

Mycorrhizal diversity of stevia (*Stevia rebaudiana* Bertoni) rhizosphere in Tawangmangu, Indonesia

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Abstract. Mycorrhizal fungi is a group of soil fungi with mutualistic symbiosis between fungi and plant roots. The diversity on mycorrhiza contributes the maintenance of plant biodiversity, ecosystem function, and plant productivity. Climate change may affects the distribution and diversity of mycorrhizal fungi, and thus the study on mycorrhizal diversity is important to develop the information about mycorrhizal function and utilization. The present study investigated mycorrhizal diversity in the rhizosphere of stevia at four locations in different altitudes and soil types. The samples taken from Tlogodlingo (Andisols 1), Kalisoro (Andisols 2), Nglurah (Alfisols 1) and Ledoksari (Alfisols 2) in Tawangmangu, Karanganyar, Central Java, Indonesia. The result showed that *Glomus* sp. and *Acaulospora* sp. were the common genus found at all locations, whereas *Gigaspora* sp. was the only species found in the acidic Alfisol soil. Statistical analysis indicated that altitude, soil pH, and P availability significantly positively correlated with mycorrhizal spore density. The increase of altitude, soil pH and P availability, also increase the mycorrhizal spore density. Mycorrhizal infectivity negatively correlated with C/N ratio.

1. Introduction

Mycorrhizal fungi is a group of soil fungi performs mutualistic symbioses between fungi and plant roots. These symbioses improve biomass and the host plants growth rate, pathogen resistance, mineral nutrient uptake, rate of photosynthesis and secondary metabolites content when compared with symbioses [1,2]. Overall, a symbiosis of mycorrhiza may improve productivity and quality of host plant particularly in the sustainable agricultural system [3].

Tawangmangu is an area located in Karanganyar District, Central Java Province, Indonesia. The altitude is from 800 to 2,000 m asl [4], mean annual rainfall 3,188 mm with mean annual rainy days is 132 days (2003-2011) [4,5,6], and thus it suitable for stevia (*Stevia rebaudiana* Bertoni) culture [7,8]. Stevia is one of a potential alternative sweeteners plant [9] in Central Java, and dominant in Tawangmangu, Indonesia.

Stevia produces secondary metabolites called *steviol glycoside*. Stevioside and rebaudioside-A are the main components of steviol glycosides [10]. According to Mandal et al. (2013)[1], mycorrhizal symbioses potentially augment the production of *steviol glycoside* main components. However, many factors can affect the success of mycorrhizal symbioses including the host plant [11], the environment, and correlation levels with other soil organisms [12]. Climate change has major impacts on the environment, especially related with biodiversity and ecosystem function changes. The association of mycorrhizal fungi and host plant is very important to cope with climate



change effects, such as water shortage delay, revegetation of degraded ecosystem [2,13,14] and also reduce symptoms of Mn^{2+} and Al^{3+} toxicity in acid soils [15].

The diversity on mycorrhiza may contribute to plant biodiversity maintenance, ecosystem function and plant productivity [16]. According to Bellgard and Williams (2011) [17], climate change may affect the distribution and diversity of mycorrhizal fungi, and thus the study on mycorrhizal diversity is important to develop the information about mycorrhizal function and utilization. In this study, we used stevia root and soil rhizosphere to determine the diversity of mycorrhiza in two soil types at four different altitudes in Tawangmangu, Indonesia.

2. Materials and Methods

2.1. Location of sampling area

This research used rhizosphere soils and roots of Stevia (*Stevia rebaudiana* Bertoni), which were collected from four different locations and two soil types in Tawangmangu (Table 1).

Table 1. Details of the sampling area.

Location	Soil type	Altitude (m asl)	Latitude	Longitude
Tlogodlingo	Andisols 1	1,772	07°40'5.28"S	111°10'43.11"E
Kalisoro	Andisols 2	1,121	07°39'49.48"S	111°8'6.77"E
Nglurah	Alfisols 1	1,049	07°40'31.59"S	111°7'55.22"E
Ledoksari	Alfisols 2	956	07°40'33.16"S	111°6'58.43"E

2.2. Sampling of rhizosphere soil and stevia root

Both rhizosphere soil and plant root samples were taken randomly at 20-25 cm depth. Approx 1-2 kg rhizosphere soil samples were collected by carefully separating the root with adhering soils. Root samples were collected by dipping the roots into the water to remove soil particles and then inserted into 50% alcohol solution.

2.3. Chemical analysis of rhizosphere soil

Soil samples were air dried and divided into two portions, to be sieved with 0.5 mm and 2 mm filter, respectively. 0.5 mm filtered soil sample was employed to measure C/N ratio, obtained from the ratio of soil organic carbon and soil total nitrogen. Soil organic carbon (C-organic) was determined by spectrophotometry and soil total nitrogen (N total) was determined by the Kjeldahl method. 2 mm filtered soil sample was prepared to measure soil pH using pH meter and phosphorus availability (P-availability) using Olsen method [18].

2.4. Mycorrhizal infectivity

The roots in alcohol solution were carefully washed with water, then cleaned roots were cut into pieces about 1 cm long, and then into a glass beaker containing KOH 10% at 70 °C for 10 minutes, rinsed with distilled water. Then neutralize the roots in 1 N HCl and then rinsed with distilled water. The cleared roots were stained with trypan blue, prepared in lacto glycerol [19]. Infection roots were viewed under the microscope, the percentage of root colonization was assessed and scored by observing the presence of arbuscular and vesicles in the observed roots.

2.5. Isolation and identification of mycorrhiza

Isolation of mycorrhiza spore was intended to measure spore density per 100 gram of soil and to assist in the identification process. Isolation of spores using wet-sieving and decanting methods, modified from Gerdermann and Nicolson [20], followed by adding 60% sucrose solution. Air-dried soil for about 100 gram was dissolved in 1 liter water and then the mixture was poured into a sieve with 250 μm , 90 μm and 45 μm filter. From this method, only the fine soil particles along with mycorrhizal spores were collected on the 45 μm sieve. Then the fine soil was placed in a centrifuge tube and added

with a sucrose solution, then centrifuged at 2000 ppm for 7 minutes. Sucrose-containing spores were poured on cleaned 45 μm sieve and immediately washed to remove the sucrose. The spores were placed on cleaned Petri dish containing distilled water and observed under a microscope. Identification of spore was done by placed the spore at the prepared glass and identified under the microscope. Identification of spore-based on the character of spore morphology including spore size, spore walls, hyphae, and spore color especially discoloration that occurs after dropping it with Melzer reagent. The spore was compared with *Species Description of INVAM (International Culture Collection of Vesicular-Arbuscular Mycorrhizal Fungi)* [21].

2.6. Statistical analysis

Standard deviations were calculated for mean values and the Pearson correlation coefficient was used to determine the relationship of altitude, soil pH, P availability, C/N ratio, mycorrhizal spore density and mycorrhizal infectivity.

3. Result and Discussion

Abiotic variables such as soil pH, C/N ratio, and phosphorus may determine the diversity of natural mycorrhizal [22]. From figure 1 it can be figured out that both of Andisols 1 and Andisols 2 were included to neutral soils at 6.6 ± 0.02 and 6.5 ± 0.11 , respectively, and Alfisols 1 and Alfisols 2 were included to acid soils with each pH was 5.5 ± 0.11 and 5.7 ± 0.07 . It can be seen too that Andisols 1 and Andisols 2 performs very low P availability (2.1 ± 0.10 ppm and 2.5 ± 0.01 ppm), while acid soils (Alfisols 1 and Alfisols 2) shows lower P (1.2 ± 0.07 and 0.7 ± 0.01 ppm).

Correlation analysis between soil pH and P availability levels resulted positive correlation as seen in table 2 with a correlation coefficient (r) 0.84. It means that the increase of pH also increases P availability. Altitude and soil pH positively correlated ($r = 0.775$), but altitude and P availability is not significantly correlated with $r = 0.488$.

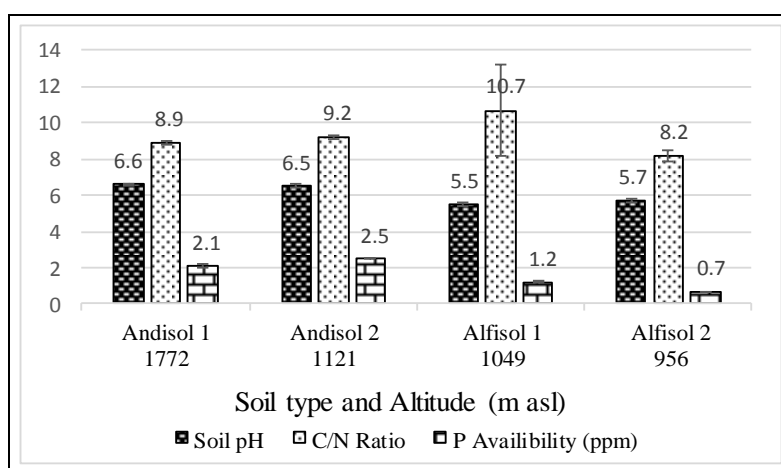


Figure 1. soil pH (H_2O), C/N ratio and P Availability of stevia rhizosphere

Figure 1 shows the Alfisol 1 soil has the highest C/N ratio (10.7 ± 2.49), and the lowest is Alfisol 2 (8.2 ± 0.31). All soil are categorized as soil with low C/N ratio. According to Wu et al. (2001) [23], low soil C/N ratio could accelerate the process of microbial decomposition of organic matter and nitrogen, which is non conducive for carbon absorption.

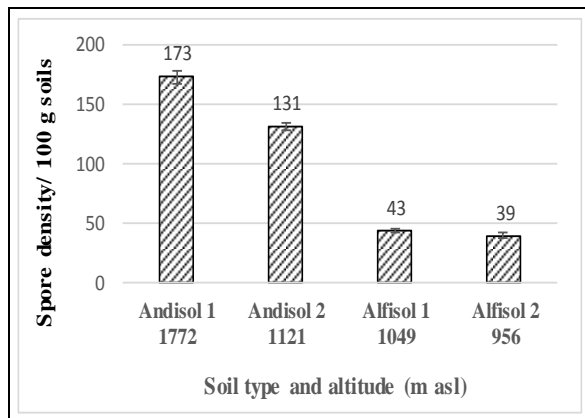


Figure 2. Mycorrhizal spore density/100 g soils at different soil type and altitude (m asl)

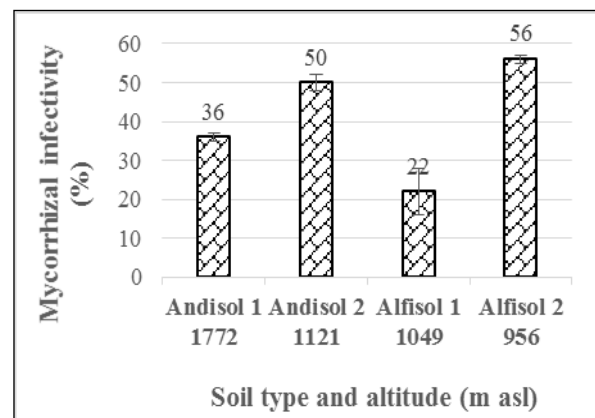


Figure 3. mycorrhizal infectivity (%) at different soil type and altitude (m asl)

Table 2. Correlation between altitude, soil pH, P availability, C/N ratio, spore density and mycorrhizal infectivity calculated using Pearson Correlation

	Pearson correlation (r)					
	Altitude (m asl)	Soil pH	P availability (ppm)	C/N Ratio	Spore density	Mycorrhizal infectivity (%)
Altitude (m asl)	1	0.775**	0.488	-0.194	0.848**	-0.07
soil pH	0.775**	1	0.84**	-0.14	0.967**	0.198
P availability (ppm)	0.488	0.84**	1	0.069	0.864**	-0.067
C/N Ratio	-0.194	-0.14	0.069	1	-0.121	-0.711**
Spore density	0.848**	0.967**	0.864**	-0.121	1	0.027
Mycorrhizal infectivity (%)	-0.07	0.198	-0.067	-0.711**	0.027	1

**Correlation is significant at level 0.01

Mycorrhiza spore's density observation resulted in various numbers as shown in Figure 2. Neutral soils (Andosols 1 and Andisols 2) had higher numbers of spore density at 173 ± 5.57 and 131 ± 2.65 , respectively, while acid soils were 43 ± 2.00 spores (Alfisols 1) and 39 ± 2.00 spores (Alfisols 2). The correlation analysis found that spore density had a positive correlation with soil pH at 0.967 and P availability at 0.864 (Table 2). This indicated that increases of pH and P availability related with increases of mycorrhizal spore density. The other study also reported that spore density significantly correlated with soil pH [24]. Figure 2 also shows higher spore density was observed at higher altitude, similarly with the correlation analysis (table 2), that altitude positively correlated with mycorrhiza spore density ($r = 0.848$).

Figure 2 and 4 shows the higher spore density and altitude irrespective with mycorrhiza diversity. The neutral soils with higher spore density had less diverse genus than the acid soils with lower spore density. The neutral soils had two types of mycorrhizal genus namely *Glomus* sp and *Acaulospora* sp., while the acid soils which have lower spore density and soil pH had three types of mycorrhizal genus, i.e. *Glomus* sp, *Acaulospora* sp. and *Gigaspora* sp. Likewise, other research of Gou et al (2012) [25] found that the lower soil pH had higher mycorrhizal diversity compared to higher soil pH on perennial

pastures with lime treatments. Figure 4 also shows that mycorrhizal less genus diversity found at higher altitude. Some studies also reported similar results, that mycorrhizal diversity is lower at higher altitude [24,26].

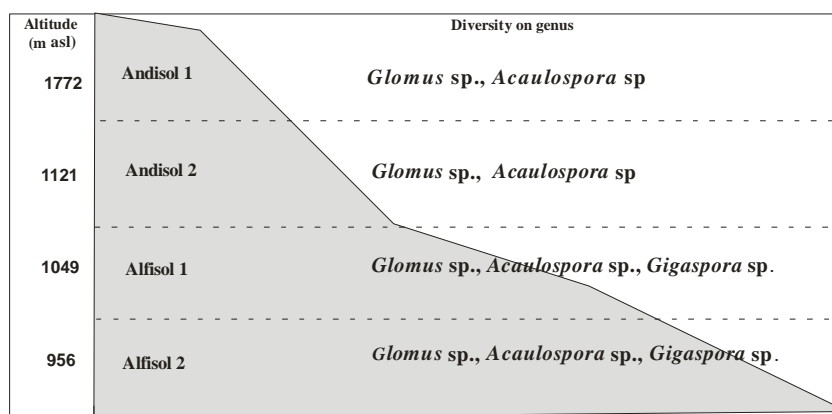


Figure 4. Mycorrhizal diversity by genus on different soil types and altitudes (m asl)

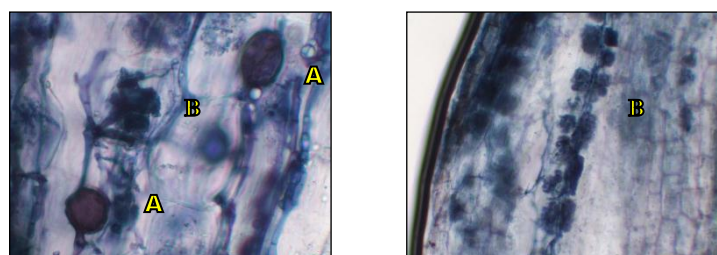


Figure 5. Mycorrhizal colonization of stevia's root. (A) Vesicle colonization form. (B) Arbuscular colonization form

Observations on root found that mycorrhizal colonization as vesicles and arbuscular structures as displayed in Figure 5. The mycorrhizal infectivity (figure 3) showed that the neutral and acid soils had various numbers. The highest infectivity belongs to Alfisols 2 at 56 ± 1.00 %, followed with Andisols 2 at 50 ± 2.00 %, Andisols 1 at 36 ± 1.00 % and the lowest were Alfisols 1 at 22 ± 6.00 %. However, there was reduction trend of mycorrhizal infectivity at a higher altitude at the same soil type. Pearson correlation resulted a negative correlation between mycorrhizal infectivity and soil C/N ratio at -0.711 (table 2). It means that the increasing of mycorrhizal infectivity related to decreasing of soil C/N ratio.

From identification of mycorrhizal spores on four different altitudes and two types of soil, including Andisols 1, Andisols 2, Alfisols 1 and Alfisols 2, obtained three different type of genus consisted of *Glomus* sp., *Acaulospora* sp. and *Gigaspora* sp.

According to table 2, *Glomus* sp. were existed in all soils types. Andisols 1 and Andisols 2 had two kinds of *Glomus* sp. each whereas the acid Alfisols 1 and Alfisols 2 only had one. The spores had their own different characteristics, such as colors consisted of hyaline, light yellow, brown to dark red-brown; the majority had globose shape but the red one had globose, subglobose to ellipsoid shapes; had smooth and rough surfaces. Each spore had special characters including an ornament, subtending hyphae and reaction with Melzer reagent. However, the main approaches of spores included in *Glomus* sp. were the thin spore walls and hyphae in spores directly stick on the spore walls with the same color [21].

Table 3. Morphology of *Glomus* sp. from different soils types
(Andisols 1, Andisols 2, Alfisols 1, and Alfisols 2)

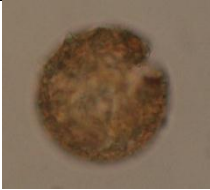
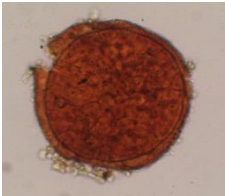
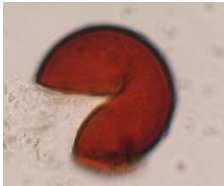
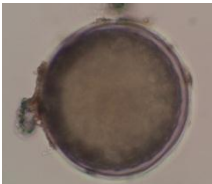

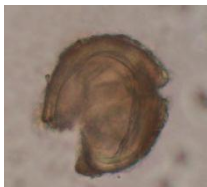

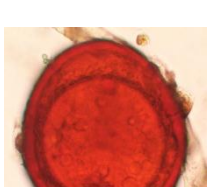

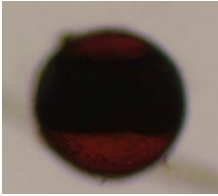
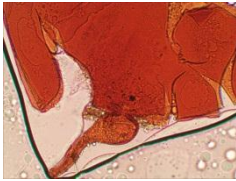
	Shape	Colour	Spores surface texture	Special characteristics	Reaction with Melzer
 <i>Andisols 1, Alfisols 2</i>	Globose	Hyalin	Rough	Have an ornament	Change from hyaline to pale yellow
 <i>Andisols 1, Andisols 2</i>	Globose	Brown	Rough	-	-
 <i>Andisols 2</i>	Globose, subglobose, ellipsoid	Dark red- brown	Smooth	-	-
 <i>Alfisols 1</i>	Globose	Hyalin	Smooth	Subtending hyphae	-

Table 4. Mycorrhizal spore morphology of *Acaulospora* sp.

	Shape	Colour	Spores surface texture	Special characteristics	Reaction with Melzer
 <i>Andisols 1</i>	Globose	Golden brown	Smooth	cicatrix	-
 <i>Andisols 2</i>	Subglobose, irregular	Subhyalin	Smooth	-	Slight change from sub hyaline to pale yellow
 <i>Alfisols 1</i>	Globose	White	Smooth	-	Change from white to orange-brown
 <i>Alfisols 2</i>	Subglobose	Red-orange	Smooth	cicatrix	-
 <i>Alfisols 2</i>	Globose	Dark orange brown	Smooth	-	A change color inside

According to table 3, *Acaulospora* sp. were existed at all type of soils. Andisols 1, Andisols 2 and Alfisols 1 had one species of *Acaulospora* sp., while Alfisols 2 had two species of *Acaulospora* sp. The characteristics of the spores are colors consisted of sub hyalin, white, golden brown, red-orange and dark orange-brown; the majority had globose shape but there was subglobose to irregular shapes, and had a smooth surface. Each spore had special characters including cicatrix, subtending hyphae and reaction with Melzer reagent. However, the main approaches of spores included in *Acaulospora* sp. were the thick spore walls and separating flexible inner spore walls when pressed slightly on prepared glass [21].

Table 5. Morphology of *Gigaspora* sp. from Alfisols 1 and Alfisols 2

	Shape	Colour	Spores surface texture	Special characteristics	Reaction with Melzer
	Globose	Dark Orange	Smooth	Swelling hyphal tip	-
					

The last identified mycorrhizal spores were *Gigaspora* sp.(Table 4), which was only found in Alfisol soils. These soils had low soil pH and very low P availability. The characteristic is dark orange color, globose shape, and smooth surface. The main approaches of spores included in *Gigaspora* sp. were the swelling hyphal tip [21].

4. Conclusion

There are three genera of mycorrhizal spores include *Glomus* sp., *Acaulospora* sp. and *Gigaspora* sp. with special characteristics at each altitude and soil type. *Glomus* sp. and *Acaulospora* sp. were found as a common in all areas, but *Gigaspora* sp. was only found in acidic Alfisol soils. Rhizosphere soil of stevia from Alfisols 2 indicated the highest mycorrhizal infectivity at 56% and diversity consisted of *Glomus* sp., *Acaulospora* sp. and *Gigaspora* sp., however, Alfisols 2 also indicated the lowest mycorrhizal spore density which was only 39 spores per 100 gram soil.

The altitude, soil pH, and P availability positively correlated with mycorrhiza spore density, where the increase of altitude soil pH, and P availability also increased the mycorrhizal spore density. Mycorrhizal infectivity negatively correlated with C/N ratio, indicated by the decrease of C/N ratio significantly increased mycorrhizal infectivity.

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