Molecular Study of Root Colonization and Diversity of Arbuscular Mycorrhizal Fungi (AMF) Associated with Lesser Yam (Dioscorea esculenta)

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Abstract. Lesser yam (Dioscorea esculenta) can be utilized as a functional food due to the high content of inulin, an ingredient of food products and prebiotics. Hence, it is important to increase and promote yam tuber production by using beneficial microbes such as arbuscular mycorrhizal fungi (AMF). This research was aimed to investigate the root colonization and diversity of AMF associated with lesser yam growing at two altitudes. Soil and root samples were collected from different altitudes, lowlands and highlands. The percentage of root colonization was measured using both the staining method and relative quantification using qPCR. The diversity of AMF was analyzed by using molecular approach T–RFLP with a specific primer pair AML1–AML2 and measured by Shannon–Wiener index. Results showed that root samples from lowlands had a higher percentage of root colonization and significant difference than highlands. A total of 17 AMF species belonging to 9 genera: Scutelluspora, Septoglomus, Sclerocystis, Ambispora, Gigaspora, Acaulospora, Claroideoglomus, Funneliformis, and Glomus were determined based on genebank database. Acaulospora was the most dominant and abundant, followed by Glomus and Gigaspora. The study indicated that these genera will be more effective used as potential AMF inoculum to improve lesser yam tuber production.

1. Introduction

In many tropical countries, Lesser Yam (Dioscorea esculenta) is widely cultivated because of its purpose as the main staple food or functional food [1]. In Indonesia, lesser yam tuber is preferably utilized as a functional food since it is potentially known as an alternative source of inulin. As soluble dietary fiber, inulin is extensively used as an ingredient in the variety of food products including prebiotics. In addition, it has low caloric value and the ability to be digested by the human body [2]. Therefore, it is essential to provide a substrate for probiotic bacteria in the colon and promote digestive health [3]. One of many types of Dioscorea sp. that grows in Indonesia and potentially contains the highest amount of inulin is Dioscorea esculenta [4]. Hence, it is important to increase and improve yam tuber production by using beneficial microbes such as arbuscular mycorrhizal fungi.

Arbuscular Mycorrhizal Fungi (AMF) are the member of Filum Glomeromycota which are able to colonize the roots of the majority terrestrial plants and develop the arbuscular structure inside cortex...
These fungi can only be cultured in the presence of their host plant and live as obligate symbionts [6]. Both of AMF and their host plants are beneficial in nutrient exchange. This association allows the AMF to obtain the carbon source from their host plants through photosynthesis while their hosts receive the water and soil nutrients such as phosphate and nitrates [7] [8]. The AMF association may also increase the host plant resistance against biotic and abiotic stresses including salinity and drought [9]. It suppresses nematode damage and additionally leads to improve yam tuber quality and weight [10]. The presence of AMF in the plant roots provide plant nutrition and their colonization potentially improve growth and yield of the plant by increasing nutrient uptake [11].

Taxonomic classification of AMF is determined and evaluated from two approaches, there are the morphological characters of AMF spores and molecular analysis targeting specific rRNA gene sequences [12]. The application of morphological characters most likely face many troubles and limitation because of the low quantity of spores as well as parasitism by soil fauna and bacteria. Aside from that, several AMF may perform vegetative reproduction without producing spores therefore it is reasonable that some AMF in the community may not sporulate at the sampling time. However, spore morphology can be high even within an AMF species [13]. Hence, it is still widely applied for the simple and efficient ways of taxonomic study. Furthermore, molecular techniques using specific primers provide proper and suitable procedures. Several primers have been developed to increase their specificities for identifying AMF and species within the Phylum of Glomeromycota [14] [15]. According to previous studies, the AMF specific primers such as AML1 and AML2 was reported able to amplify the conserved region of DNA targeting the small subunit (SSU) rRNA (Figure 1). This region has been used for AML1-AML2 specific primer pair because of less variable than the internal transcribed spacer (ITS) sequences [16].

![Figure 1. Primers for amplification of the SSU and ITS regions](image)

The diversity of AMF from yam cropping fields, particularly in Dabakala, Ivory Coast has been estimated and reported [1]. Furthermore, the study has explicated the occurrence and abundance of AMF in soils and its aim was to identify the AMF species in order to set endomycorrhizal inoculation technology for increasing yam productivity. At the four yam cropping fields investigated, the genera Glomus, Acaulospora, Scutellospora, and Ambispora were the most diversified species. Aside from these genera, Claroideoglomus, Funneliformis, and Septoglomus were also important because of their presence in all the yam cropping fields. Another finding has revealed that the identified species of AMF colonized in yam roots were determined belonging to genera Acaulospora, Glomus, Sclerocystis, and Gigaspora. Among the genera, Acaulospora was the most dominant and abundant, followed by Glomus [17]. Moreover, Zare-Maivan et al. has also reported the percentage of root colonization at different altitudes. The higher the altitudes, the more increased the percentage of root colonization [18].
In the last decades, there are several studies to explore and understand the mechanisms of the AMF community in the roots of terrestrial plants. However, there is still lack of finding and knowledge regarding the AMF community in the roots of lesser yam growing at different altitudes. This research was an effort to enrich the scientific information by studying the root colonization and diversity of AMF associated with D. esculenta using T-RFLP and q-PCR techniques. This study was aimed to obtain dominant species of AMF that are present in their plant roots. To achieve these goals, root samples of lesser yam growing at lowlands and highlands were collected, their AMF colonization in root and diversity were then investigated. This finding may help to find potential indigenous AMF as biofertilizer inoculum to promote lesser yam tuber production.

2. Methods

2.1. Samples collection
This study was carried out starting from June 2015 to March 2016. Lesser yam roots and soil samples were collected from two different altitudes of four growing locations: lowlands were represented by (1) Berbah Sleman and (2) Purwokerto Banyumas, the samples were respectively labeled as LL1 and LL2, highlands were represented by (3) Pakem Sleman and (4) Ajibarang Banyumas, the samples were respectively named as HL1 and HL2. All samples were collected in dry season. In order to keep fresh, the samples were transported to the Laboratory of Genetic Engineering, Graduate School of Biotechnology, at Universitas Gadjah Mada using an icebox and stored at -20°C for further analysis.

2.2. Analysis of root colonization using staining method
The fine roots were washed in tap water and cut into approximately a length of 0.5-1 cm. The roots were cleared in 10% (w/v) KOH by heating into waterbath at 90°C for 10 minutes and washed in water. The roots were soaked in 10% HCl at 3 minutes and washed in water twice then followed by staining overnight with 0.05% trypan blue in lactoglycerol solution with a ratio of lactic acid : glycerol : water = 1 : 1 : 1 [19]. The stained roots were mounted on a slide and examined for AMF colonization under a light microscope. The root colonization with arbuscules, vesicles, hyphae, and dark septate endophytes per sample were quantified by examining the presence of AMF at 100 times magnification [20]. Percentage of root colonization was determined using the following formula [17]:

\[ \text{Percentage of root colonization (\%)} = \frac{\text{Number of positive segments}}{\text{Number of segments observed}} \times 100 \]

2.3. Molecular analysis of total DNA plant roots
Total DNA from lesser yam roots was extracted using CTAB method. The DNA pellets were dissolved in 50 µl of TE buffer. The final DNA solution was confirmed by using electrophoresis in the 1% agarose gel and 1X TBE buffer. The electrophoresis-confirmed DNA solution was stored at -20°C for further analysis.

2.4. Amplification of the 18S rRNA gene of AMF (gene of interest)
The 18S rRNA gene of AMF was amplified using specific primer pairs modified by Lee et al. (2008): AML1 (5’- ATC AAC TTT CGA TGG TAG GAT AGA – 3’) and AML2 (5’ - GAA CCC AAA CAC TTT GGT TTC C – 3’) [21]. The forward primer AML1 had been labeled at the 5’ end with FAM fluorescent dye. All PCRs were performed in a volume of 25 µl using Dream Taq Green kit according to the manufacture’s protocol. Thirty PCR cycles were used consisting of 94°C for 15 min, followed by 94°C for 30 s, 58°C for 40 s, 72°C for 55 s and final extension at 72°C for 5 min. The PCR product (8 µl) was loaded and checked in the 1.5% agarose gel. After the confirmation, PCR product was carried out for T-RFLP analysis.
2.5. Amplification of the 18S rRNA gene of the plant (reference gene)

The 18S rRNA gene of the plant was amplified using universal primer pairs: forward 5’–GTG ACG GGT GAC GGA GAA TTA–3’ and reverse 5’–ACA CTA AAG CGC CCG GTA TTG–3’. The PCR conditions were initial denaturation at 94°C for 1 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, extension at 72°C 1 min, and final extension at 72°C for 7 min. The PCR product (8 µl) was loaded and checked in the 1.5% agarose gel.

2.6. Real-time quantitative PCR (qPCR) analysis

The qPCR assays were carried out using a Bio–Rad real-time qPCR detection system (Bio-Rad Laboratories, Munich, Germany). The qPCR mixture was set up with the components supplied in the SYBR green using a Evagreen (Bio-Rad) kit according to the manufacturer’s recommendation. A 20 µl aliquot of reaction mixture contained the following components: 5 µl master mix PCR Evagreen Bio-Rad, 1 µl forward/reverse primers (10 pmol each), 1 µl DNA template, and PCR–grade water to make up the final volume. The reference gene (18S rRNA gene of the plant) and gene of interest (18S rRNA gene of AMF) were quantified as described previously.

The percentage of root colonization was measured using the relative quantification method. Quantification cycle (Cq) values were compared between the reference gene and gene of interest, using the following formula:

\[
\text{Percentage of root colonization (\%) } = \frac{\text{Cq value of reference gene}}{\text{Cq value of gene of interest}} \times 100
\]

2.7. Terminal – restriction fragment length polymorphism (T-RFLP) analysis

The fluorescently – labeled PCR products were purified using a purification kit (ATP™) according to the manufacturer’s instruction and eluted in 50 µl of elution buffer. The purified amplicons were separately digested with \( M_{sp1} \) (10U/µl). The restriction endonuclease reaction mixture was made using the following comparison of enzyme : buffer : DNA sample = 2 : 2 : 16. The reaction mixture were incubated at 37°C for 3 h. After incubation, terminal-restriction fragments (T-RFs) from the amplified gene products were determined on a capillary electrophoresis.

2.8. Statistical analysis

The AMF species were identified by comparing with database of known TRFLP patterns. The percentage of root colonization and Shannon-Wiener index of the samples were performed and evaluated using ANOVA with Duncan test to determine and test significant differences. Significance was defined at \( p<0.05 \).

3. Result and Discussions

3.1. Physico-chemical soil conditions for AMF growth

This study highlighted an investigation of the root colonization and diversity of arbuscular mycorrhizal fungi (AMF) associated with lesser yam growing at different altitudes, highlands and lowlands. Previous studies revealed the AMF community are generally affected by the physico-chemical soil conditions. The abundance of AMF in the plant roots is typically related to some physical and chemical properties in the soil. If the soil conditions are favorable for the AMF, they can increase their abilities to supply some soluble nutrients and support the improvement of soil quality for the plant’s growth.

According to physical soil data, it could be revealed that the lowlands had a higher level of air temperature than highlands. The higher level of the oxygen at lowlands can affect the increased level of the gas pressure so that the air temperature gets increased as well. The air temperature increases as altitude decreases. In terms of chemical properties, all soil samples had pH values range from 5 to 6. The cultivated plants including lesser yam mostly can grow well in the soil which has slightly acidic
conditions. Most soil nutrients become available for plants as well as the increase of AMF and other beneficial microbes’ abilities to convert soluble nutrients in the acidic conditions [22]. Based on the pH value data, the soil in the lowlands showed the slightly lower than that in the highlands. In addition, chemical properties in the soil indicated the available phosphorus (P) and potassium (K) contents were lower in the lowlands whereas the total nitrogen (N) in soil samples was not influenced by the altitudes (Table 1).

Table 1. Physical and chemical soil properties at four sampling sites in Java, Indonesia. Sampling site abbreviations are as follows: LL1: Berbah Sleman, LL2: Karang Pucung Purwokerto, HL1: Pakem Sleman dan HL2: Ajibarang Banyumas. Air temperature data were obtained from Indonesian Agency for Meteorology, Climatology, and Geophysics.

| Sampling Site | Air Temperature (°C) | Soil pH | Altitude (ASL) | Total Nitrogen (%) | Available Phosphorous (P) (ppm) | Available Potassium (K) (me/100g) |
|---------------|----------------------|---------|----------------|--------------------|---------------------------------|----------------------------------|
| LL1           | 24-33                | 5       | 74             | 0.11               | 9.62                            | 0.98                             |
| LL2           | 24-31                | 5       | 85             | 0.13               | 10.86                           | 0.80                             |
| HL1           | 22-32                | 6       | 560            | 0.21               | 11.39                           | 1.25                             |
| HL2           | 21-31                | 6       | 556            | 0.10               | 11.01                           | 1.36                             |

The beneficial relationship between AMF and the host plants occurs when the level of available P in the soil is sufficient. Meanwhile, if the level of that is extremely low, AMF can be parasitic to the plants rather than beneficial. This can be explained that they can be competitive with each other for obtaining the available P. In contrast, when the level of that is high and above sufficient, the plants can obtain available P without the presence of AMF. Therefore, the association may not be formed in this condition [23]. The level of available P in the samples was about 9 to 11 ppm. According to previous study, it belongs to a low-medium level of available P (with the range of 4 to 15 ppm), so this soil condition was included sufficient for soluble P contents [24].

3.2. Root colonization of AMF

Root colonization by AMF is great importance for improving soil quality because of its positive effect on plant nutrient solubilization and soil structure. In this study, AMF root colonization was determined and estimated using two methods, there are staining and relative quantification techniques. The AMF root colonization in lesser yam growing at all sites showed a wide range under different altitudes. The percentage of root colonization using both methods had a higher level at lowlands than highlands (Table 2). This might be due to the low level of the water content available in the lowlands’ soil. When the air temperature at lowlands is high, the water content is available in the low level. Therefore, the association and colonization of AMF inside plant roots occur to help the plant obtaining the water. The AMF root colonization can be triggered under dry condition which is critical condition to plant survival and growth.

Table 2. Comparison of root colonization using staining and qPCR method

| Sampling Sites | The percentage of root colonization | Relative Quantification using qPCR (%) |
|----------------|-----------------------------------|---------------------------------------|
|                | Staining Method (%) | Relative Quantification |
| LL1            | 72\(^a\)                         | 82.9\(^a\)                              |
| LL2            | 68\(^a\)                         | 81.6\(^{ab}\)                           |
| HL1            | 63\(^b\)                         | 78.6\(^b\)                              |
| HL2            | 60\(^b\)                         | 72.3\(^b\)                              |
Different letters indicate significant differences between values within a given comparison (ANOVA with Duncan's Test, P<0.05)

Relative quantification describes a real time PCR experiments in which the gene of interest in one sample is compared to the same gene in other samples as well as the reference gene to the gene of interest in the same sample. A reference gene is used as an internal control for experimental variability in this type of quantification [25]. Therefore, it is able to be an appropriate technique based on the molecular approach to quantify the percentage of root colonization in the samples. As it can be seen in Figure 2, the gene of interest used in this study was a partial sequence of small subunit (18S) ribosomal RNA gene of the AMF, while the reference gene used was a partial sequence of the same region gene of the plant, a SSU rRNA gene of Dioscorea esculenta. Relative quantification was obtained by measuring comparison between Cq value of the gene of interest and Cq value of the reference gene in a sample. The calculation estimated the percentage of AMF root colonization inside the plant.

![Figure 2. Visualization of amplified partial gene: (A) 18S rRNA AMF and (B) 18S rRNA plant (M) Marker 100 bp, sample with number (1) LL1, (2) LL2, (3) HL1 and (4) HL2.](image)

The results statistically showed that the percentage of root colonization in the lowlands using relative quantification q-PCR techniques had significant differences compared to the highlands while using staining technique produced insignificant differences in the tested samples from both altitudes. As depicted in Figure 3, both methods had a positive correlation. It could be defined that two quantitative variables measuring by staining and q-PCR techniques had a linear relationship. The strength of the relationship is defined as the correlation coefficient and denoted by R value. Based on statistical analysis, the R value reached 0.86 and it simply means that the relationship between the two variables was described as a strong relationship.

![Figure 3. Correlation of root colonization between using staining and relative quantification methods](image)
In accordance with the experimental results, qPCR technique could be a reliable tool for the quantification of AMF in roots. However, it is time consuming and costly compared to the staining technique. Hence it is currently unsuitable for routine use. The staining technique remains the simple and standard technique for the quantification of root colonization. Staining and microscopic methods do not only provide data on the percentage of root colonization but also permit to visualize the presence of key features such as arbuscules and vesicles, which are the morphological criteria for AMF association.

3.3. Diversity of AMF at lowlands and highlands
A total of 17 AMF species belonging to 9 genera were successfully identified. There were Scutellospora, Septoglomus, Sclerocystis, Ambispora, Gigaspora, Acaulospora, Claroideoglomus, Funneliformis, and Glomus that could be determined based on the comparison on the genebank database. Among these genera, Acaulospora was the most dominant and abundant at all sites, followed by the genera of Glomus and Gigaspora (Figure 4). Furthermore, no difference was found between the total number of AMF genus at lowlands and highlands. Meanwhile, there were slight differences in the total number of AMF species growing at different altitudes (Figure 5). A total of 9 identified AMF genera were found at all sites, while a total number of AMF genus were estimated about 13-14 species from samples collected at lowlands and 15 species from all samples at highlands. The most abundant and dominant genus was Acaulospora found at each sampling site. This finding was also similar to data from the previous distinct studies. The genera of Acaulospora and Glomus have been reported dominant in several ecosystems and climatic conditions [25].

![Figure 4. Relative Abundance of AMF Genus at lowlands and highlands](image-url)
Figure 5. Richness of AMF species

Shannon-Weiner index is commonly used to measure diversity of AMF genus or species in a community. This index assumes all species are represented in a sample that is obtained randomly. From the data analysis, it could be obtained the samples from lowlands labeled as LL1 and LL2 reached Shannon-Weiner index at 2.97 and 2.91, respectively. On the other side, samples from highlands labelled as HL1 and HL2 had the value at 3.03 and 3.01, respectively. Furthermore, data of the diversity index statistically indicated there was no significant difference between lowlands and highlands. This might probably be explained that identified AMF were able to present and develop in lesser yam root growing in the wide range of altitudes, particularly at less than 600 above sea level (ASL). Hence, the presence of AMF could be found at both lowlands and highlands. This finding simply indicated that the diversity of AMF was insignificantly influenced by the altitudes.

4. Conclusion
This study revealed that the association between AMF and lesser yam (D. esculenta) occurred under lowland and highland conditions. The disparity of the altitude conditions may result in the difference of the diversity and the percentage of root colonization in plant roots. In this work, the higher percentage of root colonization was found at lowland instead of highland. Meanwhile, the diversity index and relative abundance of AMF genus were not affected by the altitude conditions. The genus of Acaulospora was the most dominant and abundant, followed by Glomus and Gigaspora. Therefore, we suggested that the use of these genera as potential AMF inoculum may be more effective to improve lesser yam tuber production. However, further studies should be performed in order to obtain the benefit of utilizing these genera for biofertilizer inoculum to promote lesser yam plant growth.

Author Contributions
I.N. was a major contributor performing the design of the study, conducting experiments, data interpretation, and drafting the manuscript. J.W. was helpful in supervising the research and the valuable discussion while S.S. supervised and provided the laboratory facilities and resources to support this work.

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