett et al. 1996). The condition of acid soil pH will cause the supply of nutrients needed for plants to decrease, so this is where the main role of mycorrhizae in helping the absorption of nutrients in the soil. In addition, the acidic soil pH conditions will be able to utilize the mycorrhizae in adapting to the environment and allow spores to develop more because mycorrhizae have "arcidophylis" properties that are suitable for acidic conditions.

Based on the morphological characters in this study, 3 species were identified, namely P.parasitica, P palmivora, P. citrophthora. Whereas Widyaningsih & Dwiastuti (2017) who studied Phylogenetic Relationship of Citrus in East Java Indonesian Phytopthora sp. Infected Using Polymerase Chain Reaction obtained the results that the dendogram isolates from

Banyuwangi, Jember, Ponorogo, Blitar and Tulungagung had 100% similarity coefficients, while 2 isolates from Banyuwangi had similarity around 82%. Isolates from Ponorogo numbers 3, 4, and 5 have a 100% similarity coefficient. This isolate with 21 other isolates had the smallest similarity (28%). According to Yaseen et al. (2010), P. citrophthora is the most predominant species in Syrian citrus plantations, other than P. nicotiana synonym P. parasitica. In Brazil P. parasitica is a pathogen that causes gummosis in oranges (Rosa et al., 2007).

From the results of this study, it is known that VAM fungus has the potential to increase plant resistance to disease. This is caused by the effect of induced resistance. Vigo et al. (2000) VAM infections in strawberry plants up to 55-70% can reduce the symptoms of peptic necrosis caused by Phytopthora fragaria 30-60%. Furthermore, VAM infection in the roots of tomato and cucumber plants will change morphology or anatomy, namely the formation of lignin in the endodermis on the roots so that it can become a barrier / barrier to pathogen penetration. Likewise tomato plants will increase their resistance to Fusarium oxysporium wilt (Scheffknecht et al. 2006). Harrison & Dixon (1993) reported that Medicago truncatula plants symbiosis with Glomus versiforme, the flavonoid or isoflafonoid content increased. This occurs because stimulation at the time the plant is infected with VAM will form colonization in the roots, so that the plant becomes more resistant. Garcia-Garrido & Acompo (2002) suggested that an increase in flavonoid structure indirectly contributes to plant resistance, but synthesizing chitinase and phenylalanine enzymes, ammonium lyase that is functionally useful for the endurance and histological properties of plants infected with VAM fungus will lignify in the parenchymal portion of the marked root tissue with a change in color to purple. Furthermore Vigo et al. (2000) stated that lignification is the defense of the cell wall against pathogenic infections.

5. CONCLUSION

The results of VAM identification at ICISFRI and Gadjah Mada University at the origin of samples from 18 sub-districts in East Java found 2 genera of VAM namely *Glomus* sp. , and *Gigaspora* sp. There were 3 species of pathogenic fungi Phytopthora found based on morphological observations, namely *P.parasitica, P. palmivora, P. Citrophtho*ra. VAM type *Glomus* sp. has the potential to increase resistance to root disease attacks in increasing plant resistance to Phytophthora sp.

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