Effectivity of *Azotobacter chroococcum* and arbuscular mycorrhiza fungi on physiological characteristics and growth of cocoa seedlings

Nasaruddin and I Ridwan

Department of Agronomy, Faculty of Agriculture, Hasanuddin University, Jalan Perintis Kemerdekaan KM 10 Makassar 90245 Indonesia.

E-mail: nnasaruddin@gmail.com

**Abstract.** This study aims to study the effectiveness of *Azotobacter chroococcum* bacteria and Arbuscula mycorrhiza on some physiological characteristics and growth of cocoa seedlings. The study was conducted from March to October 2015, designed in the form of a two factors experiment based on the Randomized Block Design in a screen house. Inoculation of *A chroococcum* as the first factor consisted of control, inoculation of $10^4$ CFU ml$^{-1}$ water and $10^6$ CFU ml$^{-1}$ water per tree given as much as 40 ml. Inoculation of arbuscula mycorrhiza as a second factor consisted of control, inoculation of 3.0 g, 6.0 g and 9.0 g per tree, respectively. The experimental results show that inoculation of *Azotobacter chroococcum* $10^6$ CFU ml$^{-1}$ water.tree$^{-1}$ and the arbuscular mycorrhizal fungi 6.0 g tree$^{-1}$ resulted in higher chlorophyll a, b and total leaf chlorophyll content, increased light absorption rate, leaf stomatal conductance and better seedling growth.

1. **Introduction**

Production of cocoa in South Sulawesi, as one of the main centres of cocoa in Indonesia, has decreased significantly in the past 10 years, due to aging crops, decline in the ecological quality of the land and soil fertility, the simple application of cultivation techniques, the shortage of fertilizer and increasingly the high price of fertilizer [1]. One of the efforts made to improve cocoa production and quality is the improvement of the ecology of the land using soil bio-technology (soil microbial services and natural fertilizer technology).

Effective bacteria colonize the roots commonly called Plant Growth Promoting Rhizobacteria (PGPR). PGPR has the ability to protect plant parts on the ground against viral, fungal and bacterial disease by inducing systemic resistance (ISR) [2], can accelerate germination, stimulate root and shoot growth [3], increase leaf chlorophyll levels, increase crop tolerance to drought and salt and may delay leaf aging [4]. One of the important PGPR bacteria in the soil ecosystem is the *Azotobacter chroococcum* (*A chroococcum*) known as biologic fixing agent N$_2$ by converting N$_2$ to ammonium through electron reduction and nitrous protonation [5]. Several studies have shown that inoculation of *Azotobacter* can improve the growth and absorption rate of N in annual crops such as in pepper plants [6] and vanilla plants [7].

*A chroococcum* may be symbiotic with Arbuscular Mycorrhiza and has been widely used in cultivation systems in both single inoculation and double inoculation with *A chroococcum*. Arbuscular mycorrhizas are symbiotic with plant roots and capable in increasing the uptake of N, P and K, increasing the efficiency of groundwater use, increasing the osmotic pressure value of plant cells on
soil whose moisture content is low enough so that the plant can maximizes the utilization of CO₂ and solar energy to increase the rate of vegetative growth and crop production [8]. Efforts to improve the production and productivity of long-term and sustainable cocoa crops are needed to provide plant material (seeds), especially in the rejuvenation of old crops and damaged crops. Cocoa plant growth in the field is determined by the growth of plants during the nursery. The roots of plant seedlings infected by arbuscular mycorrhizae and symbiotic with *Azotobacter* in the rhizosphere were able to adapt to extreme cropping environments [9]. Symbiosis of bacteria *A. chroococcum* with Arbuscula mycorrhizal fungi is very important for plants and can significantly increase the growth of host plants [10]. Understanding such interactions, particularly in cocoa farming areas with tropical climatic conditions and limited resources of fertilizers and simple cultivation systems are crucial in efforts to improve the production and quality of cocoa.

This study aims to determine and study the effectiveness of *A. chroococcum* and arbuscular mycorrhizae on some ecophysiological characteristics and growth of cocoa seedlings and to determine the best dosage of *A. chroococcum* and mycorrhiza *Arbuscula* for the growth of cocoa seedlings. The results of the study are expected to provide information on the potential utilization of *A. chroococcum* and *Arbuscula* Mycorrhiza which can streamline the utilization of nutrients especially N and P and for cocoa plants in the effort of rehabilitation and rejuvenation of plants.

2. Materials and Methods

The research was conducted in the form of a three factors factorial experiment based on the Randomized Block Design pattern. The first factor is the seed source consisting of clones of Sulawesi I (k1), Sulawesi 2 (k2) and Mukhtar 1 (k3). The second factor was the inoculation of *A. chroococcum* which consisted of three levels *i.e.* without inoculation as control (a0), 10³ CFUs (a1) and 10⁶ CFUs (a2), each was given 40 mL tree⁻¹. The third factor was the treatment of Arbuscular Mycorrhizae fungi (AM) *Glomus* sp in the form of a Zeolite culture, consisting of 4 levels i.e without treatment (m0), 3.0 g tree⁻¹ (m1), 6.0 g tree⁻¹ (m2) and 9.0 g tree⁻¹ (m3). Thus each experiment contained 36 treatment combinations that were repeated three times. Leaf chlorophyll and nutrient content were obtained through leaf tissue analysis. The observation of light energy absorption (%) was performed using Miniature leaf spectrophotometer, while the stomatal conductance (mmol m⁻² s⁻¹) were observed using Prometer. Data was analysed using a factorial ANOVA test, and to determine the best treatment, data was tested using honest significant difference test (Tukey’s).

3. Result

3.1. Content of Chlorophyll *a, b* and Total leaf Chlorophyll

Statistical analysis showed that seed source and AM and its interaction had no significant effect on leaf chlorophyll content. Inoculation of *A. chroococcum* and AM had significant effect on leaf chlorophyll b content but its interaction was not significant. The interaction of seed source treatment with inoculation of *A. chroococcum* and interaction of inoculation of *A. chroococcum* with AM had significant effect on total leaf chlorophyll, but other interactions were not significant.

HSD test (α 0.05) in table 1 shows that inoculation of *A. chroococcum* 10⁶ CFU mL⁻¹ tree⁻¹ per tree shows the highest leaf chlorophyll a and b content and significantly different from control. Inoculation of AM 6.0 g tree⁻¹ showed the highest leaf chlorophyll content and was significantly different from the seedlings in control treatment. Seedlings sourced from Mukhtar 1 clone seeds inoculated with *A. chroococcum* 10⁶ CFUs mL⁻¹ tree⁻¹ per tree showed the highest total leaf chlorophyll and significantly different from the other *A. chroococcum* inoculation and seeds source treatment interactions. The interaction of the inoculation treatment of *A. chroococcum* of 10⁶ CFU mL⁻¹ tree⁻¹ per tree with AM 3 g tree⁻¹ showed the highest total leaf chlorophyll and significantly different from the interaction of the other *A. chroococcum* and AM.

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Table 1. Mean of leaves chlorophyll content of Cocoa Seedlings Clone Sulawesi 1, Sulawesi 2 and Mukhtar 1 at 4 months after treatment.

| Chlorophyll types | Treatment | Inoculation of Arbuscular Mycorrhizae (AM) (M) | Mean | HSD (a) α 0.05 |
|-------------------|-----------|---------------------------------------------|------|----------------|
|                   |           | m0  | m1  | m2  | m3  |                   |                   |
| Chlo a            | 0 CFU ml⁻¹ (a0) | 0.112 | 0.117 | 0.121 | 0.123 | 0.118₃         | 0.004             |
|                   | 10⁴ CFU ml⁻¹ (a1) | 0.121 | 0.124 | 0.126 | 0.119 | 0.122₃         |                   |
|                   | 10⁶ CFU ml⁻¹ (a2) | 0.122 | 0.120 | 0.126 | 0.123 | 0.123₃         |                   |
| Chlo b            | 0 CFU ml⁻¹ (a0) | 0.291 | 0.299 | 0.302 | 0.298 | 0.297₇         | 0.003             |
|                   | 10⁴ CFU ml⁻¹ (a1) | 0.300 | 0.302 | 0.305 | 0.303 | 0.303₃         |                   |
|                   | 10⁶ CFU ml⁻¹ (a2) | 0.302 | 0.306 | 0.308 | 0.304 | 0.305₃         |                   |
| Mean              |           | 0.297ₚ | 0.302ₚ | 0.305ₚ | 0.302ₚ | 0.004         |                   |

Total Chlorophyll Seed Sources A. chroococcum (CFU ml⁻¹ water)

|                   | Mean | HSD (m) α 0.05 |
|-------------------|------|----------------|
|                   |      | 0.003          |

Arbuscular Mycorrhizae

|                   | Mean | HSD (m) α 0.05 |
|-------------------|------|----------------|
|                   |      | 0.003          |

The numbers followed by the same letter in the columns (x, y, z) and on the lines (a, b, c, d) are not significant at the 5% confidence level.

3.2. Energy Radiation Absorption and Stomatal Conductance

Statistical analysis showed that seed source, inoculation of A. chroococcum and AM treatment had significant effect on light energy absorption at wavelength 746 nm of cocoa seedlings on age of 4 months after treatment (table 2). However, the no interaction effects was found. Clonal type treatment did not affect the leaf stomatal conductance, but inoculation of A. chroococcum and AM fungi had significant effect, while treatment interaction was not significant at four months after treatment.

HSD α 0.05 showed that cocoa seedlings with seed sourced from clone Mukhtar 1 had the highest radiation absorption rate and significantly different from the seedlings of clone Sulawesi 1, but not significantly different from the clone Sulawesi 2 seedlings. Inoculation of A. chroococcum 10⁶ CFU ml⁻¹ water showed the highest percentage of radiation absorption and significantly different from control seedlings, but not significantly different from the seeds inoculated with A. chroococcum of 10³ CFU ml⁻¹ water. Inoculation of Arbuscular Mycorrhizae of 6.0 g tree⁻¹ showed the highest percentage of radiation absorption and highest stomatal conductance and significantly different from other AM dosages.

3.3. Dry weight of stem and leaves, total roots and lateral roots

Statistical analysis showed that inoculation of A. chroococcum had significant effect on dry weight of stem and leaf of cocoa seedlings, but seed source, AM inoculation treatment and interaction between treatments had no significant effect (table 3). Inoculation of A. chroococcum and AM significantly affected the total root dry weight of cocoa seedlings, while seed sources and treatment interactions had no significant effect.
Table 2. Mean of Radiation Adsorption at wavelength of 746 nm (%) and stomatal conductance (mmol m\(^{-2}\) s\(^{-1}\)) of cocoa seedlings at four months after treatment due to effect of inoculation of \(A.\) chroococcum and Arbuscular Mycorrhizae.

| Parameter          | Treatment | Arbuscular Mycorrhizae (M) | Mean | HSD (s) | α 0.05 |
|--------------------|-----------|---------------------------|------|---------|---------|
| Radiation Energy   |           |                           |      |         |         |
| Seed Source        |           |                           |      |         |         |
| Sulawesi 1 (s1)    |           | 27.67                     | 30.85| 33.98   | 31.79   | 31.07x  | 0.96 |
| Sulawesi 2 (s2)    |           | 30.76                     | 31.74| 33.45   | 33.14   | 32.27y  |       |
| Mukhtar 1 (s3)     |           | 30.03                     | 31.66| 34.73   | 32.76   | 32.29y  |       |
| A. chroococcum     |           |                           |      |         |         |
| 0 CFU ml\(^{-1}\) (a0) |          | 28.75                     | 29.78| 33.95   | 32.16   | 31.16x  | 0.96 |
| 10\(^3\) CFU ml\(^{-1}\) (a1) |        | 29.06                     | 31.81| 34.86   | 32.68   | 32.10xy |       |
| 10\(^6\) CFU ml\(^{-1}\) (a2) |        | 30.65                     | 32.65| 33.34   | 32.85   | 32.37y  |       |
| Rata-rata          |           | 29.49 a                   | 31.42b| 34.05c | 32.56b  |         |       |
| HSD (m) α 0.05     |           |                           |      |         |         |
| Stomatal Conductance|         |                           |      |         |         |
| A. chroococcum     |           |                           |      |         |         |
| 0 CFU.ml\(^{-1}\) (a0) |          | 23.76                     | 25.9 | 28.57   | 25.52   | 25.94y  |       |
| 10\(^{3}\) CFU.ml\(^{-1}\) (a1) |        | 25.23                     | 26.17| 28.04   | 26.76   | 26.55y  |       |
| 10\(^{6}\) CFU.ml\(^{-1}\) (a2) |        | 26.64                     | 29.08| 28.75   | 28.81   | 28.32x  |       |
| Rata-rata          |           | 25.21 c                   | 27.05b| 28.45a | 27.03b  |         |       |
| HSD (m) α 0.05     |           |                           |      |         |         |

The numbers followed by the same letters in the columns (x, y) and on rows (a, b) are not significantly different at the 5% confidence level.

Table 3. Mean dry weight of stems and leaves (g), total roots (g) and lateral root (g) of cocoa seedlings age four months after inoculation treatment of \(A.\) chroococcum and Arbuscular Mycorrhizae

| Parameter | Treatment | Inoculation of Arbuscular Mycorrhizae (M) | Mean | HSD (a) | α 0.05 |
|-----------|-----------|-------------------------------------------|------|---------|---------|
| Stem and  |           |                                           |      |         |         |
| Leaf      |           |                                           |      |         |         |
| 0 CFU.ml\(^{-1}\) (a0) |          | 9.53                                     | 25.66| 32.59   | 23.26   | 22.76b  | 4.15 |
| 10\(^3\) CFU.ml\(^{-1}\) (a1) |        | 18.22                                    | 24.14| 38.16   | 23.31   | 25.96ab |       |
| 10\(^6\) CFU.ml\(^{-1}\) (a2) |        | 19.23                                    | 29.13| 36.69   | 28.57   | 28.41a  |       |
| Mean      |           |                                           |      |         |         |
| HSD (m)   | α 0.05    |                                           |      |         |         |
| Total Root|           |                                           |      |         |         |
| 0 CFU.ml\(^{-1}\) (a0) |          | 1.62                                     | 4.38 | 6.13    | 3.48    | 3.90\(^a\) | 0.84 |
| 10\(^3\) CFU.ml\(^{-1}\) (a1) |        | 3.06                                     | 4.59 | 6.4     | 4.67    | 4.68\(^{xy}\) |       |
| 10\(^6\) CFU.ml\(^{-1}\) (a2) |        | 2.86                                     | 4.23 | 6.65    | 5.46    | 4.80\(^c\) |       |
| Mean      |           |                                           |      |         |         |
| HSD (m) α 0.05 |        |                                           |      |         |         |

The numbers followed by the same letters in the columns (x, y) and on rows (a, b) are not significantly different at the 5% confidence level.

HSD α 0.05 test in table 3 showed that inoculation of \(A.\) chroococcum 10\(^6\) CFU mL\(^{-1}\) water per tree had the highest dry weight of stems and leaves and total of roots weight and differed significantly with control, but not significantly different from inoculation \(A.\) chroococcum of 10\(^3\) CFU mL\(^{-1}\) water per tree. Inoculation of AM 6.0 g tree\(^{-1}\) showed the highest average of stems and leaves dry weight and the highest total roots weight and differed significantly from other AM inoculations.
4. Discussion
Growth and development of plants is a function of genetic and environmental factors. The experimental results show that the seed source has significant effect on the observed growth parameters. Seed sources from Sulawesi 1 clone seeds have a lower chlorophyll a, b and total chlorophyll content than the seed sources of clones Mukhtar 1 and Sulawesi 2. This will result in lower absorption of light energy and stomata conductance of plant leaves in cocoa seedlings of Sulawesi 1 clone compared to seedlings originating from clones Mukhtar 1 and Sulawesi 2.

Cocoa plants have a considerable genetic variation in their morphological and physiological properties [11]. In generative propagation of this plant, leaf development occurs in a rhythmic fashion and is relatively unrecognized by environmental factors, so growth is entirely under endogenous control [12], and the assimilate content of the seeds is reflected in seed size [1] but there are intra- and inter-specific variations [13]. The pattern of vegetative plant organ development will greatly determine the rate of further plant growth and plant response to the environment. Thus the size and weight of seeds will determine the next plant growth.

Microbes can act as plant growth promoting agents that produce a variety of growth hormones, as well as the various organic acids needed in the growth of root feathers and are able to improve the biological health of the soil. Plants have different responses to the changing conditions of the Rhizosphere reflected from the plant genotype. The experimental results showed that the seed source of the Mukhtar 1 clone inoculated with A chroococcum $10^6$ CFU mL$^{-1}$ water showed a better average mean of stem and leaf dry weight, roots and lateral root development compared to Sulawesi 1 and Sulawesi 2 clones.

*Azotobacter* produces growth hormone and phosphate solvent compounds that have been used as favorable plants bio-inoculant [14]. Inoculation of *A chroococcum* can reflect root development and influence linearly to plant growth as reflected in dry weight loss. Utilization of *Azotobacter* in non-legume plants, effectively promoting growth and has proven useful for woody plants on dry land [15]. *Azotobacter* is able to produce anti-bacterial and anti-fungal compounds, producing vitamins and growth hormones of IAA and GA3 in the rhizosphere so as to improve root growth which will further improve overall growth.

The experimental results showed that inoculation treatment of mycorrhizal fungi had a good effect on growth parameter. Arbuscular mycorrhiza is an association between certain fungi with plant roots by forming a complex interactional fabric. Mycorrhiza is known as soil fungi because its habitat is in the soil and is in the root zone of the plant (rhizosphere). Mycorrhizae forms a symbiotic relationship with plant roots in the same way as the nodule root bacteria [16]. AM is generally associated with more than 80% mutualism of vascular plants, including cacao plants and plays an important role in sustainable agriculture [17]. AM enhances the ability of plant root systems to absorb mineral nutrients through the expansion of mycelium, plays an important ecological role in plant nutrient uptake [18], increased soil aggregation through the production of hydrophobic glycoproteins (glomalin) exempt from extra-radical hyphae [19]. The results showed that inoculation of CMA 6.0 g.tree$^{-1}$ can increase chlorophyll a and total chlorophyll leaf, light energy absorption and leaf stomatal conductance and total dry weight.

5. Conclusion

- Cocoa seedlings from clones Mukhtar 1 and Sulawesi 2 have better ecophysiological characteristics and growth compared to clone Sulawesi 1.
- Inoculation of *A chroococcum* of $10^6$ CFU mL$^{-1}$ water per tree in cocoa seedling seeds can improve the seedling growth.
- Inoculation of Arbuscular Mycorrhizae fungi of 6.0 g tree$^{-1}$ in cocoa seedlings can improve the growth of cocoa seedlings.

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