Response of cocoa leaves morphophysiological characters to application of different microbes formulation

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Abstract. A research was carried out to study the morphophysiological response of cocoa plant leaves to different application of microbes formulation in the farmer’s cocoa plantations in Gantarangkeke village, Gantarangkeke district, Bantaeng Regency, South Sulawesi Province from July to December 2016. The experiment was set using Randomized Block Design with nine combinations of dosages and frequencies of the application of microbe formulation as treatments. The formula was sprayed on cocoa leaves using bio-mineral liquid fertilizer with dosages of 0.5, 1.0, and 1.5 mL.L⁻¹ applied once, twice and three times during the experiment. The first application was at the start of the experiment, and the second was at the age of 2 months after planting, respectively. For the three times application treatment, plants were sprayed by the formula every two months. The experiment used three replications, and each experimental unit consisted of four selected plants which were relatively uniform. Results show that the application of microbial formulations through leaves could increase some morphophysiological properties of the leaves of cocoa plants. The application of microbial formulations of 1.5 mL.L⁻¹ of water every two months during the study showed an increase in leaf area growth, leaf water content, chlorophyll a, b and total chlorophyll content of cocoa leaves.

1. Introduction

Cocoa (Theobroma cacao L.) is an important annual plantation in the world, mainly used for chocolate production, with a global production of 4.6 million tons of dry beans produced in 2017/2018 [1]. In the same year, Indonesia ranked third in the world, the number of processed cocoa beans into semi-finished products amounted to 460 thousand tons. Indonesia is currently the largest cocoa producer in Southeast Asia, following a decline in production in Malaysia 20 years ago, which produced around 86.39% produced mainly in the form of smallholder plantations.

South Sulawesi is one of the largest cocoa producers in Indonesia, with a total production of 124.77 thousand tons from an area of 210.62 thousand hectares [2]. However, in the last ten years, cocoa production in South Sulawesi has continued to decline due to continuously used of old plants, decreased ecological quality of land and soil fertility, application of conventional cultivation techniques, scarcity of fertilizers and increasingly high fertilizer prices [3]. One effort that can be done to improve the production and quality of cocoa is to improve land ecology through the use of soil biotechnology (soil microbial services) and natural fertilizer technology that can improve soil fertility and improve plant growth [4].
Bio-mineral is one type of liquid fertilizers that can be applied to improve soil fertility, either through the plant leaves or through the soil. The Bio-mineral liquid fertilizer contains various types of beneficial microbes such as *Azotobacter* sp., *Rhizobium*, phosphate solvent bacteria, *Lactobacillus*, *Azospirillium*, cellulosic bacteria, *Pseudomonas*, and *Trichoderma* (Table 1). The recommended dosage is between 1-2 ml per liter of water.

**Table 1. Content of microbes in Bio-mineral liquid fertilizer.**

| Types of microbes       | Content per milliliter (ml) |
|-------------------------|-----------------------------|
| *Azotobacter* sp.       | $3.70 \times 10^5$          |
| *Rhizobium* sp.         | $3.70 \times 10^7$          |
| Phosphate solvent bacteria | $1.18 \times 10^7$          |
| *Lactobacillus* sp.     | $5.50 \times 10^5$          |
| *Azospirillium* sp.     | $5.50 \times 10^6$          |
| Selulotic bacteria      | $3.55 \times 10^7$          |
| *Pseudomonas* sp.       | $3.55 \times 10^6$          |
| *Trichoderma* sp.       | $3.55 \times 10^5$          |

Rhizo sp here in the soil as a plant growing medium is a component of the entire ecosystem which is an area of biological and chemical activity in the soil, influenced by compounds released by the roots intensively and is a food for soil microorganisms [5]. Bacteria that effectively colonize roots are commonly called Plant Growth Promoting Rhizobacteria (PGPR) or plant growth stimulant Rhizobacter. PGPR has the ability to protect parts of plants above the ground against viral, fungal and bacterial diseases with induced systemic resistance (ISR) [6], can accelerate germination, stimulate the growth of roots and shoots [7], increase leaf chlorophyll levels, increase plant tolerance to drought and salt and can delay leaf aging [8].

This study aims to determine the effect of microbial administration on several morphophysiological characteristics of leaves of cocoa plants. The results of the study are expected to be used as a solution to improve the growth and production of cocoa plants and as information material for farmers about the use of microbes useful in the cocoa cultivation system.

### 2. Methods

The research was carried out in the form of experiments in the farmer's cocoa plantations in Gantarangkeke Village, Gantarangkeke Subdistrict, Bantaeng Regency, South Sulawesi Province. The research located at an altitude of 400 meters above sea level (asl), with temperatures ranging from 23-30 °C. Experiments were conducted from July to December 2016 and set based on the Randomized Block Design. The treatment used was the application of bio-mineral liquid fertilizer on the cocoa plant leaves with a dose of control or without dryland (m0), sprayed once at the start of the experiment with dosages of 0.5 mL.L$^{-1}$ water (m1), 1.0 mL.L$^{-1}$ (m2) and, 1.5 mL.L$^{-1}$ water (m3), sprayed twice at the beginning and at the age of 2 months after planting with dosages of 0.5 mL.L$^{-1}$ water (m4), 1.0 mL.L$^{-1}$ water (m5), and 1.5 mL.L$^{-1}$ water (m6), sprayed three times during the experiment with application of the formula every 2 months with dosages of 0.5 mL.L$^{-1}$ water (m7), 1.0 mL.L$^{-1}$ water (m8), and 1.5 mL.L$^{-1}$ water (m9). The experiment was repeated three times, and each experimental unit consisted of four relatively uniform selected plants. Data were analyzed using a non-factorial Analysis of Variance (ANOVA) followed by Tukey’s test at a level of $p \leq 0.05$. 
3. Results and discussion

3.1. Results
Statistical analysis shows that the treatment of microbial application through spraying on leaves has a very significant effect on the total leaf area formed and total chlorophyll leaves, and has a significant effect on relative leaf water content, stomatal density, chlorophyll a content and chlorophyll b level, but has no significant effect on leaf mass area (table 2).

Table 2. Effect of the application of microbes through cocoa leaves on the specific leaf area (SLA) (cm$^2$), Leaf Mass Area (LMA) (g/cm$^2$), relative Leaf Water Content (LWC) (%), Stomatal Density (n/cm$^2$), Chlorophyll a, b, and leaf total chlorophyll content (µmol.m$^2$).

| Treatment | Parameter Observed | SLA Formed (cm$^2$) | LMA (g/cm$^2$) | Relative LWC (%) | Stomatal Density (n/cm$^2$) | Chlorophyll a (µmol.m$^2$) | Chlorophyll b (µmol.m$^2$) | Total Chlorophyll (µmol.m$^2$) |
|-----------|--------------------|---------------------|----------------|------------------|---------------------------|-----------------------------|-----------------------------|--------------------------------|
| m0        |                    | 171.8 a             | 0.0069         | 49.3 A           | 349.9 a                   | 489.1 a                     | 282.1 a                     | 714.4 a                      |
| m1        |                    | 217.9 bc            | 0.0078         | 54.1 A           | 341.1 a                   | 493.2 ab                    | 286.5 ab                    | 717.2 a                      |
| m2        |                    | 194.9 bc            | 0.0076         | 57.6 Ab          | 391.9 ab                  | 496.9 abc                   | 292.0 ab                    | 722.5 ab                      |
| m3        |                    | 220.2 c             | 0.0074         | 58.4 Ab          | 375.4 ab                  | 493.4 ab                    | 287.5 ab                    | 721.6 ab                      |
| m4        |                    | 232.6 cd            | 0.0074         | 52.9 Ab          | 491.1 c                   | 495.3 ab                    | 289.9 ab                    | 728.5 bc                      |
| m5        |                    | 226.6 c             | 0.0076         | 55.6 Ab          | 468.4 bc                  | 500.0 bc                    | 296.0 bc                    | 735.6 c                       |
| m6        |                    | 255.4 d             | 0.0077         | 54.4 Ab          | 456.9 bc                  | 491.5 ab                    | 286.0 ab                    | 718.4 bc                      |
| m7        |                    | 252.6 d             | 0.0076         | 59.7 b           | 472.6 c                   | 502.8 c                     | 299.7 c                     | 736.6 c                       |
| m8        |                    | 219.8 c             | 0.0081         | 52.5 ab          | 459.2 bc                  | 499.2 bc                    | 295.0 bc                    | 730.8 bc                      |
| m9        |                    | 252.6 d             | 0.0076         | 59.7 b           | 472.6 c                   | 502.8 c                     | 299.7 c                     | 736.6 c                       |
| Tukey’s at |                   | 23.0 ns             | 6.4            | 57.3             | 9.3                       | 12.0                        | 12.0                        |

Numbers followed by different letters in the column were significantly different at the level of 95% (Tukey’s p≤=0.05). m0 = control; m1, m2, and m3 = application of bio-mineral liquid fertilizer with dosages of 0.5, 1.0, and 1.5 mL.L$^{-1}$, respectively at the beginning of trial; m4, m5, and m6 = application of bio-mineral liquid fertilizer with dosages of 0.5, 1.0, and 1.5 mL.L$^{-1}$, respectively at the beginning of trial and at 2 months after planting; m7, m8, and m9 = application of bio-mineral liquid fertilizer with dosages of 0.5, 1.0, and 1.5 mL.L$^{-1}$, respectively every 2 months. ns= not significant.

Figure 1. Regression analysis relationship between dosage of microbial formulations with Stomatal Density (A), Chlorophyll a (B), Chlorophyll b (C) and Total Leaves Chlorophyll (C). Symbol (●) bio-mineral applied once, (○) bio-mineral applied twice, (▲) bio-mineral applied every two months.
Figure 1). The higher the \( \text{f} \) fed, \( \text{f} \) would ease the relative leaf water content, leaf stomatal density, and growth hormones such as IAA and GA, and cytokinin (table 2). The dose of microbial formulations has a significant effect on chlorophyll a, b and total chlorophyll leaves content. Regression analysis showed that microbial formulations that applied in once, twice and three times application were linearly correlated to leaf stomatal density, chlorophyll a, b and total chlorophyll content of leaves. The higher the dose of microbial formulations, the better the effect on leaf stomata density, chlorophyll a, b and total chlorophyll content of the leaves (figure 1).

3.2. Discussion

The results of the experiment show that the treatment of microbial formulations sprayed on leaves could increase the leaf area formed, increase the relative leaf water content, leaf stomatal density, and the level of chlorophyll a, b, and total chlorophyll leaves content. However, the treatment did not significantly affect the leaves LMA (table 2). The dose of the microbial application was positively correlated linearly with stomata density and leaf chlorophyll content (figure 1). The higher the microbial dose given, the higher the microbial density and the higher the chlorophyll content of the leaves. The treatment of microbial application 1.5 mL.L\(^{-1}\) of water applied three times during the experiment every two months showed higher average leaf area formed, relative leaf water content, stomatal density, and chlorophyll a, b and total chlorophyll content of leaves. The application of microbial formulations on plant leaves beside affecting the morphophysiological characters of leaves, other microbes applied to the leaves will fall to the ground and can improve soil fertility by adding soil microbes. The effect can be seen from the highly significant effect of the application frequency treatments and linearly correlates positively with stomatal density and chlorophyll content of plant leaves.

The application of the microbial formulation tested in the experiment was the PGPR microbial group. The results obtained in the recent study also found in the previous studies of various plants which showed that PGPR inoculation could improve yields and growth of the plants under drought stress as well as under normal condition [9][10]. The results of the study of Dey et al. [11] show that PGPR can increase plant growth under stress conditions by producing ACC deaminase, producing plant growth regulators such as IAA, GA, and cytokinin [12]. Furtherly, they can improve symbiotic nitrogen [13] and help in dissolving "mineral phosphate" and other nutrients [14].

The results show that the application of microbial formulations could improve the relative water content of the leaves. The response is probably due to PGPR microbes that can increase the efficiency of water use in plants. The application of PGPR in plants has a significant effect on the level of water use in plants [15]. Besides that, the application of PGPR microbes can induce the synthesis of hormones and antibiotics or lytic enzymes [13]. Utilization of PGPR, such as Azotobacter in non-legume plants, effectively increases growth and has proven to be beneficial for woody plants in dryland [16]. Azotobacter can produce anti-bacterial and anti-fungal compounds, produce vitamins and growth hormones such as IAA and GA\(_3\) in the rhizosphere so that it can increase root growth, which allows higher levels of plant water uptake hence will further improve the relative water content of the leaves.

The application of microbial formulations has a significant effect and has a linearly positive correlation with stomata density. The experimental results are in line with the results of research from Agami et al. [17] in basil (Ocimum basilicum L.) plants which found that leaves developed in conditions of higher water availability had greater stomata density and size. The application of microbial formulations has a significant effect on the chlorophyll a, b and total chlorophyll content of leaves. The increase is probably due to the application of PGPR microbial formulations that can directly facilitate plant growth and development through various mechanisms such as nutrient uptake or increasing nutrient availability through nitrogen fixation, mineralization of organic compounds, dissolution of mineral nutrients, and production of phytohormones [16]. This mechanism affects plant growth activities directly and varies according to microbial strains and plant species. Direct
enrichment of mineral absorption occurs due to an increase in individual ion flux at the root surface with the presence of PGPR. Chlorophyll and carotene concentrations increased significantly in all bacterial strain treatments because PGPR acts to increase the direct growth of the plants. The microbes tend to increase accessibility and concentration of nutrients by improving their supply for plant growth and productivity [18]. Plants absorb nitrogen from the soil in the form of nitrates (NO₃⁻) and ammonium (NH₄⁺), which are important nutrients for growth. Nitrate is usually the dominant form of nitrogen available in aerobic soil, where nitrification occurs and is absorbed by plants [13]. Some PGPRs can dissolve phosphates, increasing the number of phosphate ions available in the soil, which can be easily taken by plants [19]. Nitrogen is the main constituent of plant leaf chlorophyll so that it indirectly induces the synthesis of chlorophyll a, b and total chlorophyll. Similar results were reported by Heidari et al. [20] that suggested that the inoculation of bacterial strains such as Pseudomonas sp., Bacillus lentus, Azospirillum brasilens, increased the chlorophyll content in basil (Ocimum basilicum L.) under water stress.

4. Conclusion
Application of microbial formulations through leaves could increase some morphophysiological properties of the leaves of cocoa plants. The treatment dose of the application of microbial formulations of 1.5 mL.L⁻¹ of water three times during the study showed an increase in leaf area growth, leaf water content, chlorophyll a, b and total chlorophyll content of cocoa leaves.

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