Application of AMF (Arbuscular Mycorrhizal Fungi) and Organic Fertilizer to Increase the Growth, Biomass and Bioactive Content of Centella

O Trisilawati¹, B Hartoyo², N Bermawie¹, E R Pribadi¹

¹Department of Ecophysiology, Indonesian Spices and Medicinal Crops Research Institute, Indonesian Agency for Agricultural Research and Development (IAARD), Indonesia
²Institute for Agricultural Technology of Central Java, Indonesian Agency for Agricultural Research and Development (IAARD), Indonesia

Corresponding author: trisilawati03@gmail.com

Abstract. Centella (Centella asiatica L. Urban) is one of medicinal plants beneficially for health. The development of herbal medicine and health food industry, require the good quality of raw materials, safety for consumption and prepared continuously. AMF (Arbuscular Mycorrhizal Fungi) is a potential biofertilizer widely used on cultivated plants, for increasing biomass and bioactive content. Organic fertilizer as source of nutrients might be synergized with the development of AMF. The aim of the research was to identify the effect of AMF and organic fertilizer treatments to the growth, biomass yield and asiaticoside content of CASI 016 centella accession on andosol soil. Research was conducted for six months, by using randomized complete block design with two factors and three replications. First factor was AMF inoculation, without AMF and with AMF treatment; second factor was organic fertilizer application, consisted of a). control, b). manure, c). rock phosphate, d). Ash husk, e). manure + rock phosphate, f). manure + ash husk, g). manure + rock phosphate + ash husk. Parameters observed were plant growth, fresh and dry weight of leaf, stolon and root, asiaticoside content, and the uptake of N, P, K nutrients. Result showed that AMF and organic fertilizer treatments could increase centella growth parameters and biomass. Moreover, AMF could increase asiaticoside content of leaves, with the increment of 0,1% to 0,6%. Combination between AMF and organic fertilizer resulted the higher uptake of N, P, K nutrients per hectare.

Keywords: AMF, organic fertilizer, centella, bioactive content

1. Introduction

Centella asiatica L.or pegagan is a tropical medicinal herbaceous plant belonging to the Umbelliferae family (Apiaceae), and commonly known as gotu cola, indian pennywort, and ji xue cao [1]. This plant is one of the potential medicinal plants to be developed. Industrial needs for gotu kola raw materials
reached 100 tons/year, but only 4 tons can be supplied per year [2]. All parts of the plant except roots could be used for traditional medicines.

*Centella asiatica* accumulates large quantities of pentacyclic triterpenoid saponins, essensial oil, amino acids, and other chemical compounds, collectively known as centelloids. These terpenoids include asiaticoside, centelloside, madecassoside, brahmoside, brahminoside, thankuniside, scfeffoleoside, centellose, asiatic-, brahmic-, centelic-and madecassic acids [3] [4]. It is commonly used in the Ayurvedic system of medicine to treat various diseases. The extract is used for revitalizing the body and blood vessels, strengthening the body's tissue structure, brain tonic, helping the digestive process, as a laxative, giving rise to appetite, multiplying red blood cells, healing mild disorders in the liver and spleen, anti-infection, anti-poisons, fever, art decay and antiysphilis [1].

Development of the herbal medicine and health food industries required agriculture product that were free from chemicals contamination, good quality and safe for consumption. Therefore, in the medicinal plants cultivation, the use of chemical inputs must be minimized and replaced with more safety and environmentally friendly inputs, such as organic and bio fertilizers.

Utilization of organic fertilizers can maintain land sustainability (physical, biological and chemical soil properties) and improve land productivity, also reduce the environmental impact of chemical fertilizers usage. AMF (Arbuscular Mycorrhizal Fungi) is a potential biofertilizer widely used on cultivated plants, for increasing nutrient availability, plant yield and bioactive content [5] [6] [7].

The aim of the research was to observe the effect of AMF and several type of organic fertilizer treatments to the growth, biomass yield and asiaticoside content of *Centella asiatica* (CASI) 016 centella accession on Andosol soil.

2. Materials and method

Research was conducted in the green house of Gn.Putri Research Garden (1500 m asl.) in Cipanas, West Java, for six months. Soil fertility analysis of Andosol soil from Gn. Putri Research Station showed that soil pH was very acid (4.45), C organic content was high (3.2%), total Nitrogen content was low (0.19%), C/N ratio was high (16.84), available Bray P was low (1.22 ppm), exchangeble cations were low: Ca (4.28 me/100g soil), Mg (0.75 me/100g soil), K (0.25 me/100g soil), and Na (0.23 me/100g soil), Al saturation was high (0.41 me/100g soil), Cation Exchange Capacity was high (20.16 me/100g soil), low Base saturated (27.33%), and sandy clay loam soil.

Research design used was Randomized block design, factorially, three replications and two factors. First factor was AMF inoculation, without AMF and with AMF treatment (200 spores/plant), and the second factor was organic fertilizer application, consisted of a). control, b). Manure (cow dung), c). rock phosphate, d). Ash husk, e). Manure + rock phosphate, f). Manure + Ash husk, g). Manure + rock phosphate + Ash husk. Seven organic fertilization treatments were used: (a) control with no fertilizer, (b) manure (75 g/polybag), (c) rock phosphate (1 g/polybag); (d). ash husk (0.66 g/polybag), (e) manure + rock phosphate powder, (f) manure + ash husk and (g) manure + rock phosphate powder + ash husk. Andosol soil as growth medium (5 kg/polybag) has been sifted 2 mm in size and sterilized by fumigation using a basamid dose of 0.004% w/w (40 g basamid for 1 t of soil) for two weeks. Seeds source comes from 2-3 segments of stolon cuttings CASI 016 accession, and the healthy parent of centella plant. Centella seedlings were planted in polybag (10 x 15 cm) for 1 month, with sterile media (mixed of soil and organic fertilizer = 1:1).

Parameters observed in this study were growth parameters (number of leaves on origin plant, thickness of largest leaf of the origin plant, primary stolon number, number of leaves and node on the
longest primary stolon, and diameter of the longest leaf stalk of the origin plant) at 1 to 13 WAP (Weeks After Planting), fresh and dry weight of leaf, stolon and root, asiaticoside content, and the uptake of N, P, K nutrients (harvested time).

3. Result and discussion

3.1. Plant Growth

AMF inoculation and application of organic fertilizer treatments could significantly increase centella growth parameters.

Inoculation of AMF increased significantly the leaf number of origin plant, primary stolon number, number of leaves and node on the longest primary stolon, and diameter of the longest leaf stalk of origin centella (Figure 1 and Table 1). Generally, effect of the treatment was significant at 7 WAP until 11 WAP and 13 WAP. AMF have the ability to increase the surface area of roots by effectively producing intraradical and extraradical mycelium for better utilization and effective transportation of nutrition and water [8]. The hyphal diameter ranges 2–20 μm with capacity of absorbing nutrients from area of 25 cm around the roots for translocation [9].

At 11 WAP the organic fertilizer application was significantly affected the three growth parameters, namely: the longest diameter of leaf stalks and the largest leaf thickness of the parent plant, and the number of primary stolon. (Table 2). Organic fertilizer significantly increased primary stolon number, larger leaf thickness and diameter of the longest leaf stalk of origin plant. At 11 WAP, application of Manure mixed with rock phosphate or single manure and rock phosphate showed significantly the highest primary stolon number, thickness of largest leaf and diameter of the longest leaf stalk of the origin plant compared to the control, and better than other organic fertilizer treatments. Applications of husk ash seemed had less positive response to increase centella growth. In general, husk ash has a fairly good macro nutrient content, that were 0.2% – 2.85% P2O5, 0.58% - 2.50% K2O, 0.20% - 1.50% CaO, and 0.12% - 1.96% MgO [10].

Table 1. Effect of AMF application to the diameter of the longest leaf stalk and the thickness of largest leaf of the origin plant

| Treatment | Diameter of the longest leaf stalk of the origin plant (mm) | WAP |
|-----------|----------------------------------------------------------|-----|
|           |                                                          | 3** | 5** | 7** | 9   | 11* |
| NonAMF    | 1.33 b                                                   | 1.06 a | 1.42 b | 1.48 b | 1.50 b |
| AMF       | 1.46 a                                                   | 0.75 b | 1.62 a | 1.64 a | 1.59 a |

Note: Numbers followed by the same letter in the same columns are not significantly different at 5% DMRT.
Figure 1. Effect of AMF application to the leaf number of origin plant, number of leaves and node on the longest primary stolon, and primary stolon number.

Table 2. Effect of organic fertilizer to several growth parameters at 11 WAP

| Organic fertilizer treatment | primary stolon number | Thickness of largest leaf of the origin plant (mm) | Diameter of the longest leaf stalk of origin plant (mm) |
|-----------------------------|-----------------------|---------------------------------------------------|------------------------------------------------------|
| Control                     | 2.83 b                | 0.478 b                                           | 1.407 b                                              |
| Manure                      | 3.44 a                | 0.593 a                                           | 1.605 a                                              |
| rock phosphate (RP)         | 3.44 a                | 0.578 a                                           | 1.627 a                                              |
| Ash husk                    | 3.17 ab               | 0.578 a                                           | 1.558 a                                              |
| Manure + RP                 | 3.61 a                | 0.585 a                                           | 1.585 a                                              |
| Manure + Ash husk           | 3.50 a                | 0.543 ab                                          | 1.513 ab                                              |
| Manure+RP+Ash husk          | 3.61 a                | 0.542 ab                                          | 1.510 ab                                              |

Note: Numbers followed by the same letter in the same columns are not significantly different at 5% DMRT
3.2. Plant Harvested

AMF inoculation had significant effect on the root length of origin plant, fresh and dry weight of root, fresh and dry weight of stolon, and also fresh and dry weight of biomass (Table 3). The increment of those parameters due to AMF inoculation around 6 to 25%, that were: root and stolon dry weight (25%), root fresh weight (22%), biomass dry weight (17%), root length of original plant (15%), fresh weight of stolon (10%) and biomass weight (6%).

Arbuscular mycorrhizal colonization increased root, stem and leaf weights, leaf area, root length and specific leaf area, and it decreased root length/leaf area ratio, root/shoot weight ratio and specific root length of micropropagated transplants of *Prunus cerasifera* L. Arbuscular mycorrhizal colonization will stimulate the formation of extensive root which formed higher root volume. In general, AMF colonization in plant roots will change the root system architecture, so root weights will be increased [11].

Application of organic fertilizer increased significantly the root length of the parent plant, fresh and dry weight of root, stolon fresh weight, fresh and dry weight of leaves, fresh and dry weight of centella biomass (Table 4 and 5). Generally, the application of manure + rock phosphate + ash husk showed highest yield parameter, following by manure + ash husk, and manure + rock phosphate, respectively.

Rock phosphate is a source of P nutrients, containing 1.04% N, 9.83% P, 0.11% K, 21.82% Ca and 0.19% Mg. Phosphate rock as a natural P can be used as a slow release fertilizer to replace chemical P fertilizers which are expensive and sometimes difficult to obtain [12]. Ash husk is one of the agricultural wastes used as an organic fertilizer, a better and cheaper alternative K source. The application of ash husk should be combined with other organic fertilizers. The combination of 5 t manure with 2 t ash husk/ha could increase the highest soybean yield [13]. Application of 2 t ash husk/ha had the same effect with of 150 kg KCl/ha. Combination of 5 t manure with 2 t ash husk/ha could also increase the highest soybean yield.

| Table 3. Effect of AMF inoculation to centella yield parameters |
|---------------------------------------------------------------|
| No | Parameters                  | NonAMF | AMF   |
|----|------------------------------|--------|-------|
| 1  | Root length of original plant | 30.92 b | 35.63 a |
| 2  | Root fresh weight/plant      | 29.60 b | 36.23 a |
| 3  | Root dry weight/plant        | 5.42 b  | 6.77 a |
| 4  | Stolon fresh weight/plant    | 46.16 b | 50.90 a |
| 5  | Stolon dry weight/plant      | 9.00 b  | 11.21 a |
| 6  | Biomass fresh weight/plant   | 80.51 b | 85.44 a |
| 7  | Biomass dry weight/plant     | 15.60 b | 18.31 a |

Note: Numbers followed by the same letter in the same columns are not significantly different at 5% DMRT.
### Table 4. Effect of organic fertilizer to centella root

| Organic fertilizer treatment | Root length of origin plant | Root weight/plant (g) |  |
|-----------------------------|-----------------------------|-----------------------|---|
|                             |                             | fresh                | dry |
| Control                     | 29.28 c                     | 24.54 c              | 4.49 c |
| Manure                      | 29.73 c                     | 30.92 abc            | 5.99 abc |
| rock phosphate (RP)         | 32.23 bc                    | 35.57 ab             | 5.59 ab |
| Ash husk                    | 33.14 abc                   | 29.61 bc             | 5.51 bc |
| Manure + RP                 | 34.20 abc                   | 35.84 ab             | 6.48 ab |
| Manure + Ash husk           | 37.54 a                     | 35.51 ab             | 6.33 ab |
| Manure+RP+Ash husk          | 36.84 ab                    | 38.44 a              | 7.32 a  |

Note: Numbers followed by the same letter in the same columns are not significantly different at 5% DMRT

### Table 5. Effect of organic fertilizer to stolon, leaf and biomass weight of centella (g)

| Organic fertilizer treatment | stolon fresh weight | leaf fresh weight | leaf dry weight | Biomass fresh weight | Biomass dry weight |
|------------------------------|---------------------|-------------------|----------------|----------------------|-------------------|
| Control                      | 40.47 c             | 30.18 b           | 5.65 b         | 70.65 d              | 13.74 b           |
| Manure                       | 51.11 ab            | 35.89 a           | 7.16 a         | 86.92 abc            | 17.41 a           |
| rock phosphate (RP)          | 46.79 abc           | 34.49 ab          | 7.13 a         | 81.28 bc             | 17.29 a           |
| Ash husk                     | 45.92 bc            | 32.12 ab          | 6.32 ab        | 78.04 dc             | 15.96 ab          |
| Manure + RP                  | 49.13 ab            | 35.22 a           | 7.36 a         | 84.35 abc            | 18.37 a           |
| Manure + Ash husk            | 52.28 ab            | 36.31 a           | 7.12 a         | 88.59 ab             | 17.95 a           |
| Manure+RP+Ash husk           | 54.02 a             | 37.01 a           | 7.24 a         | 91.04 a              | 18.01 a           |

Note: Numbers followed by the same letter in the same columns are not significantly different at 5% DMRT

### Table 6. Effect of AMF inoculation and organic fertilizer application to the asiaticoside content of centella

| Treatment | G0 | G1 | G2 | G3 | G4 | G5 | G6 |
|-----------|----|----|----|----|----|----|----|
| Organic fertilizer/AMF       | %  |
| NonAMF                              | 1.72| 1.65| 1.36| 1.31| 1.24| 1.54| 1.78 |
| AMF                                  | 2.16| 2.07| 1.46| 1.93| 1.71| 2.00| 1.83 |

Note: G0= Control, G1= Manure, G2= Rock Phosphate (RP), G3= Ash husk, G4= Manure+RP, G5= Manure+ash husk, G6= Manure+RP+ash husk
3.3. Asiaticoside content
AMF could increase asiaticoside content value of centella at all organic fertilizer treatment (Table 6). High value of asiaticoside content was showed by mycorrhizal centella without organic fertilizer treatment (2.16%), AMF+manure (2.07%), AMF+manure+ash husk (2%), respectively. The increment of asiaticoside content on mycorrhizal centella plant ranged from 0.1 to 0.62%. Inoculation of AMF on several organic fertilizer treatment, such as control, manure, ash husk, manure + ash husk resulted in higher increment of asiaticoside content, that were 0.44%, 0.42%, 0.62%, and 0.46%.

There were many studies on the regulation of secondary metabolites in mycorrhizal infected plants. Analysis of extracts obtained from Trifolium repens roots and shoots revealed that compositions of the flavonoid mixtures varied in the presence or absence of the arbuscular fungus (Glomus intraradices). Types of flavonoid such as quercetin, acacetin and rhamnetin accumulated in roots of inoculated plants, whereas they were not detected in non-inoculated plants [14]. In ginger, inoculation with S. herogama and G. decipiens resulted in larger yields of oleoresin, corresponding to 3.48% and 1.58% of rhizome fresh biomass respectively [15].

The beneficial effect of AM symbiosis in improving nutrient supply, especially P, might increased the levels of asiaticoside. AMF may participate in the biosynthesis regulation of secondary metabolites in plants. Moreover, AMF induced the accumulation of mycorradicin via non-mevalonate methylerythritol phosphate pathway (MEP pathway) [16].

3.4. Nitrogen, Phosphorus and Potassium uptake
Inoculation of AMF was significantly affected the uptake of P and K per plant (Table 7), moreover the uptake of N, P, K per ha improved quietly on the value of 8.54%, 18.84%, and 18.88% (Table 8). External hypha of AMF might reduce the distance for nutrients to enter plant roots, increase nutrient absorption and nutrient concentration on the absorption surface, and chemically changed nutrient properties to easily absorbed by roots [17]. The AMF hyphae are extended beyond the accessibility limit of roots to transport immobile nutrients (P, Cu, Zn and partly NH₄) in soil which are not taken up by non-inoculated plants [18].

Organic fertilizer application significantly increased the uptake of N (18% to 45%), P (14% to 39%), K (23% to 43%). The highest N, P and K uptake showed on manure treatment, followed by manure + rock phosphate, and manure + ash husk, and rock phosphate, respectively (Table 9).

Interaction between AMF and organic fertilizer affected significantly the N, P, and K uptake of centella/plant (Table 10). Mycorrhizal centella resulted the highest increment of P (59.1%) and K uptake (58.5%). Application of manure+ash husk on mycorrhizal centella resulted higher uptake of N, P and K per hectare (with the increment of 10.3%, 40.3%, and 14.1%) compared to non mycorrhizal plant (Table 11). Moreover, the application of rock phosphate on mycorrhizal centella increased the uptake of N, P and K per hectare with the increment of more than 30%. Organic fertilizer as source of nutrients might be synergized with the development of AMF. Organic fertilizers as sources of nutrients such as residual crops and slow-dissolved mineral fertilizers such as rock phosphate do not suppress the development of AMF, even stimulate the development of AMF [19].
Table 7. Effect of AMF inoculation to P and K uptake of centella per plant

| Treatment     | P uptake | K uptake |
|---------------|----------|----------|
| Non AMF       | 0.015 b  | 0.231 b  |
| AMF           | 0.016 a  | 0.252 a  |

Note: Numbers followed by the same letter in the same columns are not significantly different at 5% DMRT.

Table 8. Effect of AMF inoculation to N, P and K uptake of centella per hectare

| Treatment     | N uptake | P uptake | K uptake |
|---------------|----------|----------|----------|
| NonAMF        | 29.99    | 2.92     | 45.56    |
| AMF           | 32.55    | 3.47     | 54.16    |

Table 9. Effect of organic fertilizer to N, P and K uptake of centella per plant

| Organic fertilizer | N uptake | P uptake | K uptake |
|--------------------|----------|----------|----------|
| control            | 0.119 d  | 0.012 c  | 0.188 b  |
| Manure             | 0.173 a  | 0.016 ab | 0.270 a  |
| rock phosphate (RP)| 0.158 abc| 0.015 ab | 0.231 a  |
| Ash husk           | 0.140 cd | 0.014 bc | 0.237 a  |
| Manure + RP        | 0.167 ab | 0.017 a  | 0.270 a  |
| Manure + Ash husk  | 0.144 bc | 0.017 a  | 0.250 a  |
| Manure+RP+Ash husk| 0.160 abc| 0.017 a  | 0.245 a  |

Note: Numbers followed by the same letter in the same columns are not significantly different at 5% DMRT.

Table 10. Effect of AMF inoculation and organic fertilizer to N, P and K uptake of centella

| Treatment       | N uptake | P uptake | K uptake |
|-----------------|----------|----------|----------|
| control         | 0.119 d  | 0.010 e  | 0.154 d  |
| Manure          | 0.192 a  | 0.016 abc| 0.269 ab |
| rock phosphate (RP)| 0.140 cd | 0.013 cd | 0.200 ed |
| Ash husk        | 0.126 d  | 0.012 de | 0.231 abc|
| Manure + RP     | 0.182 ab | 0.014 bcd| 0.286 a  |
| Manure + Ash husk| 0.152 bcd| 0.016 abc| 0.258 ab |
| Manure+RP+Ash husk| 0.154 bcd| 0.017 abc| 0.220 bc |

Note: Numbers followed by the same letter in the same columns are not significantly different at 5% DMRT.
Table 11. Effect of AMF inoculation and organic fertilizer to N, P and K uptake of centella per ha

| Treatment                      | N uptake kg/ha | P uptake kg/ha | K uptake kg/ha |
|--------------------------------|----------------|----------------|----------------|
|                                | NonAMF AMF    | NonAMF AMF    | NonAMF AMF    |
| control                        | 22.87 25.12    | 1.92 3.06      | 29.69 47.05   |
| Manure                         | 37.19 32.46    | 3.13 3.38      | 52.32 57.07   |
| rock phosphate (RP)            | 27.34 36.78    | 2.63 3.58      | 39.25 54.20   |
| Ash husk                       | 25.49 33.70    | 2.47 3.41      | 46.52 52.85   |
| Manure + RP                    | 36.18 33.02    | 3.72 3.14      | 56.58 55.66   |
| Manure + Ash husk              | 28.86 31.83    | 3.01 4.22      | 48.97 55.86   |
| Manure + RP + Ash husk         | 31.97 34.91    | 3.44 3.49      | 45.57 56.46   |

4. Conclusion
Application of AMF significantly affected growth parameter of Centella, included leaf number of origin plant, primary stolon number, number of node on the longest primary stolon, number of leaf on the longest primary stolon, and diameter of the longest leaf stalk of the origin plant. Growth increment of mycorrhizal centella was 43-78%. Moreover, application of AMF significantly increased Centella yield (fresh and dry weight of stolon, root and biomass), asiaticoside content (with the increment of 0.1% to 0.6%), and the uptake of P and K nutrient.

Organic fertilizer application significantly increased number of primary stolon, leaf thickness, diameter of the longest leaf stalk of the origin plant, fresh and dry weight of stolon, leaf and biomass, and the uptake of N, P, K nutrient.

Combination of organic fertilizer (ash husk, rock phosphate, manure+ash husk, manure+ash husk+RP) with mycorrhizal centella resulted higher N, P, K uptake per hectare.

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