Physiological responses of clove seedlings applied with different microbial consortium in the rhizosphere and phyllosphere

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Abstract. Rhizosphere and phyllosphere serve as habitat for many kinds of plant-associated microbial. This study aims to determine the effect of application of microbial consortium in the rhizosphere and phyllosphere of clove seedlings on the plant physiological parameters. The study was conducted as a factorial experiment with two factors based on the randomized blocked design. Application of microbial consortium of Azotobacter sp., Lactobacillus sp., Bacillus subtilis, and Trichoderma sp. in the rhizosphere was set as the first factor, consisted of four levels, namely control (0 mL), 4, 6, and 8 mL. The second factor was the application of the microbial consortium of Gliocladium sp. and Beauveria bassiana in the phyllosphere consisted of four levels, namely control (0 mL), 2, 4, and 6 mL. The physiological parameters observed included observations on the components of leaf stomata, light intensity, and leaf chlorophyll. The results show that the best microbial consortium treatment that gave better physiological response of the clove seedlings was 8mL/plant in the plant rhizosphere, and 6mL/plant in the plant phyllosphere. However, there were no significant difference on the effect of the application of these microbial consortiums in the rhizosphere and the phyllosphere applied simultaneously compared to controls, meaning that the treatment application was 8mL / plant or 6mL / plants. Some influence directly and indirectly from the microbial consortium both in the rhizosphere and the phyllosphere was on the physiological parameters of the light and leaf chlorophyll components, but not significant for the stomata component.

1. Introduction
To restore the glory of clove farmers, the Directorate General of Plantation is developing clove cultivation in 2019 with an area of 18,800 ha. In fact, in 2020-2024 the development is targeted to increase by an area of 4,200 ha / year. Meanwhile, the need for clove seedlings in 2020-2024 is estimated to reach 1,994,000 seedlings / year [1].

The problem in providing clove seedlings, namely death due to stress of transplanting from seeding to polybags. According to Arif et al. [2], transplanting causes a risk of damage due to stress of adaptation process to the environment and physiological stress due to damage to vegetative organs, especially roots, which can cause slow growth and many deaths.

Another cause of death of clove plants is sunburn where the light intensity is too high. Sunburn cause an increase in leaf temperature so that the transpiration rate increases, this results in reduced leaf area or...
fallen leaves followed by death and 3.5% of plants die due to drought in July, inhibition of nutrient absorption and further pest attacks namely aphids which suck food from young leaf buds[3]. Efforts to obtain good quality of clove seeds require appropriate environmental engineering for growth. Environmental engineering in increasing the ability of plants to adapt to the environment can be done by utilizing microbes [4]. Microbial applications in the form of a consortium can reduce the risk of failure of microbial utilization in the field [5]. Several microbial consortia such as Azotobacter sp., Lactobacillus sp. can be used in decomposition of organic compounds to fertilize the soil [6]. Bacillus subtilis can form biofilms and siderophores that can overcome metal boundaries in environmental conditions [7], Trichoderma sp. is known to be found as the dominant soil micro flora in a wide variety of habitats. The mechanism of Trichoderma as a soil-borne pathogen control agent can be through mechanisms of parasitism, competition for space and nutrition, forming a suitable environment for plants, forming growth-promoting substances, as well as antibiosis and induction of plant resistance [8].

In the phyllosphere, the response to the application of Gliocladium sp. can increase the content of chlorophyll a and b [9], and Beauveria bassiana increase the fertility of soybean plants in field conditions through spraying on the leaves (Phyllosphere) of plants [11]. Studies on the effect of the microbial consortium in the rhizosphere and phyllosphere on the plant physiological response of clove seedlings have not been widely carried out. As cloves have a fairly high economic value, it is necessary to study the ability of the microbial consortium applied in the rhizosphere and phyllosphere to prevent plant death due to physiological stress during planting in the field.

2. Methodology

2.1. Study site.
This research was carried out from March to June 2020 in Tanete Village, Tompobulu Sub-District, Gowa Regency, South Sulawesi Province, which located at an average altitude of 1000 m above sea level, air temperature ranges from 22-30°C with an average humidity of 60-80%, wind speed 19 km/hour and is at the coordinate point position of 05 ° 20 ′ 36 ″ SL and 119 ° 40 ′ 05 ″ EL.

2.2. Experimental design.
This research was conducted in the form of a factorial experiment, 2 factors arranged based on the randomized block design pattern. The first factor was the application of the microbial consortium in the rhizosphere (G) using Azotobacter sp., Lactobacillus sp., Bacillus subtilis, and Trichoderma sp. microbes, which consisted of 4 test levels, namely G0 = 0 mL/plant, G1 = 4 mL/plant, G2 = 6 mL/plant, G3 = 8 mL/plant. The second factor was the application of the microbial consortium in the phyllosphere (P) using Gliocladium sp. and Beauveria bassiana microbes, which consisted of 4 test levels, namely P0 = 0 ml/plant, P1 = 2 ml/plant, P2 = 4 ml/plant, P3 = 6 ml/plant. From these two factors, 16 treatment combinations were obtained. Each treatment combination consisted of 2 plants which were repeated 3 times resulted in 96 plants used.

2.3. Plant materials and growth media preparation.
Plant materials used in the study was 18 months old Zanzibar cultivar cloves and then transplanted into polybags with a size of 15 cm x 20 cm. Soil used for growing media was cleaned from particles that can disturb plants such as stones and other plant root remains. Subsequently, the soil were mixed with compost with a ratio of 1: 2. Compost application as a basic fertilizer was carried out to provide stimulation to the nutrient requirements of the plants.

2.4. Microbial consortium application to clove plants.
The application of the microbes to the clove plant rhizosphere was carried out by spraying a 10 ml of microbial solution onto soil surface from a distance of 5 cm. Microbial application to the clove phyllosphere was carried out by spraying the leaves and stems of plants with a sprayer. Applications were carried out once a month for three months.
2.5. Plant physiology observation.

2.5.1 Leaf stomata components. Observation of stomata was carried out using an electron microscope, by taking preparations sample using nail polish, garnish and clear tape, the selected clove leaves, namely the third leaf from the shoot was then applied with nail polish evenly in the lower surface of the leaves, after 15 minutes, clear tape was attached to the nail polish and left for 15 minutes. Afterwards, the tape was then placed onto deg glass and given a label. Observation of leaf stomata components consisted of stomata density and stomata opening area. Stomata density was calculated using equation 1:

\[
\text{Density area} = \frac{\text{Number of stomata}}{\text{Field of view}}
\]  

(1)

Where field of view is calculated using equation 2:

\[
\text{Field of view} = \frac{\pi r^2}{\text{mm}^2} \text{ or } \frac{\pi r^2}{100 \text{cm}^2}
\]  

(2)

The density of stomata was observed under a microscope using 40 times magnification with a field of view diameter of 0.52 mm², while for non-stomata area was observed using a magnification of 100 times with a field of view diameter of 0.52 mm².

The area of the stomata opening was then calculated using the equation 3:

\[
\text{Stomata opening area} = \pi r_1 r_2
\]  

(3)

Where:
\[
\pi = 3.14;
\]
\[
r_1= \text{the length of the stomata opening};
\]
\[
r_2= \text{the width of the stomata opening}.
\]

2.5.2 Light energy component. Observation of the components of sunlight energy, namely the amount of radiation scope, the amount of transmitted radiation, the amount of reflected radiation, and the amount of absorbed radiation were measured using the Miniature Leaf Spectrometer C1-710 / 720 (figure 1A). Observation was carried out at the end of experiment after three times treatment application by selecting the third leaf from the shoot (figure1B).

2.5.3 Chlorophyll components observation. Observation of leaf chlorophyll components was observed using a Content Chlorophyll Meter (CCM 200+) (Figure 2A) on the 3rd leaf from the shoot, at four months after three times treatment applications. Observations were made (Figure 2B) on the chlorophyll a, chlorophyll b, and total leaf chlorophyll content, using the equation 4 as follows:
Leaf chlorophyll content = $a + b(CCI)^c$ \hspace{1cm} (4)

Where: \(a, b, c = \) constant (table 1);
CCI = Leaf chlorophyll index.

Table 1. Constant values a, b and c.

| Parameter  | y = a + b (CCI)$^c$ |
|------------|---------------------|
|            | A       | B   | C    |
| Chl a      | -421.35 | 375.02 | 0.1863 |
| Chl b      | 38.23   | 4.03  | 0.88  |
| Chl$_{tot}$| -283.20 | 269.96 | 0.277 |
| $\alpha$   | -3.50   | 3.96  | 0.027 |

Source: Gonçalves 2008-[12].

![Figure 2](image1.png)

(A) Content Chlorofil Meter (CCM 200+) tool, B. Usage of tools in the field.

2.6. Data analysis

The data from the observations were analysed using the F test at the 5% level, if there was a significant effect between treatments, a further test was carried out using the LSD (Least Significant Difference) at the 5% level.

3. Results and discussion

3.1. Effect of microbial consortium application on density and area of stomata.

There was no significant difference between the treatment of the microbial consortium in the rhizosphere and in the phyllosphere nor the interaction between the two treatments on the leaves stomatal parameters. This shows that the simultaneous application in the rhizosphere and phyllosphere of the clove plants does not have an effect on the density and area of stomata openings. However, the results of the observation that shows the best average stomatal opening area was the application of microbes at 8 mL per plant in the rhizosphere or 6 mL in the phyllosphere (table 2). The overly large opening of the stomata can cause excess transpiration, forcing roots to absorb more water from the ground and when the groundwater concentration is low it can cause drought stress therefore the best stomata opening area is chosen.

Table 2. Average of stomata opening area and stomata density of clove leaves with the treatment of microbial consortium in the rhizosphere and phyllosphere.

| Parameter           | Microbial Consortium in the Phyllosphere (P) |
|---------------------|---------------------------------------------|
|                     |                                             |


Microbial Consortium in the Rhizosphere

| (G) | 0 mL (p0) | 2 mL (p1) | 4 mL (p2) | 6 mL (p3) |
|-----|------------|------------|------------|------------|
| Stomata Opening Area | 0 mL (g0) | 0.0037 | 0.006 | 0.0046 | 0.0051 |
| | 4 mL (g1) | 0.0038 | 0.006 | 0.0045 | 0.006 |
| | 6 mL (g2) | 0.0045 | 0.0044 | 0.0075 | 0.0061 |
| | 8 mL (g3) | 0.0056 | 0.0051 | 0.0063 | 0.0057 |
| Stomata density | 0 mL (g0) | 231.9 | 243.9 | 268 | 284.2 |
| | 4 mL (g1) | 248.1 | 303.1 | 279.5 | 264.3 |
| | 6 mL (g2) | 255.4 | 271.7 | 285.8 | 286.3 |
| | 8 mL (g3) | 253.4 | 276.4 | 291 | 281.1 |

Stomatal opening area is influenced by the potassium (K) element where K plays an important role in regulating osmotic and turgor pressure, which in turn will affect cell growth and development and also opening and closing of the stomata [13]. There is a significant relation between K concentration and Nitrogen availability in plant [14]. In fertile soils the K content in the tissue is almost the same as N [15]. Plants that are supplied with nitrogen (N) have a higher K concentration and plant water content [16] thus directly or indirectly the stomatal opening area is influenced by microbial activity because microbes have the potential to act as nitrogen fixers and biofertilizers. Azotobacter sp. as non-symbiotic nitrogen fixing microorganisms, nitrogen fixation occurs because of the nitrogenase enzyme [17], and Bacillus subtilis is categorized as a Plant Growth Promoting Rhizobacter (PGPR) bacterium, affecting plants directly or indirectly by fixing nitrogen [18]. An increase in P and K uptake can occur with the activity of P-solvent bacteria and K mobilizers such as in Bacillus [19, 20]. Lactobacillus sp. also have a role in dissolving soil phosphate and potassium [21] as well as Trichoderma sp., a fungi generally used for decomposing soil organic matter, which contains several nutrients such as N, P, S and Mg in addition to other nutrients needed by plants for their growth [22].

In the phyllosphere, microbes can protect plants such as Gliocladium sp. that is able to adhere firmly along the leaf bones, epidermal cells and into the stomata [23]. Similarly, Beauveria bassiana where its conidia have germinated and hyphae elongated on the leaves, stalks, stems, and adhere to the cuticle of the plant epidermis but do not enter the stomata [24], hence able to minimize the level of nutrient loss and minerals in the leaves through washing due to prolonged and repeated rainfall. Minerals that are easily washable in young leaves are Na and Mn with a loss of more than 25% over 24 hours while the slightly leached minerals with a loss of between 1-10% are Ca, Mg, K, and Sr [25, 26]. K transport occurs in the xylem and phloem vessels. The direction of transport of xylem sap goes in sync with the flow of transpiration from roots to leaves. While the direction of transport of phloem sap to actively growing tissues such as young leaves (flush) [16].

The next parameter, the observation of the best average stomatal density (table 2) is shown in the application of the microbial consortium of 8mL treatment in the rhizosphere and 6 mL in the phyllosphere. The higher the stomata density, the higher the number of stomata [27]. The stomata density determines the conductance of the stomata in regulating the gas diffusion process [28]. Increasing stomatal density indicates higher number of stomata, thereby resulted in increased rate of translocation of water and mineral salts, regulating leaf temperature by releasing heat and water from leaves and regulating optimum turgor pressure in cells, and increasing the transpiration and CO₂ absorption for photosynthesis. In this condition, the risk of sunburn due to high level of radiation intensity on the clove seedlings might be overcome [29].

3.2. Effect of microbial consortium application on light and chlorophyll parameters.
The results of microbial consortium application in the rhizosphere and in the phyllosphere treatment on the parameters of the amount of light received and reflected were significantly different from the control
at the 0.05% Turkey’s, but there was no interaction. The best treatment based on the 0.05% Turkey’s test for parameters of the amount of light received (Scope), light reflection, and light absorption (table 3) is the treatment of 8mL/plant of microbial consortium in the rhizosphere or 6mL/plant in the phyllosphere. For light transmission, there was no significantly difference in the Turkey’s 0.05%, but gave the best average results for treatment of 8mL/plant in the rhizosphere or 6mL/plant in the phyllosphere.

Table 3. Average amount of light received by leaves (watts / cm² / second), amount of light reflected (%), amount of light transmitted (%), amount of light absorbed (%) by treatment of the microbial consortium in the rhizosphere and phyllosphere.

| Parameter          | Microbial consortium in the Rhizosphere (G) | Microbial Consortium in the Phyllosphere (P) | Average | Turkey’s [G] 0.05 |
|--------------------|---------------------------------------------|---------------------------------------------|---------|------------------|
| Total Scope        | 0 mL (g0)                                   | 9148.9                                     | 9725.6  | 10591.6          |
|                    | 4 mL (g1)                                   | 9895.6                                     | 11429.7 | 11058.2          |
|                    | 6 mL (G2)                                   | 9948.7                                     | 11299.5 | 11521.3          |
|                    | 8 mL (g3)                                   | 10259.8                                    | 10366.6 | 11987.4          |
| Average            | 9813.2 y                                    | 10705.4 xy                                 | 11505.5 x | 11289.6 x       |

| Reflected Light (%) | 0 mL (g0)                                   | 14.10                                      | 14.60   | 15.0 b           |
|                    | 4 mL (g1)                                   | 15.00                                      | 15.50   | 16.4 ab          |
|                    | 6 mL (g2)                                   | 14.70                                      | 16.20   | 18.50            |
|                    | 8 mL (g3)                                   | 17.60                                      | 17.10   | 18.2 a           |
| Average            | 15.3 y                                     | 15.8 xy                                   | 17.9 x  | 17.0 xy          |

| Light Transmitted (%) | 0 mL (g0)                                   | 14.55                                      | 15.15   | 15.09            |
|                      | 4 mL (g1)                                   | 14.93                                      | 15.82   | 16.60            |
|                      | 6 mL (g2)                                   | 16.55                                      | 15.66   | 16.88            |
|                      | 8 mL (G3)                                   | 16.28                                      | 17.02   | 17.41            |
| Average              | 15.35                                      | 15.15 xy                                  | 16.66   | 17.05            |

| Light Absorbed (%)   | 0 mL (g0)                                   | 6.16 c                                     | 6.42 c  | 7.09 c           |
|                      | 4 mL (g1)                                   | 6.66 c                                     | 7.01 c  | 7.62 c           |
|                      | 6 mL (g2)                                   | 7.48 bc                                    | 6.99 c  | 6.92 c           |
|                      | 8 mL (g3)                                   | 7.08 c                                     | 7.25 bc | 9.16 a           |
| Average              | 6.52                                       |                                           |         |                  |

The results of leaf chlorophyll index, chlorophyll a, chlorophyll b, total chlorophyll shown in table 4. There is no significant interaction between the treatment of the microbial consortium in the rhizosphere and the phyllosphere on the parameters. This result indicates that the application of the microbial consortium in the rhizosphere and the phyllosphere simultaneously does not provide significant values compared to control on the chlorophyll components. So that if it is given separately, it gives significantly different results at the Turkey’s 0.05%, this can be seen in all parameters of the chlorophyll index, chlorophyll a, b and total chlorophyll (table 4). The treatment that was significantly
different to control, namely 8 mL/plant of the microbial consortium in the rhizosphere or 6 mL/plant in the phyllosphere.

**Table 4.** Average chlorophyll index, chlorophyll a, chlorophyll b, and total chlorophyll of cloves seedling leaves on microbial consortium in the rhizosphere and phyllosphere treatment

| Parameter                      | Microbial consortium in the Rhizosphere (G) | Microbial Consortium in the Phyllosphere (P) | Average | Tukey’s \[G\] 0.05 % |
|--------------------------------|--------------------------------------------|---------------------------------------------|---------|-----------------------|
| Index of Chlorophyll           |                                            |                                             |         |                       |
|                               | 0 mL (g0)                                 | 116.3                                       | 119     | 120.4                 | 121.9 | 119.5 b                |
|                               | 4 mL (g1)                                 | 119                                         | 121.3   | 121                   | 123.4 | 121.2 ab               |
|                               | 6 mL (g2)                                 | 118.8                                       | 124.1   | 121.8                 | 122.4 | 121.8 ab               |
|                               | 8 mL (g3)                                 | 119.2                                       | 122.5   | 122.3                 | 128.1 | 123.0 a                |
|                               | Average                                   | 118.3 y                                    | 121.7 x | 121.4 x               | 123.9 x |                       |
| Tukey’s \[P\] 0.05%           |                                            |                                             |         |                       | 2.8   |                       |
| Chlorophyll a                  |                                            |                                             |         |                       |
|                               | 0 mL (g0)                                 | 488.3                                       | 492.4   | 494.2                 | 496.2 | 492.8 b                |
|                               | 4 mL (g1)                                 | 492.2                                       | 495.4   | 495                   | 498.3 | 495.2 ab               |
|                               | 6 mL (g2)                                 | 491.9                                       | 499.2   | 496.2                 | 496.9 | 496.1 ab               |
|                               | 8 mL (g3)                                 | 492.4                                       | 497.1   | 496.8                 | 504.8 | 497.8 a                |
|                               | Average                                   | 491.2 y                                    | 496.0 x | 495.5 x               | 499.0 x |                       |
| Tukey’s \[P\] 0.05%           |                                            |                                             |         |                       | 3.8   |                       |
| Chlorophyll b                  |                                            |                                             |         |                       |
|                               | 0 mL (g0)                                 | 303.2                                       | 308.7   | 311.3                 | 314.3 | 309.4 b                |
|                               | 4 mL (g1)                                 | 308.5                                       | 313     | 312.5                 | 317.2 | 312.8 ab               |
|                               | 6 mL (g2)                                 | 308.2                                       | 318.7   | 314.1                 | 315.2 | 314.0 ab               |
|                               | 8 mL (g3)                                 | 308.8                                       | 315.4   | 315                   | 326.6 | 316.5 a                |
|                               | Average                                   | 307.2 y                                    | 314.0 x | 313.2 x               | 318.3 x |                       |
| Tukey’s \[P\] 0.05%           |                                            |                                             |         |                       | 5.5   |                       |
| Total Chlorophyll              |                                            |                                             |         |                       |
|                               | 0 mL (g0)                                 | 725.1                                       | 731.7   | 734.7                 | 738.1 | 732.4 b                |
|                               | 4 mL (g1)                                 | 731.4                                       | 736.7   | 736.1                 | 741.5 | 736.4 ab               |
|                               | 6 mL (g2)                                 | 731                                         | 743.1   | 738                   | 739.2 | 737.8 ab               |
|                               | 8 mL (g3)                                 | 731.8                                       | 739.5   | 739.1                 | 752.3 | 740.7 a                |
|                               | Average                                   | 730.2 y                                    | 314.0 x | 313.2 x               | 318.3 x |                       |
| Tukey’s \[P\] 0.05%           |                                            |                                             |         |                       | 6.3   |                       |

Numbers followed by the same letter in columns (a, b) and rows (x, y) are significantly different based on Tukey’s test with a confidence level of 0.05.

Light energy is one of the determining factors in the photosynthesis process, plants receive high enough light energy (scope) but not all of the total light energy can be absorbed in the photosynthesis process, the amount of light energy in the scope is reflected, transmitted, and released in the form of heat energy only about 0.5-3.5% of the light energy is used by plants for the photosynthesis process. This is consistent with the statement of Nasaruddin and Yunus [16] that the amount of solar radiation reaching the earth’s surface is only about 20% of the total radiation emitted, and 98% of the total light received is returned to the atmosphere in the form of energy. Only about 2% of solar energy is used by plants in the process of photosynthesis. The photosynthetic process takes place in the plastids of cell organisms called chloroplasts. Inside the chloroplast contains chlorophyll pigment which is green as the
main light-absorbing pigment and carotenoid as a complementary pigment. High-level plants contain two kinds of chlorophyll, namely chlorophyll a and chlorophyll b, while the most carotenoids found in plants are b carotene and lutein. Chlorophyll is not effective in absorbing green light so that more is reflected and transmitted.

The protein-chlorophyll complex is an important component of photosynthesis. Light radiation received by plants in photosynthesis is absorbed by chlorophyll and additional pigments, which are protein-chlorophyll complexes [30]. The factors that influence the formation of chlorophyll include light, genes, elements of N, Mg, Fe as catalysts in the synthesis of chlorophyll [31,32]. Microbes help plants in providing nitrogen. It is known that Azotobacter sp., Bacillus subtilis, Lactobacillus sp. and Trichoderma sp help in the provision of nitrogen nutrients for plants [33, 34, 35, 36, 37].

The phyllosphere microbes, Gliocladium sp. not only able to adapt to life in plants but also succeeded in mimicking its chemical profile by producing the same few metabolites. Extraction results of Gliocladium sp. in liquid culture showed the presence of fatty acids and other lipids [38]. Lipids play a role in cytokine hormone signalling [39]. One of the functions of cytokines, which is to stimulate chloroplast development and chlorophyll synthesis [40].

While Beauveria bassiana is a cosmopolitan anamorphic fungus that infects hundreds of insect species that immigrate to the plant surface via wind-borne, B. bassiana conidia attached to the plant phyllosphere without any interaction but interact with immigrating insects [41] so that B. bassiana acts as a entomopathogens of shell lice and aphids that interfere with light absorption are ultimately disturb photosynthesis, this is consistent with the results of research by [42]. B. bassiana protects and reduces the attack rate of shell lice (Coccus viridis) and aphids (Aphis gossypii) in young seedlings, because the stems are still soft, making it easier to absorb fluids and obtain plant nutrients as food, therefore the plant becomes stunted and new leaves are slow to grow thus in the end the plants dry up and wither. Also, shell lice (C. viridis), aphids (A. gossypii) excrete honey dew. The presence of honey dew that is released can be seen by the presence of ants or black sooty dew. The appearance of this sooty moisture causes the leaf surface to be covered so that it will hinder the photosynthesis process.

4. Conclusion

Many microbes can manipulate the level of plant hormones and synthesize their chemical profiles, which contribute to plant growth, increased immunity and pathogenesis. From the recent study it can be concluded that the best microbial consortium treatment that gave better physiological response of the clove seedlings was 8mL/plant of Azotobacter sp., Lactobacillus sp., Bacillus subtilis, and Trichoderma sp in the plant rhizosphere, and 6mL/plant of Gliocladium sp. and Beauveria bassiana in the plant phyllosphere. However, there were no significant difference on the effect of the application of these microbial consortiums in the rhizosphere and the phyllosphere simultaneously compared to controls, meaning that the treatment application was 8mL / plant or 6mL / plants. Thus the differences in the application of the microbial consortium in the rhizosphere and the phyllosphere affect the physiology of clove seedlings directly or indirectly thus economically, giving one consortium microbes treatment is more effective.

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