Research article

Native Endomycorrhiza With Tolerance to Heavy Metal Contamination in Organic Culture Media

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Abstract.
Endomycorrhizal fungi are spora-carrying organisms that can survive in heavy metal-contaminated environments. The goal of this study was to investigate endomycorrhizal fungi from heavy metal-affected areas and determine an effective mix of organic culture media to increase the number and diameter of endomycorrhizal spores. In Sorowako, Indonesia, endomycorrhizal fungi were isolated from the rhizosphere of: rice husk charcoal, sand, zeolite (KM1); rice husk charcoal, sand, sawdust (KM2); rice husk charcoal, sand, cocopeat (KM3); rice husk charcoal, sand, rice soil (KM4); rice husk charcoal, sand, cold magma (KM5); rice husk charcoal, sand, cold magma (KM6); and rice husk charcoal, sand (KM7). The results of the first phase of research revealed that three endomycorrhizal genera (44.44%–75.86% *Acaulospora* sp, 9.52%–44.44% *Gigaspora* sp, and 3.38%–19.05 % *Glomus* sp) can adapt to and resist conditions contaminated with Hg, Cd, Ni, Pb, As, Cr, Mn, Fe, Cu, Co, and Sn, namely as a carrier medium. It was concluded that a combination of organic media was recommended, but that this must decompose first.

Keywords: Fungi, mycorrhizal, organic waste, rhizosfer

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1. Introduction

Endomycorrhizae is one of the obligate soil microorganisms. This fungus has the ability to be mutually symbiotic with 90% of the host plant species. However, it is largely determined by environmental conditions and the species of endomycorrhizal fungi. Endomycorrhizae that have ability to live in numerous environmental conditions, mainly on lands infected with heavy metals. There a number of endomycorrhiza fungi are resistance to heavy metal stress, among them, are Glomus mosseae, Glomus intraradices, and several other important Glomus species. The protection mechanism carried out by endomycorrhizae against toxic heavy metal contamination can be carried out by precipitate the heavy metals in hyphae [1], filtration effect [2], biochemical deactivation [3]

or heavy metal decomposition mechanism with external hyphal secretion [4]. However, using heavy metals tolerant endomycorrhiza is a crucial for surviving in heavy metal contaminated soils. [2].

One of the obstacles to minimizing the use of endomycorrhizal technology in forestry, mining, and agricultural industries is lack of endomycorrhizal fungi that high levels adapted to heavy metals and have not yet been commercially produced on a large scale [5], [6] The manufacture of endophytic mycorrhizal inoculants is quite simple. The human resources avaibility, high-quality starter inoculum, host plants, production equipment, and material of carrier, is the most important thing in the process of endomycorrhiza production [6], [7].

Zeolite, sand, peat, paddy soil, and clay are usually used as carrier medium [8], [9], however, these carriers of media have problems if they are mobilized in large numbers because have heavy mass.

It is not common to use organic materials as carriers for endophytic mycorrhizal inoculants. A number of studies have shown that endophytic mycorrhizas have a positive interaction with the organic matter in heavy metal contaminated soil, dry and saline soil [10]-[12]. Therefore, it is hoped that providing a lightweight, inexpensive, porous, homogeneous and easily available carrier medium for heavy metal-resistant endomycorrhizal fungi can become the goal of this research.

2. Methodology

The research was conducted in two steps. In the first step, samples taking off the rhizosphere *Spathoglottis-plicata*, Sumasang-sp (domestic name) and *Polypodium-glycyrrhiza*, on coordinate point 2^{O}31'7,6"S:121^{O}22'50,7"E. Rhizosfer *Melastama-affine*, *Chromolaena-odorata*, and *Nephrolepis-exaltata*, on coordinate point 2^{O}31'53,5"S:121^{O}22'35,4"E, Sorowako, Indonesia; using methods from [13]. Continued, endomycorrhizal spores were isolated using the wet sieving technique from the soil [14] using a stratified sieve with mesh sizes of 325,0; 40,0 and 50,0 µm in the Laboratory of Microbiology, Center for Environmental and Forestry Research and Development,-Makassar,-Indonesia. Spores was screened followed a guidebook the International-Culture Collection-of Vesicular-Arbuscular Mycorrhizal-Fungi by-INVAM.

The measured soil heavy metals concentration carried out in the chemistry laboratory, Polytechnic- of Ujung-Pandang,-Makassar, using X-Ray Florence-Spectrophotometer. The soil heavy metal levels is shown in table 1.

The second step is to conduct research in the Agricultural Technology Laboratory of Muhammadiyah University (3O59‘S and 119O39‘E) and the Microbiology Laboratory, Center for Environmental and Forestry Research and Development Makassar. Proceed (5˚05‘S and 119˚30‘E). The research designed by a completely random design with five kind mixture of the organic medium (Figure 2), that is; rice husk charcoal, sand, and zeolite (KM1, as control); rice husk charcoal, sand, and sawdust (KM2); rice husk Charcoal, sand, and cocopeat (KM3); rice husk coal, sand, and rice soil (KM4), rice husk coal, sand, and cold magma (KM5), the ratio is 1:1:1. Before putting the nutrient medium composition into a medium tank up to 1000 cm3, it is first homogenized and sterilized. The chemical and physical analysis of organic medium mixture was conducted in the Soil Science Laboratory of the Faculty of Agriculture, Hasanuddin University.

Figure 1: The combined organic medium is used for the physical form of AM Gigaspora sp. propagation Indigenous, Sorowako.

The inoculant used by AM Gigaspora sp is from previous research. Each 50 grams of propagation material contains 2530 spores. Disinfectant solution with 2% concentration was drowned to corn seed for 510 minutes to disinfect the surface, and then the seeds are air-dried. Plant in a culture tank contain a mixture of culture media, collect corn seeds and provide propagation material AM Gigaspora sp. Store the plants to 60 days old and then capture for 30 days.

The wet filtration was used and sucrose gradient centrifugation to separate the spores of AM Gigaspora sp from 50 g of combined medium [15]. Electron microscope use with a magnification of 40 to calculate the number and measure the diameter of the spores. Analysis of variance (ANOVA) was used to analyze the data observed from amount and diameter of AM Gigaspora spores and Duncan’s test if the treatment factors were significantly affected [16].

3. Result and Discussion

3.1. Exploration Of Native Endomycorrhiza

Laboratory testing results showed that the two coordinate points in the post-harvest area were polluted by metals Hg, Cd, Ni, Pb, As, Cr, Mn, Fe, Cu, Co and Sn, soil and plants exceeding the critical limit (Table 1) . This can put pressure on large organisms and soil microorganisms to end their life cycles, but some organisms can adapt to environments with high heavy metals levels.

TABLE 1: Heavy metal elements as pollutants in the post-mining area of Sorowako.

Heavy metals	Coordinat point		Critical lmit	
(ppm)	2^{O}31'57,6"S:121^{O}22'50,7	2^{O}31'53,5"S:121^{O}22'35,4	Soil	Plant
Mercury (Hg)	<17,2	<8,4	0,3- 0,5[b]	-
Cadmium (Cd)	<4,6	<7,8	0,50[a]	5-30[b]
Nickel (Ni)	9.207	23.859	32-100[d]	5-30[b]
Lead (Pb)	<41,4	<83,6	100[a]	50[b]
Arsenic (As)	261	486	10[e]	-
Chromium-(Cr)	26.458	38.754	2,5[a]	5-30[b]
Manganese-(Mn)	4.134	10.739	1500[a]	-
Iron-(Fe)	454.631	920.358	100.000[C]	50[a]
Copper-(Cu)	87.3	221	60-125[a]	20-100[b]
Cobalt-(Co)	1.578	3.005	10[a]	15-30[b]
Stannum-(Sn)	<14,0	<15,9	-	-

[a]Sources : Ministry of State for Population and Environment Republic of Indonesia and Dalhousie University Canada. 1992. [b]Alloway, 1995, [c]Mengel and Kirdy, 1987, [d]Pendias, and Pendias, 2000, [e]Soil Research Institute, 2002.

Several of the literature provides a chain of "classic" ecological ideas that describe the technique of growing tolerance or resistance in a community. Resistance indicates the capacity of microorganisms to delay or eliminate the impact of pollutants that are normally effective at inhibiting them, whereas tolerance indicates the capacity of microorganisms adaption in the high levels of contaminants. As said by [17], [18], the mechanisms involved greatly determine the level of microorganisms tolerance and resistance to contaminated consequences of heavy metals. Briefly, metallic tolerance may be described as a phenomenon wherein microorganisms increase resistance to stress because of exposure to metallic that may cause poisoning [19].

All organisms can acquire the heavy-metals resistance by reduce metallic uptake or efflux increase,-generate and secrete-of organic-acids out of cell-; or when the organism survives in high concentrations of internal metals, through "tolerance." This mechanism metals are sequestered within the cell by synthetic ligands [20] [21].

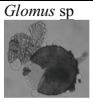
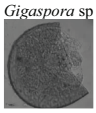
Coordinat point	type of spore	Morphological Description	Spore Diameter (µm)	
			Indigenous	INVAM
2°31'57,6"S:121°22'50,7"E	*Glomus* sp 	Spores shaped of the round, color of hyaline, found remaining of the hyphae, spores have one layer of spores walls.	77.5 x 77.5	70-220
	Gigaspora sp 	Spores shaped of the round, color of clear yellow, spores have one layer of spores wall, a smooth surface without any decoration.	203 x 203	206-358
	Acaulospora sp 	Spores shaped of the round, color of yellowish-brown, the color of the middle spores is darker than the outside, spores have two walls.	60 x 60	74-289
2°31'53,5"S: 121°22'35,4"E	*Glomus* sp 	Spores shaped of the round, color of hyaline, found remaining of the hyphae, spores have one layer of spores cell walls	82.5 x 82.5	70-220
	Gigaspora sp 	Spores shaped of the round, color of creamy yellow, spores have one layer of spores wall, a smooth surface without any decoration.	260 x 260	206-358
	Acaulospora sp 	Spores shaped of the round, color of yellowish-brown, the color of the middle spores is darker than the outside, spores have two walls.	80 x 80	74-289

Figure 2: The morphology of AM spores isolated from the heavy metal polluted area on Sorowako, East Luwu Regency, South Sulawesi Province, Indonesia.

Endomycorrhizal spores obtained from the rhizosphere of several host plants at two coordinated points revealed three endomycorrhizal genera that are resistant- and adaptable- to areas with high heavy metals concentrations: *Glomus*-sp, *Gigaspora*-sp. and *Acaulospora*-sp. (Table 2) abundance of spores was different from the rhizosphere of each host plant (Table 3), which can follow the second tolerance mechanism.

Table 2: Abundance of AM spores for 1000 mg of rhizosphere sample.

Coordinate point	Pioneer plant rhizosfer	Amount of Spora		
		GLS	GGS	ACS
2^{O}31'57,6"S:121^{O}22'50,7"E	*Polypodium glycyrrhiza Sumasang sp Spathoglottis plicata*	2 2 0	0 2 1	7 8 1
2^{O}31'53,5"S:121^{O}22'35,4"E	*Chromolaena odorata*	0 0 0	0 1 0	24 1 13
	Melostama_affine Nephrolepis_exaltata			

Note: GLS, *Glomus*-sp; GGS, *Gigaspora*-sp; ACS, *Acaulospora*-sp

Endomycorrhizae fungi have key role in the plant stabilization process of toxic heavy metals. Plants that have associations with endomycorrhizae accumulate heavy metal residue by store the heavy metals in vesicles and in fungal hyphae so that the metal do

not move and do not disturb phosphate absorption and any micronutrients. Endomycorrhizal fungi also secrete organic acids that increase phosphate solubility in the soil. Organic acids produced by fungi convert unusable forms of phosphorus into usable forms. Glomalin is sortable metal glycoprotein produced by Endomycorrhizal fungi that function to restrain toxic metals to be immobile. Beside that, metallothionine is a protein that secreted by certain endomycorrhizal fungi, which contribute to immobilized heavy metals in rhizosphere [2]. Endomycorrhizal external mycelium also produces Glycoprotein (Glomalin), a protein that sensitively binds with heavy metal. Several studies and reviews have shown that endomycorrhizae from areas contaminated with heavy metals have become tolerant of heavy metal toxicity and adapt well to these areas. The evolution of metal tolerance has been shown to be rapid in endomycorrhizae. As stated by [11], that within a year or two tolerant strains of some endomycorrhizae have been able to grow and develop..

Local AM isolates that occur naturally in metal-contaminated areas are more tolerant than isolates from unpolluted areas. The local AM isolates also efficient to infect roots of plant in heavy metal-contaminated areas. Therefore, it is necessary to collect and reproduce heavy metal tolerant of indigenous isolates to demonstrate the AM symbiosis effect in the recovery of contaminated soil. It was further suggested that phytoremediation in contaminated areas could potentially be approached by introducing plants-mycorrhizal fungi symbiosis. It is therefore very important for us to combine tolerant plants with specific AM fungal isolates according to heavy metal concentrations in the future phytoremediation research.

3.2. Propagation of Native Endomycorrhiza

The statistical analysis revealed a significant effect on amount of spore and diameter of spore by the mixture organic medium treatments. Based on Duncan's test, the KM4 treatment showed the largest amount of spores than the other treatments (Figure 3A), while on variable of diameter spore, there is no difference in KM4 treatment with KM3 treatment (Figure 3B).

The result showed that the KM4 treatment has a positive impact on *Gigaspora* sp. cycle life. According to the physic characteristics of the culture medium, the mixture of organic culture medium is ranging from sand to loam (Table 1). The sandy has larger pores, which may correspond to the development of Gigaspora sp. that larger than Acaulospora sp. and Glomus sp. [22]. However, each of mycorrhizal spore have different growth and development.

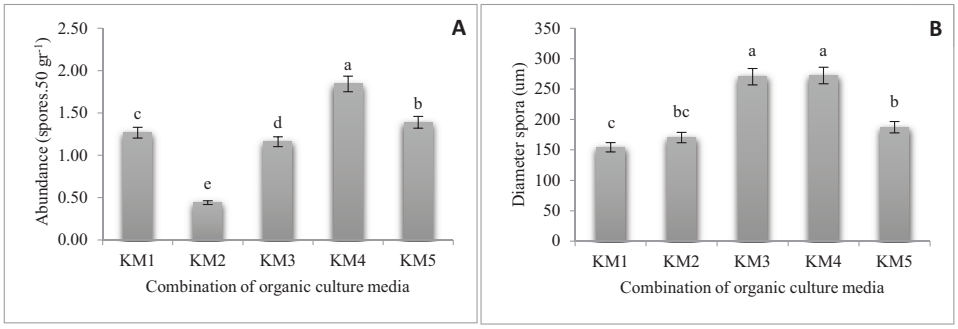


Figure 3: The amount and diameter spores of AM Gigaspora sp in the organic medium combination. The displayed spore frequency data has been converted to logarithmic form.

AM Gigaspora sp. are the bigger (205600μm) spores than AM Glomus sp. and Acaulospora sp. They are fashioned from spherical hyphae tip after which extend round the biggest length that appears and finally turn out to be spores [23]. The suspension adheres to the outer floor of the spore wall. It is characterised via way of means of the life of a round suspension without a bacterial shield [24]. Gigaspora exists in KM3 mixture and KM4 mixture, with >250 μm diameter, whilst it's far nevertheless tremendously small in different medium combinations. It is believed that the fashioned spores have now no longer but reached their most length and are nevertheless growing. spores are an vital factor as a supply of inoculum [25]. The Gigaspora inoculum is spores [6], whilst the Glomus and Acaulospora inoculums are spores, mycorrhizas, and extra-root hyphae [26]. Acaulospora species require much less time to provide spores withinside the equal surroundings than Gigaspora species [27]. In addition, Gigaspora species typically shape mycelium of their energetic shape and bring fewer spores than Acaulospora species [27] [28]. The distinction withinside the variety of AM spores is likewise as a result of the scale of the spores. Due to the bigger spore length of AM Gigaspora, it takes longer to provide spores, so lower than Acaulospora [29], [30].

In addition, the spore number is also affected by medium chemical properties. Soil chemistry has significant influence to AM spore growth and development. The CEC value is related to the medium fertility [31]. A medium with a high CEC can provide better nutrients than a low CEC [32] [33], and CEC can also provide better nutrients in the rice husk-charcoal medium, sand, and soil combination (Table 1). Wiwin [34] also stated, soil, sand and rice husk charcoal is improved AM development, but the study did not deliver any details about mycorrhiza.Top of Form

The KM2 treatment showed low of Gigaspora sp spores number and did not showed value of diameter spore; this may be due to the high concentrations of P in sawdust (Table 1). High Phosphate content can inhibit the development of AM. The increase of P level can increase the colonization of AM to a certain extent, but this has a negative

impact at higher levels [35] [36]. High Phosphate content can slowly mycelial growth, colonization and sporulation [37] [38]. The high phosphorus content in the medium mix reduces the permeability of cell membranes to carbohydrates, thereby disrupting AM's phosphate supply [38], [39]. There any reason such as the existence of parasitic fungi that was able to inhibit plant roots development and increase the media temperature on sawdust and cocopeat treatment. High C/N was describe the decomposition process on the mixture medium. Therefore, compost made of water hyacinth (80%) and zeolite is a good mycorrhizal carrier material [40] [41]. However, organic media mixture is lighter and easy to transport (Table 4).

TABLE 3: Soil physic and chemist of mixture organic media for Gigaspora sp.

Combination of Media culture organic	Physical properties		Chemical properties							
	Texture	Media weight per 10 cm^3 (g)	pH (H_2O)	CEC cmol(+)kg^{-1}	BS (%)	C/N	N (%)	P (ppm)	K cmol(+)kg^{-1}	Mg cmol(+)kg^{-1}
KM1	Sandy	9,4	6,7	7,0	73,0	9,0	0,2	9,7	0,4	1,3
KM2	Sandy	6,5	6,7	9,6	65,0	10,0	0,2	11,3	0,4	1,4
KM3	Sandy	6,9	6,3	8,9	47,0	13,0	0,2	8,1	0,4	1,1
KM4	Loamy and sandy	9,8	6,1	12,2	36,0	16,0	0,1	10,3	0,5	0,9
KM5	Sandy	10,8	6,4	6,9	73,0	8,0	0,2	12,3	0,5	1,5

Organic matter formed from decomposition of plant and animal tissues. Organic matter can enhance the availability of nutrients to plants and reduce the activity of microorganisms in soil [42]- [44]. Several studies stated that organic materials known can support AM inoculants growth and development [45] [46].

The compatible host plants [12], medium [6], and environment [47] are important thing when propagating AM inoculant. It is important to take this into consideration, because AM is mandatory, and each AM's requirements for these factors are not always the same [25]. The propagation of the inoculum must have a high infection ability, rapid host roots colonization, and high spore production [48]. All plants can be infected, but potency levels of each plant and AM are different. AM can infects and colonizes some plant species with the greatest colonization response [49]-[51]. AM Gigaspora sp. work by invade the host plant cortex cell, combine and transfer the nutrients from soil to root plant and provide carbon (C) and phosphorus (P) for plants use, resulting in increased of plant biomass, spore numbers, and root infestation [52]-[54].

4. Conclusion

The found 44.4% to 75.9% for spora AM Acaulospora sp; 9.5% to 44.4% for spora AM Gigaspora sp; and 3.4% to 19.1% for spora AM Glomus sp which is able to adapt and survive in areas polluted by heavy metals (Hg, Cd, Ni, Pb, As, Cr, Mn, Fe, Cu, Co and Sn). The mixture of medium (rice husk charcoal : sand : cocopeat and rice husk charcoal : sand : rice soil) cause improvement of AM Gigaspora spores. The mixture of organic medium potentially used as carrier of AM Gigaspora sp. in Sorowako.

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