**THESIS**

**ISOLATION AND POTENTION OF LOCAL PONOROGO *Rhizobium sp.* TO INCREASE NITROGEN ABSORPTION**

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# ABSTRACT

**Isolation and Potention of Local Ponorogo *Rhizobium sp* to Increase Nitrogen Absorption**

**Topan Adi Pondra**

**37.2016.63.1590**

Rhizobium is a bacterium that plays a role in fixing nitrogen in legume plants. Previous research stated that local Rhizobium gave better growth in legume plants compared to conventional Rhizobium. The purpose of this research was to isolate and see the effect of local *Rhizobium sp*. Ponorogo isolated from plant roots. The research was conducted in the agrotechnology practicum and laboratory from January to June 2020. Laboratory tests were to determine the characterization and growth of local Rhizobium bacterial isolates that isolated from soybean plant roots. Isolates were grown on Yeast Extract Mannitol Agar (YEMA) media added with Congo Red (CR) and Bromthymol Blue (BTB) dyes. Bacterial growth was observed by inserting one bacterium into ± 50 ml of YEM broth. Bacterial growth was observed using a spectrometer every 2 hours. Field tests were conducted to see the isolates effectiveness on the soybean growth and Nitrogen uptake. The research design used was a completely randomized design with 6 treatments and 5 replications. The method used for the analysis of N plants is the Kjehdal method. From the research results, it was found that the bacterial isolates had a round colony shape, flat edges (overall), yellow, milky white and yellowish white. Rhizobium bacteria growth in liquid YEM medium was still stable until the 7th day with a slight decrease and increase in absorbance. In the Mannitol agar extract Yeast medium, the fastest bacterial growth was found in the Rhizobium Mlarak 3 isolate. However, soybeans treated with Rhizobium Mlarak 3 isolate gave higher N uptake than controls and other isolates.

Keywords: *Rhizobium sp*, Growth, Soybean, N plants.

**THESIS**

**Isolation and Potention of Local Ponorogo *Rhizobium sp.***

**to Increase Nitrogen Absorption**

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# STATEMENT OF AUTHENTICITY OF THESIS

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Hereby declare that as follows :

1. Thesis entitled: **Isolation and Potention of Local Ponrogo *Rhizobium sp* to Increase Nitrogen Absorption**
2. The research conducted is the work of the author himself.
3. Any idea or quotes from other people’s work in the form of publications or other forms in this thesis, has been recognized in accordance with standard disciplinary procedures.
4. The author also recognizes that this thesis can be produced thanks to the guidance and support of the supervisor namely: **Muhammad, S.P., M.P** and **Umi Isnatin S.P.,M.P.**

If later in this thesis there are things that indicate that academic fraud has been committed, the author is willing to obtain a bachelor's degree that the author has withdrawn in accordance with the provisions of the S1 study program in Agrotechnology, Faculty of Science and Technology, Darussalam University Gontor.

Ponorogo, 28 Oktober 2020

Topan Adi Pondra

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# PREFACE

Praise to Allah SWT for His guidance, the writer can complete the thesis with the title *Isolation and Potential of Local Ponorogo*Rhizobium sp.*to Increase Nitrogen Absorption*. Sholawat and greeting to the great prophet Muhammad SAW who has brought his people from the dark ages to the times that are bright with science. The author completes this thesis aiming to obtain a Bachelor's degree in Agriculture from the Agrotechnology Study Program, Darussalam Gontor University. The purpose of this study was to obtain the best local Ponorogo Rhizobium isolate. These local Rhizobium bacteria are used to increase the uptake of nitrogen nutrients in plants. With the presence of Rhizobium bacteria, farmers do not need to use synthetic chemical fertilizers for plant nitrogen requirements. Chemical fertilizers have a lasting impact on soil damage. The benefits of this research have both practical and academic benefits. The practical benefit of this research is that the local Ponorogo Rhizobium bacteria can be used as biological fertilizer in the future. And the academic benefits of this research are as learning material for academics.

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May the goodness of all parties receive abundant rewards by Allah Subhanahu Wa Ta 'ala Aamiin. Finally, I apologize for all the shortcomings that the author made in completing this thesis. The author realizes that this thesis is far from perfect. Therefore, the authors hope to get suggestions and criticism to make the thesis better.

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# CHAPTER 1: INTRODUCTION

## 1.1 Background

Allah created his creatures in this world, nothing is in vain, including the microorganisms that are in the soil. One of the soil microorganisms that can increase plant growth and yield is *Rhizobium sp* bacteria. *Rhizobium sp* bacteria helps to increase plant growth by providing nitrogen elements that can be absorbed by plants[[1]](#footnote-1). Rhizobium bacteria symbiosis with the roots of legume plants by forming root nodules.

Several microorganisms including Rhizobium has different effectiveness depending on the region. Differences in effectiveness can be caused by the differences in soil fertility, geographic location, climate, and host plants in the area[[2]](#footnote-2). The Rhizobium bacterial isolates obtained could be reused to increase the N uptake in legume plants. Soil with added microorganisms has a tendency of high soil fertility. In addition, microorganisms can provide efficiency in fertilization and are environmentally friendly[[3]](#footnote-3).

Nitrogen (N) elements are required for vegetative growth. Nitrogen is the largest elemental contained in the plant and used as an establishment of amino acids and chlorophyll. Nitrogen has very important role for manykind physiological processes. Leaf growth, stems and other vegetative growth is heavily dependent on the nitrogen element. Deficiency of nitrogen can inhibit growth, causing chlorosis, and inhibits late growth of shoot. Nitrogen deficiency can be known by colour degradation from green to yellow[[4]](#footnote-4).

The high nitrogen requirements in plants growth force the farmers to use synthetic chemical fertilizers. The main purpose of farmers using synthetic fertilizer is because the nutrients can be available quickly to the plant. The negative impact of synthetic chemical fertilizers usage is it make the aggregate of soil being harder and plant’s root difficult to penetrate the soil.

ظَهَرَ الْفَسَادُ فِي الْبَرِّ وَالْبَحْرِ بِمَا كَسَبَتْ أَيْدِي النَّاسِ لِيُذِيقَهُمْ بَعْضَ الَّذِي عَمِلُوا لَعَلَّهُمْ يَرْجِعُونَ

means: "has seen damage on land and by sea caused by human actions, God wants them to feel some of their (due) of their deeds, so they return (to the right path)." (Q.S Ar-Rum: 41)

The ayat explains the damage that has been made by humans both in the land and sea. The use of excessive chemical fertilizer is one of the damages that are on land due to human being. If the use of synthetic chemical fertilizers is performed for long periods, the soil will be damaged, so the harvest from farmers will decrease. The phenomenon is one of the damage consequences caused by synthetic chemical fertilizers used.

To reduce damage caused by synthetic chemical fertilizers, farmers switch to more environmentally friendly, it is compost fertilizers. However, the disadvantage of the compost fertilizer is unable to provide N nutrient quickly, due to the compost fertilizers must go through the decomposition process to process the nutrient for being absorbed by the plant. The amount of compost fertilizer used for plant cultivation is larger than synthetic chemical fertilizers. Therefore, compost fertilizer applications are still combined with synthetic chemistry (inorganic).

The inefficient use of compost can be overcome by adding biological fertilizers. The addition of rhizobium biofertilizer provide more effective N nutrient. Rhizobium provide nitrogen for plants at 15-20 days after infection and is marked by the appearance of root nodules. The combination of compost and Rhizobium has more impact because compost provides a better environment for microbial growth. Also, Rhizobium bacteria have a long-term impact by providing Nitrogen for the next growing season, thereby increasing plant growth[[5]](#footnote-5).

The research cannot be separated from the functions and benefits of the research. One of the Maqosid sharia from this study is *hifdzul maal* (safeguarding property). *Hifdzul maal* from the research is saving money spent on buying synthetic chemical fertilizers, because farmers spend more money to buy synthetic chemical fertilizers than Rhizobium fertilizer.

Comparison for the cost needed to buy Rhizobium biological fertilizer and synthetic chemical fertilizer is described as follows. According to previous research, it was stated that the application of Rhizobium bacteria 450 gr/ha with a mixture of 60 kg/ha Petrobio biofertilizer gave the best results the number of pods and dry weight of seeds[[6]](#footnote-6). The price of 50 gr Rhizobium bacteria is Rp. 20,000.00, therefore, if 450 grams are needed, it will costs (450/50) X Rp. 20,000 = Rp. 180,000.00. Meanwhile, the price of Petrobio biofertilizer is IDR 55,000 per 5 kg. For 60 kg needs, the cost required is (60/5) X IDR 55,000.00 = IDR 660,000.00. So the total cost required is Rp. 180,000.00 + Rp. 660,000.00 = Rp. 840,000.00. The cost needed to buy Rhizobium biological fertilizer is Rp. 840,000.00 for 1 ha cultivation area.

The calculation of the cost needed to buy synthetic chemical fertilizers as follows. For areas with moderate nutrient classes, the need for fertilizers is very high. The number of chemical fertilizers needed for soybean growth is Urea (152 kg/ha), SP-36 (80 kg/ha), and K fertilizer (190 kg/ha)[[7]](#footnote-7), respectively. The cost required is very large. The price of 152 kg of urea fertilizer is Rp 273,600.00 or Rp 1800.00. The price of 80 kg SP-36 fertilizer is Rp. 480,000.00 or Rp 6,000/kg. The price of 190 kg KCL fertilizer is Rp 1,368,000.00 or Rp 7,200.00/kg. Then the total cost required is Rp 2,121,000.00.

From the calculation above, the difference in costs incurred is very large, namely Rp. 2,121,000.00 - Rp. 840,000.00 = Rp. 1,281,600.00. If the use of Rhizobium biological fertilizer can be applied, farmers can save costs. Cost savings incurred to buy fertilizers is one of the maqasid of sharia, namely hifdzul maal. If farmers can save money spent on buying fertilizers, then the opportunity for farmers to develop their businesses will be even greater. This is because farmers can fulfill their needs to buy the facilities and infrastructure needed for developing their business.

One of the *Rhizobium sp* bacteria hosts is soybean plants. Soybean itself is a plant whose seeds are used as food. the need for soybeans in Indonesia is very high. The reason is because soybeans are used as a food ingredient. The high demand for soybeans is not accompanied by a yields increase. The soybean cultivation in Indonesia has decreased due to land area reduction and land fertility degradation.

Increased soybean crop production can be done by extensification and intensification. Extensification is increasing crop yields by expanding cultivated land, while intensification is utilizing existing space with intensive care so that the yield produced is equivalent to a large area of ​​land. Giving *Rhizobium sp* in soybean cultivation is one way of intensification. Adding the Rhizobium sp bacteria can increase N uptake in soybean plants. By increasing N uptake, soybean vegetative growth will be better. Increased vegetative growth of plants will provide good photosynthetic results in plants, these photosynthetic products will later be accumulated into plants and soybean yields.

## 1.2 Problem Formulation

The research on the isolation of *Rhizobium sp* bacteria from soybean roots has several problem formulations that will be discussed.

1. What are the characteristics of *Rhizobium sp* bacteria found in the roots of

soybean plants planted in Ponorogo Area?

2. How is the growth of Rhizobium sp bacteria in Yeast Extract Mannnitol Agar and Yeast Extract Mannitol Broth media isolated from soybean plant roots?

3. How much is the total N content of soybean plants inoculated by Rhizobium sp from the isolation results?

## 1.3 Research Purpusoses

The purpose of this study is to answer the above questions related to.

1. Knowing the characteristics of Rhizobium sp bacteria that grow on the roots of soybean plants.

2. Knowing the growth of *Rhizobium sp* bacteria isolated from soybean plants in Yeast Extract Mannnitol Agar and Yeast Extract Mannitol Broth media isolated from soybean plants.

3. Knowing the total N content from plants inoculated by *Rhizobium sp*.

## 1.4 Scope of problem

The limitation of this problem is only limited to the Rhizobium sp bacteria which is symbiotic with soybean plant roots. So that other types of bacteria that are not in symbiosis with soybean plant roots will be ignored in this study. Then the isolated bacteria will be tested again on soybean plants to determine the effect of giving Rhizobium sp on the growth and uptake of N in soybean plants.

## 1.5 Benefits of Research

The results of this study will have two benefits from an academic and practical perspective.

### 1.5.1 Academic Use

The results will be used as a reference by further research that discussing the same theme. This research can be used as learning theory by the students and the academics.

### 1.5.2 Practical Uses

The results of this study can be used as a biofertilizer for soybean plants to increase yield production.

## 1.6 Research Hypothesis

The hypothesis H0 is Rhizobium sp bacteria has no significant effect on increasing growth and nitrogen uptake in soybean plants. Meanwhile, the hypothesis H1 is Rhizobium sp has a significant effect on increasing growth and uptaking the nitrogen nutrients.

# CHAPTER II: LITERATURE REVIEW

## 2.1 Classification of *Rhizobium sp*

*Rhizobium sp* are bacteria that can symbiosis with the roots of legumes. This study uses soybean (*Glycine max*) which is one of the legume plants. Infected legume roots will form root nodules on soybean plants. The taxonomy of the *Rhizobium sp* bacteria is as follows[[8]](#footnote-8).

Division : Protophyta

Class : Scizomycetes

Order : Eubracialis

Family : Rhizobiaceae

Genus : Rhizobium

Species : Rhizobium sp.

## 2.2 Characteristics of *Rhizobium sp*

Rhizobium are bacteria that can symbiosis with the roots of legume plants that play a role in providing nitrogen. Infected plant roots will form nodules. These bacteria bind free nitrogen in the air and convert it into ammonia (NH3). These compounds are then converted into amino acids which are necessary for plant growth. These bacteria get carbohydrates from plants which are used as an energy source.

From the macroscopic appearance, Rhizobium bacteria have milky white colonies, not transparent, circular, convex, semitranslusent colony forms, and have a colony diameter of 2-4 mm. Rhizobium bacteria colonies can be seen on agar media for yeast-mineral salts-mannitol within 3-5 days. Microscopically, rhizobium bacteria are rod-shaped, aerobic, gram negative with a size of 0.5 - 0.9 X 1.2 - 1.3 µm, are motile in liquid media and have one polar or subpolar flagella. *Rhizobium sp* require a temperature of 25 - 300C and a pH of 6 – 7 pH as optimum growth environment[[9]](#footnote-9). Rhizobium bacteria are chemoorganotropic, which means they can use a variety of carbohydrates and organic acids as a carbon source. These bacteria attack the root hairs of legume plants by means of intracellular symbiosis.

## 2.3 Mechanism for the Formation of Root Nodules

Rhizobium bacterial infection in legume plants has two different mechanisms. First, bacteria enter through the root hairs. Second bacteria enter through the crack in the roots. Root hair infection almost occurs in all legume crops, one of which is soybean. There are four processes for root nodules infection, namely Rhizobia colonializing in the Rhizosphere, the root surface attaching, root hair branching and root hair bending[[10]](#footnote-10).

Prior to the infection process, legume plants produced aromatic compounds capable to stimulate the Nod Rhizobia gene through the protein NodD regulator. One of the aromatic compounds that stimulate rhizobia are flavonoids, (isoflavones, chalcones, flavonols, flavones, and anthosiadins)[[11]](#footnote-11). Among the rhizobes that can take advantage from the degradation products of flavonoids as a single carbon source and free energy is the Bradyrhizobium bacteria.

Rhizobium bacteria that entered the roots will occupy the space between the root hair wall and the epidermal junction with the cortex cells. The space filled by Rhizobium bacteria will form a zone of intracellular infection. After entering into the cell, the Rhizobium bacteria carry out an intercellular infection and multiply in the cell. The formation of nodules occurs due to repeated division of plant cells in the infected roots. and every cell that divides contains some rhizobia. After cell division stops the bacteria turning into bacteroids, these changes are accompanied by metabolic changes[[12]](#footnote-12).

In bacteroids there is a nitrogenase enzyme which produces an enzyme for nitrogen fixation. Leghemoglobin is also found in bacteroids, which play a role in protecting the oxygen-labile nitrogenase enzyme. Oxygen is used for bacteroid respiration. Root nodules form at 10-14 days under controlled conditions and free from nitrogen nutrients while in field conditions it can be seen when the plants are 21-28 days old[[13]](#footnote-13).

## 2.4 Nitrogen Fixing Process

Nitrogen tethering occurs in bacteroids that are assisted by the nitrogenase enzyme. Nitrogenase enzymes consist of two types of protein, namely proten molybdenum-Iron-Sulfur (Mo-Fe-S) and Protein Iron (Fe). Nitrogen fixing activity in root nodules is influenced by the transfer of oxygen into the nodules. and photosynthesis results from plants for nodules. The fixation process by the nitrogenase enzyme requires ATP and low pressure reductants. The resulting photosynthesis will be oxidized by Rhizobium bacteria to provide energy and the necessary compounds. Nitrogen fixation in the air requires Feredoxin which acts as an electron donor. It takes six electrons to reduce N2 to 2NH3.

N2 + 6H + + 6e ~ + 12ATP + 12H20> 2NH3 + 12ADP + 12Pa

Nitrogen tethering only occurs when plants actually need available N. If there is N available in the soil for plants, the tethering carried out by Rhizobium bacteria will be inhibited and stop. The results of N anchoring in root nodules with the help of nitrogenase enzymes will be used for plant growth with the help of enzymes found in plants.

The red pigment present in root nodules between the bacteroid and the membrane sheath surrounding it. The amount of red pigment in root nodules depends on the ability of Rhizobium bacteria to bind oxygen[[14]](#footnote-14). This oxygen is used for the respiration process. The enzyme that plays a role in the reduction of N2 to ammonia (NH3) is nitrogenase.

Some Rhizobium species are only effective on certain legume crops. One drawbacks of using Rhizobium is its specific nature in infecting host plants. So that each species of Rhizobium infects only certain legume plants. If there is a symbiosis between Rhizobium and plant roots, root nodules will be formed[[15]](#footnote-15). Rhizobium sp bacteria in peanuts are different from Rhizobium sp in soybeans. Several types of Rhizobium that have symbiosis with soybeans are *Rhizobium japonicum* and *Bradyrhizobium japonicum*. In order to survive the bacteria must be able to live saprophytic with the host plant. In addition, Rhizobium must be able to adapt to complex and competitive soil conditions.

The number of nodules also affects the N uptake in plants. Based on previous research, the addition of Rhizobium bacteria at a dose of 5 g / kg combined with straw mulch gave the largest number of root nodules so that the availability of plants for the nitrogen was fulfilled. More nodules that are formed indicate the activity of Rhizobium bacteria[[16]](#footnote-16). Other research also supports that the effectiveness of Rhizobium bacteria can be seen from the number of root nodules formed. The addition of bacterial isolates was able to increase the number of root nodules in legume plants[[17]](#footnote-17).

Isolation of local Rhizobium bacteria has greater potential in supporting plant growth compared to introduce bacteria from different areas. Strains for biological fertilizers should be isolated from certain areas and inoculated back into the environment so that the use of biological fertilizers has more impact[[18]](#footnote-18). This opinion is also supported by research conducted in Kenya, that two local Rhizobium sp isolates were more effective than Commercial Rhizobium. The effectiveness was indicated by the dry weight and the number of nodules of the soybean plant which was higher than commercial Rhizobium sp[[19]](#footnote-19). The disadvantage of Rhizobium bacteria is that they cannot survive in acidic and toxic soil conditions such as aluminum and pesticides[[20]](#footnote-20). Rhizobium bacteria grow better in alkaline soil conditions. If the bacterial inoculation is carried out in acid soil conditions, dolomite lime is needed so that the soil acid content decreases and the bacterial grow better.

Rhizobium sp are heterotrophic bacteria, where the energy source comes from the oxidation of organic compounds such as sucrose and glucose. It was a reason why bacteria need a host plant as a place to grow. Bacteria get a source of energy from plants while plants get nitrogen from the fixation carried out by bacteria.

## 2.5 The Role of Rhizobium as Nitrogen Fixing

Most of the plant's need for nutrients is obtained naturally. Carbon, Hydrogen, Oxygen and Nitrogen are readily available in nature. However, the element Nitrogen as the main element of protein formation in plants cannot be absorbed directly by plants, even though the availability of Nitrogen is around 80% in the air. To meet the need for nitrogen, some plants use soil microbes to fulfill these elements, one of which is the Rhizobium bacteria which can be in symbiosis with plant roots.

The ideal place for soil microbes is the rhizosphere zone because in this zone there is a lot of organic material that can be utilized by microbes. In this zone, plants also produce organic substrates such as hormones, lectins and enzymes that break down organic compounds. Each plant has a specific protein (inducer) which is useful for attracting bacteria[[21]](#footnote-21). One of the compounds that act to attract Rhizobia to form nodules is daidzein and genistein. Both of these compounds are included in the isoflavone group[[22]](#footnote-22).

Rhizobium bacteria can increase legume crop production by providing 10-25% of plant nitrogen needs. Provision of liquid fertilizer at a dose of 0.0625 ml of Rhizobium bacteria every 25 cm2 can increase the growth and yield of *Phaesolus vulgaris* plants[[23]](#footnote-23). For soybean plants, giving bacteria inoculum can significantly increase the growth and yield of soybeans compared to plants without Rhizobium application[[24]](#footnote-24).

The advantages of using Rhizobium bacteria can increase the efficiency of use and there is no damage to the soil, if it has become a biofertilizer, the price is relatively cheap, application is relatively easy and can increase nutrients, especially nitrogen[[25]](#footnote-25). The addition of Rhizobium biofertilizer to soybean plants can reduce fertilizer use and increase plant weight, root nodules and seed dry weight[[26]](#footnote-26).

The positive impact of using Rhizobium inoculants in the long term is to provide nitrogen availability in the cultivated soil. Some of the tethered nitrogen remains stored in the roots and nodules and then degraded into the soil in the form of ammonium (NH4 +) and NO3- nitrate[[27]](#footnote-27).

## 2.6 Growth of Rhizobium sp bacteria on Growth Media

Rhizobium bacteria have long been used as objects of research, because of their ability to fix nitrogen. In previous studies, many *Rhizobium sp* bacteria were affixed to Yeast Mannitol Agar (YEMA) media. Due to the potential of *Rhizobium sp* bacteria which can bind nitrogen, many industries have made Rhizobium sp bacteria as a biofertilizer. In addition, many studies have explained the ratio of media concentrations for the growth of these bacteria. From the research results, the concentration of propagation media that gives good bacterial growth will be obtained but in cheaper price. This optimization can reduce production costs for the *Rhizobium sp*.

To identify Rhizobium bacteria, YEMA media was added with Congo Red (CR) and Bromthymol Blue (BTB) dyes. Congo red dye has a role in distinguishing Rhizobium bacteria from other bacteria. The characteristic of Rhizobium bacteria is that it does not absorb red color when given the Congo Red dye. Bromthymol Blue dye functions in the classification of the growth rate of Rhizobium bacteria. If the bacteria turns blue, it means that the bacteria has slow growth (Slow Growing). And if the bacteria changes color to yellow, it is included in fast growing[[28]](#footnote-28).

The more effective bacteria propagation is done on liquid YEM media. Based on the research that has been done, the addition of 6 grams / liter of yeast extract and 12 grams / liter of mannitol provides optimal bacterial growth compared to higher concentrations of yeast extract and mannitol[[29]](#footnote-29). The fewer materials used, the smaller the production costs required.2.7 N-fixing bacteria other than *Rhizobium sp.*

There are many nitrogen fixing bacteria, not only limited to Rhizobium sp. There are nitrogen fixing bacteria that can symbiosis with the roots of the host plant and some are not able to symbiosis with plant roots. one of the bacteria that can be used for N besides Rhizobium is Azotobacter.

Azotobacter bacteria is one of the aerobic nitrogen fixing bacteria that does not have symbiosis with plant roots. Azotobacter characteristic are gram negative and moves with flagella. The cell shape of the Azotobacter bacteria ranges from stem to round[[30]](#footnote-30). Although the Azotobacter bacteria are aerobic, they can also live in environments with low oxygen levels. Apart from being an N-fastener, Azotobacter sp can also produce the IAA hormone which can stimulate root growth and act as phosphate solubilizing bacteria[[31]](#footnote-31).

In addition to the Azotobacter bacteria, there is also the *Azospirilum sp* bacteria which is one of the Plant Growth Promoting Rhizobacteria (PGPR) bacteria. These bacteria have broader interactions with various plant roots. Azospirilum sp bacteria is known as an associative Nitrogen fixing bacteria because the energy source of Azospirilum sp bacteria comes from the root exudate of the host plant. This exudate contains sugars, amino acids, vitamins, malate, and several other compounds. These bacteria have a curved cell shape and vibrinoids with a negative gram[[32]](#footnote-32).2.8 Description of Soybean Plant Varieties Detam 4 Prida. Soybean is a grain crop that is used as a food source in several countries in the world. Soybeans are used as a source of protein for humans and this protein is equivalent to animal protein[[33]](#footnote-33). Basically, the soybean plant is a plant that can symbiosis with various kinds of soil microbes, including the mycorrhizal arbuscular fungi and *Rhizobium sp*, so that this plant does not need a lot of additional fertilizer for growth. This is because the microbes in the soil help the roots the soybean roots to absorb nutrients.

Due to the soybean planting season in Indonesia, planting is carried out in the second dry season with a rice-rice-soybean cropping pattern. Although soybean plants are somewhat resistant to water deficiency, filling the pods is a crucial phase. If the soybean plant lacks water in this phase it will cause crop failure with empty pods. In this study, the soybean used was the Detam 4 variety. The Detam 4 variety soybean was the crossing result between two different lines. This type of soybean only grow flowers once in a period (determinate)[[34]](#footnote-34). The first flower on Detam 4 soybean begins to flower at the age of 36 days and it is estimated that the seeds can be harvested at 76 days old.

This Detam 4 variety has triangular and green leaves. This soybean plant has a height of about 53.2 cm. This variety has purple flowers, brown pods, and black seed coat. In one plant there are approximately 55 pods. For the weight of each 100 seeds is about 11 grams and the average yield is 2.5 tons/ha. The advantage of this variety is that it is resistant to pod-sucking pests and rust disease. In addition, this variety have early age and tolerant to drought[[35]](#footnote-35).

# CHAPTER III: METHODOLOGY

## 3.1 Time and Place

The time of the research was carried out in January - August 2020. Sampling bacteria was carried out in two different places, namely Demangan Village, Siman District and Bajang Village, Mlarak District. Bacterial Identification and isolate testing was carried out in the Agrotechnology lab at the Darussalam Gontor University.

## **Tools and Materials**

The tools used are a spectrometer, small shovel, knife, plastic container, microscope, optilab, measuring cup, petridish, test tube, pipette, spatula, LAF (Laminar Air Flow), fume hood. The materials used were 1 gram of soybean root nodules, rubber, plastic, YEMA media, Congo Red dye, and Brom Thymol Blue.

## Research Method

### 3.3.1 Sampling Method

Soybean plant roots were taken by making holes around the roots with 15 cm in radius and 20 cm in depth. The roots of the soybean plant were pulled out slowly. Root nodules were taken by cleaning the roots from the soil using water. Extracted root nodules can be stored overnight in the refrigerator. Long-term storage obtained by using a dry glass tube and then stored in the refrigerator[[36]](#footnote-36). The characteristic of an active nodule is the appearance of a red to brownish color when the root nodule is split.

### 3.3.2 Media Creation

The medium used was YEMA (*Yeast Extract Manitol Agar*) media consisting of K2HPO4 (0.5 g), MgSO47H2O (0.2 g), NaCl (0.1 g), CaCO3 (3 g), 10 g Mannitol, yeast extract 3 g, agar 20 g, aquadest 1000 ml and pH 6.8[[37]](#footnote-37). The media was put into an erlenmeyer tube then stirred using a Hot Plate until well blended[[38]](#footnote-38).

### 3.3.3 Sterilization of Equipment and Materials

The finished media was covered with cotton and plastic wrap. Meanwhile, the Petri dish is coated with paper and then wrapped in plastic. YEMA and Petridish media were put into an autoclave at a temperature of 1210C for 15 minutes[[39]](#footnote-39).

### **3.3.4 Isolation of Rhizobium Bacteria from Plant Roots**

Isolation of bacteria was done by taking root nodules on soybean plants then sterilized using 70% alcohol by shaking in an Erlenmeyer tube for 1 minute. Furthermore, sterilization was done using 5.25% NaClO solution which was diluted 100 ml of Aquades until 10% in concentration for 15 minutes.

The next procedure is the root nodule were rinse 3 times using distilled water for 1 minute per rinse. In the next step, the root nodule weighing 1 gram was crushed using a mortar and then added 10 ml of distilled water. The yield of the scour was diluted to 10-7. The results of the 10-7 dilution were pipetted 1 ml using a micropipette to be grown in YEMA media. After that, it was incubated at room temperature 27 - 280C for 3 days. The colony morphology of the growing bacteria was observed to be separated and grown on YEMA media by streaking it until there was actually one bacterial colony[[40]](#footnote-40).

### 3.3.5 Identification **of *Rhizobium Sp***

**1. Bacterial Color Changes on Media**

In bacterial characterization tests the bacteria were grown in the YEMA media added by the 0,025 gr Congo Red (CR) dye and 1 ml Brom Thymol Blue (BTB) 1% with a medium volume of 1000 ml [[41]](#footnote-41). Then the color change of bacteria was observed carefully.

**2. Rhizobium Bacteria Morphology Observations**

This observation was done visually by looking directly at growing bacterial colonies. The observations for bacterial colonies morphology were made in the forms, diameters, edges, elevation, optics, and pigmentation. Observations on the colony colors were done directly by looking at the colony colors on the Petridisk. The observation of shapes, edges, elevation, and optics were done directly to observations of colony colors. To measure the diameter of the bacterial colony, the caliper and magnifying glass on *Colony Counter* were used.

**3. Gram Staining Bacterial Testing**

Gram Color is aimed for observing the morphology of Rhizobium sp bacterial cells from the shape and color of grams. For gram test, as much as 10 μL 10-3 was taken by pipette and placed above the preparation glass. Then it was drained by skipping glass preparations on the fire. A gram of bacterial staining was done in several stages. The first coloring was Violet Crystal for 30 seconds then it was rinsed using Aquades. Then the iodine solution was added and let stand for 1 minute and rinsed again using Aquades. Furthermore, it was cleaned using alcohol 70% to wash violet crystal color and rinsed using Aquades. Next the object was dotted with Safranin and incubated for 45 seconds. Further, it was rinsed using Aquades and dried. Furthermore, the object was ready to be observed using a microscope[[42]](#footnote-42). Cells that can preserve blue or purple color are called gram's positive bacteria and grams that bind red or pink the color of safranin are called gram-negative bacteria.

### 3.3.6 The Curve of bacterial growth in YEM Broth

One loop of rhizobium bacteria was inserted into 50 ml YEM Broth in Erlenmeyer. The next step was shaking bacteria for 24 hours. The bacteria was taken 2 ml to measurements the absorbance using a spectrometer with 600 μm in wavelength. The Observations were done every 2 hours until the last measurement was 26 hours. After the measurement, the next stage was making the bacterial growth curve.

### 3.3.7 Propagation of Rhizobium Bacteria in YEM Broth

1 ml of YEM broth media containing Rhizobium bacterial strains cultured into 100 ml of YEM broth in 250 ml Erlenmeyer. Then the bacteria were shaked at 125 rounds/min, in room temperature for 3 days[[43]](#footnote-43).

### 3.3.8 Observation of Amount of Bacterial Colony

Bacteria grown in the YEM Broth was taken 1 ml to be grown in the YEM agar with a 10-7 dilution series calculated by TPC (Total Plate Colony) method. The countable colony is a plate containing 30 -300 bacterial colonies. The Calculation of bacterial colony was done using Colony Counter tool. The Calculating of the number of bacteria was done using the formula below..

### 3.3.9 Rhizobium Bacteria Ammonia Content Test

Bacteria culture in the YEM broth were taken by 0.5 ml, then 4,5 ml of distilled water was added. After homogeneous, 0,1 ml *Nessler’s Reagent* was added. The formation of yellow to brown indicates the existence of ammonia content in bacteria culture. The concentration of Ammonia was measured using a spectrometer with 500 nm wavelength[[44]](#footnote-44).

### 3.3.10 **Testing Field of Rhizobium Bacteria Strain in Soybean**

1. **The Design of Research**

The research design used in this research was complete random design with 6 treatments and 5 replications.

1. **Steriliation of soil**

Soil sterilization was done to remove the microorganisms in the ground. The Microorganisms in the soil were eliminated, in order to not affect the growth of the soybean plant and to ensure that Rhizobium bacteria was the only factor that affects the growth of soybean. The sterilization was done by heating the soil by steamed in a large pan at 1210C. Heating is the most effective and efficient sterilization. This way was done by utilizing the hot steam that will kill the microbes that exist in the ground. Prevoius research showed that sterilization with steam techniques is one of the efficient sterilization other than burning or oven[[45]](#footnote-45).

**3. Inoculation of Rhizobium Bacteria Isolates**

Bacterial inoculation for soybean plants was taken from YEM broth containing the bacterial culture. Inoculation was carried out by diluting the liquid culture containing bacteria. The seeds have had been germinated until 7 days. then inoculated with 1 ml of liquid culture containing bacteria. After that, the seeds were planted into polybags with a total of 4 seeds per polybag. After one week, two plants with same height were left. Soil moisture was maintained by giving water every day[[46]](#footnote-46).

The parameters observed were plant height, shoot wet weight, root wet weight, shoot dry weight, root dry weight and total plant N. After the plants were 50 days old, the plants were harvested to take their leaves for N analysis. The accumulation of N in these plants will provide data on the results of the fixation of Rhizobium bacteria.

**4. Observation of Plant Height**

Observation of plant height was measured once a week. plant's height was measured using a ruler. Measurements were made from the base of the plant to the highest canopy[[47]](#footnote-47).

**5. Root Length Observation**

Root of the plant was taken by tearing the polybags and watering the soil until it was separated from the soybean roots, then drying it to the air. The length of the soybean root was measured using a ruler from the base to the end of the longest root.

**6. Soybean Plant Head Wet Weight**

Observation of wet weight was carried out by weighing the canopy of the soybean plant which was still fresh from the base of the stem to the top of the plant. this wet weight was weighed uses analytical balances for more accurate weighing results.

**7. Soybean Plant Canopy Dry Weight.**

To determine the dry weight, the soybean plants water content must be removed using an oven. The soybean crown was put into a paper bag and then dried at 700C for 48 hours. Then was weighed using analytical scales[[48]](#footnote-48).

**8. Water Content of Plants**

To calculate the water content of plants using the following formula[[49]](#footnote-49).

Water Content∶ (BB-BK) / BB X100%

Information :

BB: Wet weight of plants before oven

BK: Dry weight after oven

**9. Root Nodule Calculation**

The Observation of root nodules was carried out by uprooting plants in the final vegetative phase. The plants that had been extracted were then observed for the number of nodules present in the roots[[50]](#footnote-50).

**10. Determination of Total Plant N with a Spectrophotometer**

The N test in this plant was done by oxidizing the parts of the plant that will be tested using a solution of sulfuric acid (H2SO4). N test with a spectrometer was done using indophenol blue. To be able to determine the N content in plant samples, the plants were digested first using H2SO4 and a mixture of selen.

*Sample Digestion*

The sample to be used in the N-total analysis comes from the soybean leaves. The sample was digested and weighed 0.250 g, then put into the digestion tube, And added 1 gram of selen mixture and 2.5 ml H2SO4. After that, flatten it and leave it overnight. Blanks were prepared by inserting 1 gram of selen and 2.5 H2SO4 into the digestion tube, then heated into the digestion block at a temperature of 3500C and the digestion process was done when white steam comes out.

*Standard Making*

1. Standard 0

The blanks that had been made were diluted using 50 ml distilled water.

2. Standard principal 1000 ppm N

Powder (NH4) 2SO4 weighed at 4.7413 g into a 1 l volumetric flask then add distilled water to the limit of 1 liter and stir until homogeneous.

3. Standard 20 ppm

As much as 2 ml of 1000 ppm N standard staple was pipetted and poured into a 100 ml volumetric flask and added 100 ml of standard 0

4. Making a standard series of 0-20 ppm N

Standard N 20 ppm were pipetted as much as 0,1,2,4,6,8 and 10 ml and pour it into the reaction tube. Standard 0 was added to 10 ml in size. This series has concentrations of 0,2,4,6,8,12,16 and 20. Then it should shaked until homogeneous.

5. Na-phenate solution

100 grams of NaOH powder was dissolved in 500 ml distilled water in a 1 l volumetric flask. Then 125 g of phenol powder was added and stired until dissolved added up to 1 l of distilled water.

6. Tartrate buffer solution

50 grams of NaOH powder was dissolved into 500 ml of distilled water, and added 50 g of K powder, Na tartrate and stir until dissolved and added distilled water to 1 l.

*Measurement of N on a Spectrometer*

A total of 1 ml of sample was put into a test tube, added 9 ml of distilled water and shaked it with a tube shaker, Pipette into the test tube 2 ml of dilute extract and standard series. Then 4 ml of tartrate buffer and Na-Phenate was added, following by 4 ml of 5% NaOCl, shaked it, let it stand for 10 minutes, then measured it with 636 nm wavelength.

The Calculation formula with Spectrophotometer.

N content (%) = ppm curve x ml extract / 1000 ml x 100 / mg example x fp x fk

= ppm curve x 50 / 1,000 x 100/250 x 10 x fk

= ppm x curve 0.2 x fk

Information:

ppm curve = sample rate obtained from the relationship curve between the standard series level and the reading after correcting the blank.

100 = conversion to%

fp = dilution factor (10)

fk = moisture content correction factor = 100 / (100 -% moisture content)[[51]](#footnote-51)

### 3.3.11 Data Analysis

Analysis of data was done using ANOVA (*Analysis of Variance*) with a level of 5%. If there is a difference, then it was going to proccess with the Smallest Significant Difference Test (LSD). Data analysis was done using Microsoft Excel software.

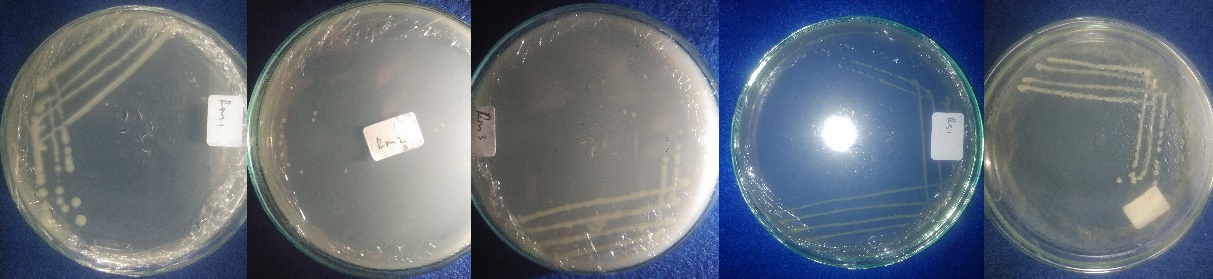
# CHAPTER IV: RESULTS AND DISCUSSION

## 4.1 The Characteristics of *Rhizobium sp*

The results of bacterial isolation on the roots of soybean plants in two different places showed that there were three isolates isolated from soybean taken in the Mlarak area and two isolates from the Siman area. Colony observations were carried out on the fifth day after growingof bacterial isolates in YEMA media. From the characterization of bacteria, it was found that each isolate had the following colony characteristics.

**Table 1**.Characteristics of Rhizobium Bacteria

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Number** | **Characteristics** | ***Rhizobium sp* Strains** | | | | |
| ***Rhizobium* Mlarak 1** | ***Rhizobium* Mlarak 2** | ***Rhizobium* Mlarak 3** | ***Rhizobium* Siman 1** | ***Rhizobium* Siman 2** |
| 1 | Colony Diameter (mm) | 2 – 4 | 4,5 – 6 | 3 – 6 | 2,5 - 3 | 2 – 3 |
| 2 | Color | Yellowish | Yellowish | Yellowish | yellow | Milky |
| 3 | Optic | Opaque | Opaque | Opaque | Opaque | Opaque |
| 4 | Form | Round | Round | Round | Round | Round |
| 5 | Elevation | Convex | Convex | Convex | Droplet | Convex |
| 6 | Margin | entire | entire | entire | entire | entire |



**Figure 1.** The Rhizobium growth on YEMA media. Rhizobium Mlarak 1, 2, 3, Rhizobium Siman 1 and 2 (left-right).

From the results of observations on bacterial colonies, different characterizations were obtained for each isolate. Rhizobium isolates isolated from the roots of soybean plants in the Mlarak area supposed to have a larger bacteria colonies diameter than bacteria isolated from the Siman area. Diameter sizes of Rhizobium Mlarak 1, 2, and 3 are 2 - 4 mm, 4.5 - 6 mm, and 3 - 6 mm, respectively.

Bacteria isolated from the roots of cowpea (Vigna unguiculate) colony have 2-5 mm in size with yellowish-white color during three days incubation period. However, some isolates have a 2-3 mm in size with an incubation period of 7-10 days.

For colony color characterization, Rhizobium bacteria generally have different colors. The Rhizobium Mlarak 1, 2, and 3 isolates have the same color, namely yellowish-white color. The Rhizobium Siman 1 isolate has a yellow color colony and the Rhizobium Siman 2 isolate has a milky white color. In several studies, it was stated that the colors of the Rhizobium bacteria colonies were milky white[[52]](#footnote-52), yellowish white[[53]](#footnote-53) and yellow[[54]](#footnote-54). Previous research also stated that Rhizobium isolates isolated from edamame soybean cultivation land also had a milky white color[[55]](#footnote-55).

Characterization of Rhizobium based on optics is the ability of light to penetrate bacterial colonies. Major bacterial colonies can transmit light but in quite a small number. Previous studies have shown that Rhizobium bacteria isolated from soybean plants have opaque characterization, which is light that penetrates small amounts of bacterial colonies[[56]](#footnote-56).

For the colony form, each isolate has the same form, which was round. Rhizobium bacteria isolated from soybean plants have a round shape, convex elevation, and have entire edges[[57]](#footnote-57). The characteristics of these bacteria are also supported by other studies which state that isolated Rhizobium bacteria have colony morphology with a round shape, entire edges, and a convex elevation[[58]](#footnote-58).

**Figure 2.** The Bacterial Isolates, a) Rhizobium Mlarak 1, b) Rhizobium Mlarak 2, c) Rhizobium Mlarak 3, d) Rhizobium Siman 1, e) Rhizobium Siman 2 (left-right).

Microscopic observations carried out with a microscope showed that the bacteria isolated from the nodules of soybean plants had a rod cell shape and were gram-negative. From the results of the gram staining, the crystal violet dye was not completely dissolved when given iodine and alcohol, which were indicated by the presence of blue on the surface of the bacteria. However, the inside of the bacteria was pink which indicates gram-negative bacteria.

In addition to the rod-shaped bacterial cells, there were also bacteria with round cells (*Coccus*) and gram-positive. It was suspected that there is not only one type of bacteria in one plant. In addition, each root nodule contains not only one type of strain but various types of bacterial strains. Previous studies showed that bacterial isolation from the root nodules of Fenugreek (Trigonella foenum-graecum) showed different cell shapes, ranging from stem to round with gram-positive and negative[[59]](#footnote-59). Another study stated that the bacteria isolated from the roots of legume plants had a round cell shape (*Coccus*) and were gram-positive[[60]](#footnote-60). This study is in line with the microscopic observations that have been carried out on Rhizobium bacteria in soybean plants.

## 4.2 The Colony Color in Congo Red and Brom Thymol Blue Media

Color testing by adding Congo Red and Brom Thymol Blue dyes has been used extensively in previous studies. This technique is a simple technique to determine Rhizobium bacteria and the growth acceleration seen from the color change. This color test was intended to determine Rhizobium bacteria with other bacteria on Congo red media. Generally, Rhizobium bacteria colonies did not absorb the red color on YEMA and Congo Red media[[61]](#footnote-61). Rhizobium does not absorb the red color from congo red because it does not have β-D-Glucan polysaccharide bonds[[62]](#footnote-62). Meanwhile, congo red is a dye used to detect β-D-Glucan content.

Bacterial growth on YEMA and BTB media is intended for the classification of the growth rate of Rhizobium bacteria. The chemical formula of BTB (Bromthymol Blue) itself is C27H28Br2O5S. Bromthymol Blue turned to yellow when it reacted with acids and blue when it reacted with alkaline. In previous research, it was shown that indicator plastics made of BTB show a greenish color change when reacting with NH3 vapor[[63]](#footnote-63). Meanwhile, NH3 itself has weak alkaline properties.

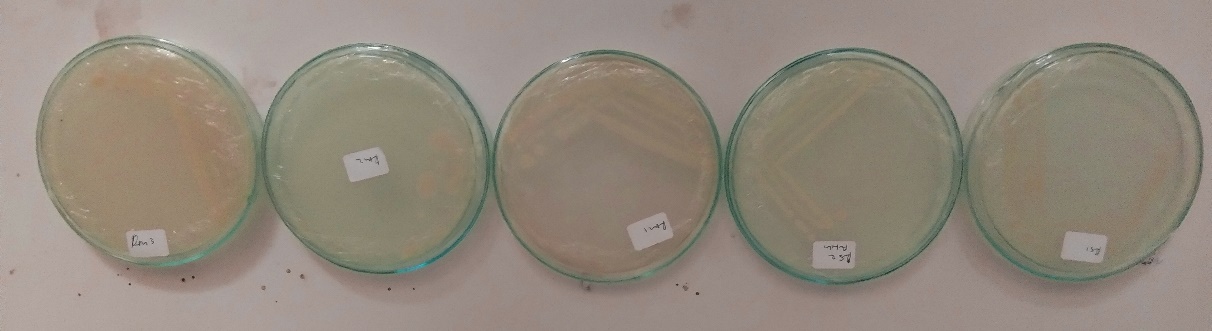
Each isolate has a media color change to blue and yellow. The color change to yellow indicates that the bacteria absorbed acid and was grouped as the fast-growing group. Bacteria isolated from an environment with a low pH (acid) if grown in a medium with an optimum pH will provide better bacterial growth[[64]](#footnote-64). Isolates that reacted with alkaline were indicated by turning blue. Bacteria with fast growth will produce yellow colonies with creamy or yellowish edges within 3 days of the incubation period[[65]](#footnote-65). While the slow growth bacteria remain white and have a smaller colony size within 7-10 days.

A color change in the color of the media to yellow indicates that the bacteria have the ability to grow quickly and produce acid[[66]](#footnote-66). In this research, it was found that the isolates that were able to change the medium to yellow were Rhizobium Mlarak 1 and 3 isolates. Meanwhile, the isolates that gave blue color to the media were Rhizobium Mlarak 2, Rhizobium Siman 1 and 2 isolates.

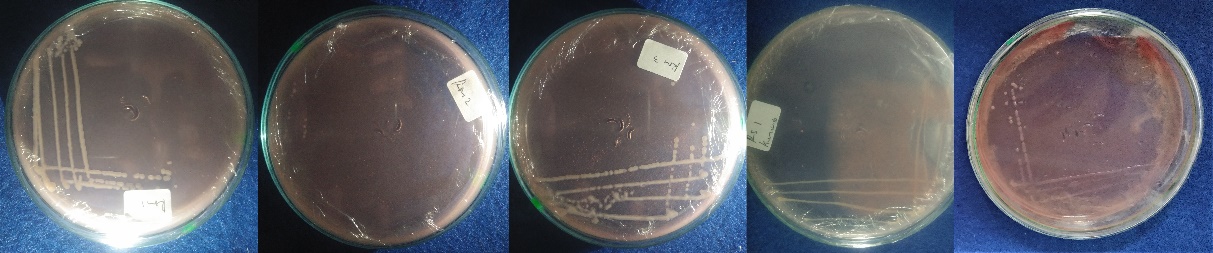
**Table 2.** TheGrowth of bacterial colonies on YEMA + CR and YEMA BTB media

|  |  |  |  |
| --- | --- | --- | --- |
| **Num** | **Rhizobium Strain** | **CR (*Congo Red*)** | **BTB (*Brom Thymol Blue*)** |
| 1 | *Rhizobium* Mlarak 1 | White | Yellow |
| 2 | *Rhizobium* Mlarak 2 | White | Blue |
| 3 | *Rhizobium* Mlarak 3 | White | Yellow |
| 4 | *Rhizobium* Siman 1 | Yellow | Blue |
| 5 | *Rhizobium* Siman 2 | White | Blue |

Bacterial isolates grown on agar + Congo Red media showed no change in the color of the bacterial colony to red. Rhizobium bacteria colonies did not absorb or slightly absorb red color when grown on YEMA + Congo Red media[[67]](#footnote-67). Congo red dye is often used to determine the degradation of β-D-Glucan on agar media for bacterial selection[[68]](#footnote-68). The agar medium absorbs the congo red color because it has polysaccharide chain in form of β-D-glucan bonds[[69]](#footnote-69).hile most of the Rhizobium bacteria do not contain β-D-glucans so that the congo red dye is less absorbed into the Rhizobium bacteria colony[[70]](#footnote-70).



**Figure 3.** The Rhizobium growth on YEMA + BTB media. Rhizobium Mlarak 1, 2, 3, Rhizobium Siman 1 and 2 (left-right).



**Figure 4.** The Rhizobium Growth on YEMA + CR Media. Rhizobium Mlarak 1, 2, 3, Rhizobium Siman 1 and 2 (left-right).

So, the results of the BTB test for bacteria obtained can be grouped into two types of growth, there were slow-growing and fast-growing. Isolates included in the fast-growing group were Rhizobium Mlarak 1 and rhizobium Mlarak 3 isolates, while isolates included in the slow growth Rhizobium Mlarak 2, Rhizobium Siman 1, and Rhizobium Siman 2.Based on previous research, it was found that bacteria that had slow growth were Bradyrhizobium sp, which were marked with change in blue color on YEMA + BTB media. meanwhile, Rhizobium sp. has a fast growth was indicated by a yellow color change in YEMA + BTB medium[[71]](#footnote-71).

## 4.3 The Growth of Bacteria on Liquid YEM Media.

Rhizobium bacterial growth test was carried out using a spectrophotometer with a wavelength of 600 µm. The test was carried out by observing the absorbance of bacteria grown in liquid YEM media every 2 hours by taking 2 ml of liquid culture containing bacteria. Bacteria were grown using a shaker and incubator at 28°C.

**Figure 5.** The Absorbance of Rhizobium Isolates on YEM Broth.

From the absorbance test, it was obtained a bacterial growth curve as seen from the turbidity of the media. The cloudier media indicates bacterial growth. The first phase in bacterial growth is the lag phase. In this phase, the bacteria adapt to the surrounding environment and cell division has not increased significantly. Rhizobium Mlarak (RM) 1 isolate has a 0-4 hours lag period. Meanwhile, Rhizobium Mlarak 2, Rhizobium Mlarak 3, and Rhizobium Siman 1 have a 0-6 hours lag period and Rhizobium Siman 2 has a 0-8 hours. During the lag period. There is an increase and decrease in the number of bacterial cells. This can be caused by the occurrence of bacterial competition against existing nutrients before the increase in the number of bacterial cells in the lag phase. The growth of Rhizobium leguminosarium STDF-Egypt 19 also experienced a decline before increased constantly[[72]](#footnote-72).

The next stage of bacterial growth is the log phase. In this phase, the bacteria experience rapid growth. All tested isolates took 14 hours, from 8 to 22 hours. In this phase, the bacteria experienced increased regular growth until the 22 hours. After 24 hours, all Rhizobium isolates did not show a significant increase, this indicates that the peak growth of all Rhizobium isolates occurred at 24 hours The weakness of measuring bacteria using a spectrophotometer is that it cannot differentiate between living and dead bacterial cells[[73]](#footnote-73). After the first to seventh days, all Rhizobium isolates did not show a significant increase or decrease. Although the curve shows the stable growth of Rhizobium bacteria, the bacteria that are still alive and have died cannot be distinguished. From the bacterial growth curve, it can be determined the optimum bacterial harvest time, which is at 24 hours. Because the peak of bacterial growth occurs at this time before growing constantly.

## 4.4 The Colony Growth on YEMA Media (*Yeast Extract Mannitol Agar*)

The growth of bacterial colonies on YEMA media has different numbers. this is due to the ability of each isolate to form colonies. The table of bacterial growth in YEMA is shown in the graph below. Colony growth obtained from the Mlarak and Siman areas has a different growth rate every day.

**Figure 6.** The Growth of Rhizobium Mlarak (1011) on YEMA

Isolates taken from Mlarak showed different growth. The highest growth was found in Rhizobium Mlarak 3 isolates with the amount of 72 X 1011 CFU on the 3rd day. Meanwhile, in Rhizobium Mlarak 1 and 2, the number of colonies was not much different on the same day, it were 21.9 X 1011 CFU and 23 X 1011, respectively.

**Figure 7.** The Rhizobium Siman (109) growth graph on YEMA media

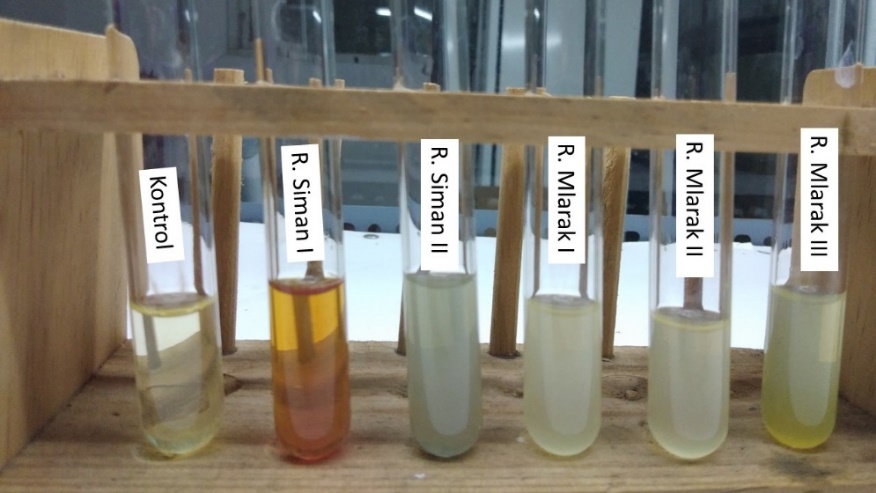
Bacterial isolates taken in the Siman area had slower growth than Rhizobium bacteria isolated from the Mlarak area. Rhizobium Siman 1 showed growth on the first day with the number of colonies 1.66, X 109 with a growth peak of 10.70 X 109 on the 4th day. Meanwhile, Rhizobium Siman 2 isolate showed growth on the 3rd day with the number of colonies 4.6 X 109 and on the 4th day experienced a significant increase with the number of colonies 48 X 109.

The relationship between bacterial size and growth time of Rhizobium bacteria on YEMA media has an effect. As could be seen from the size of the Rhizobium Mlarak bacterial isolate, it has a larger colony diameter than Rhizobium Siman. The growth time between rhizobium bacteria isolates Mlarak and siman was also different. Previous studies have shown that bacteria with a diameter of 2-5 mm have a 3 days growth period which is classified as fast growth. While bacteria with a diameter of 2-3 mm have a of 7-10 days growth period and has been classified as slow growth[[74]](#footnote-74). All isolates grown on YEMA media are still classified as fast growing bacteria because the bacteria have grown for less than 7 days based on previous references.

## 4.5 The Test of Bacterial Ammonia Content

This qualitative test aims to determine the ammonia content produced by bacteria in broth media. From the ammonia content test, it was found that the bacteria culture turned yellow when Nessler's reagent was added, which indicated that the ammonia content was produced by Rhizobium bacteria in the broth culture media. The equation is as follows[[75]](#footnote-75):

**NH4+ + 2[Hgl4] + 4OH → [HgO.Hg(NH2)l] + 7H + 3H2O**



**Figure 8.** The bacterial broth culture + Nessler's reagent

Nessler's reagent will change color to brown yellow when it reacts with a solution containing ammonia. The resulting yellow color occurs because of the colloid dispersion in the alkaline ammonia solution[[76]](#footnote-76). The picture showed that Rhizobium Siman 1 produces a dark yellow color that indicates a reaction between Nessler's reagent and ammonia produced by bacteria. As it is known that Rhizobium bacteria can bind free nitrogen to be converted into ammonia (NH3). This ammonia in plants will be converted into amino acids and was used for plant growth[[77]](#footnote-77).

## 4.6 The Plant Height

Plant height is one of the parameters observed in vegetative observations of plants. Ponorogo local Rhizobium isolate did not significantly affect the height of soybean plants. The results of the Analysis of Variance (ANOVA) showed that there was no significant difference for each treatment with an F value of 0.94 with F Table 2.62. However, as can be seen from the average of each treatment, Rhizobium Siman 2 isolate showed the highest height with 58.86 cm after 5 week. The difference in the height of plant between Rhizobium Siman 2 inoculation treatment with control was 7.51 cm.

**Figure 9.** The graph of soybean plant height per week

In the first week after inoculation, the highest average of soybean was in the control treatment (21.77 cm). The height of this control plant can be caused by the absence of competition in nutrition uptake between bacteria and plant roots for nutrition. From previous research, it was found that between plants inoculated by Rhizobium sp bacteria and without inoculation with the same NPK fertilizer dose gave higher control plant height was higher than plants inoculated by Rhizobium sp bacteria at 15 DAS and 30 DAS[[78]](#footnote-78).

In the second week, soybean plants inoculated with Rhizobium gave higher plant height than control plants. This week the plants inoculated with Rhizobium Mlarak 3 (27.24 cm) gave the best plant height compared to other treatments, but the average difference between Rhizobium Mlarak 3 plants and control plants was not much different, which was only 0.94. cm. In plants with control treatment and Rhizobium Siman 1 had the same plant height, namely 26.3 cm. However, the height of the soybean plants inoculated with Rhizobium Mlarak 1 (25.68 cm) was still lower than the control. This can be caused by the absence of symbiosis between Rhizobium bacteria and soybean plant roots. Meanwhile, Rhizobium itself will form nodules when the plant is 15-20 days old[[79]](#footnote-79).

In the third week, the highest plants were still found in soybean plants inoculated by Rhizobium Mlarak 3 (34.13 cm), while the control had a plant height of 32.57 cm. However, the soybean plants inoculated with Rhizobium Mlarak 1 (31.13 cm) were still under control.

In the fourth week, all soybean plants inoculated with Rhizobium bacteria had higher plant height than the control. Plant height that exceeds control indicates a symbiosis between Rhizobium bacteria and plant roots that have been formed. This symbiosis can be characterized by the appearance of nodules of the plant roots. In the fourth week, soybean plants inoculated with Rhizobium Siman 2 (47.92 cm) had the highest plant height compared to other treatments.

The fifth week is the last observation of the plant because the soybean plant has appeared flowers that indicate that the vegetative growth of the plant ends. The average plant height for each treatment shows different results if sorted from lowest to highest plant height are as follows. Control plant height (51.35 cm), Rhizobium Siman 1 (54.3 cm), Rhizobium Mlarak 1 (54.76 cm), Rhizobium Mlarak 2 (56.06 cm), Rhizobium Mlarak 3 (57.81 cm) and Rhizobium Siman (58.86 cm). From the observation the best soybean plant height from the first week to the last week were soybean plants inoculated by Rhizobium Siman 2 and Rhizobium Mlarak 3 bacteria because these isolates gave a higher plant height than other treatments.

Although the soybean plant height inoculated by the local Rhizobium bacteria Ponorogo gave a better plant height than the control. As explained above, ANOVA did not give significantly different results for each treatment every week. Previous studies have shown that the second and last week did not give significantly different heights for soybean plants[[80]](#footnote-80). Rhizobia treatment of soybean plants did not provide a large difference in the average plant height, but the height of soybean plants given *Rhizobia sp* isolate was higher than the control[[81]](#footnote-81).

The availability of water in the soil also affects plant height. Lack of water in plants can inhibit plant growth. The water deficit for a long time will cause plant growth failure[[82]](#footnote-82).

## 4.7 The Root Length

Plant roots have an important role in absorbing nutrients in the soil. If the plant roots are good, the absorption of nutrients by the plant is also fulfilled. One of the roles of Rhizobium bacteria is to help plants absorb nutrients, especially Nitrogen[[83]](#footnote-83). From the observations, soybean plants that have the shortest to longest root lengths are as follows. Soybean plants with control treatment (35.63 cm), Rhizobium Mlarak 3 (37.42 cm), Rhizobium Mlarak 2 (38.2 cm), Rhizobium Siman 1 (38.8 cm), Rhizobium Mlarak 1 (39.01 cm) ) and Rhizobium Siman 2 (39.65 cm).

The control treatment had the shortest root length compared to plants inoculated with local Rhizobium sp. Rhizobium Siman 2 gave the highest plant root length compared to other isolates with an average of 39.65 cm. Analysis of variance (ANOVA) showed no difference in each treatment with an F value of 0.75 and an F Table of 5% 2.62.

**Figure 10.** The root length graph

This study showed that the addition of Rhizobium bacteria inoculant could lead to an increase in the average root length, although the variance did not show that it was significantly different for each treatment. Giving Rhizobacteria and endophytic bacteria to soybean plants of Davros variety can increase the root length of soybeans, but it does not significantly affect the root length of soybean plants[[84]](#footnote-84).

Besides the Rhizobium effect, root length can be influenced by the content of organic matter and the type of soil used in soybean cultivation. Applying compost to the soil can improve soil properties, thereby increasing root growth. Root growth tends to lead to a source of nutrients and water[[85]](#footnote-85). The high organic matter content in compost will bind water more tightly. That makes root growth rapidly. The replenishment of compost can also increase the activity of microorganisms in the soil. Good root growth will provide better plant growth. The greater the root volume, the more active the nutrient absorption by the root plant.

## 4.8 The Branches Number

The number of branches in the soybean plant provides a close relationship with soybean production. Generally, the more branches that are produced, the more pods will produce seeds. From the results of research conducted that Rhizobium Siman 2 and Rhizobium Mlarak 1 produced the highest average number of branches 4.3. The lowest number of branches was produced by Rhizobium Mlarak 2. However, after being analyzed using Analysis of Variance (ANOVA) between treatments did not give significantly different results on the number of branches of soybean plants.

**Figure 11.** The graph branches number

The average number of branches from smallest to largest is as follows. Rhizobium Mlarak 2 (3,4), control (3,5), Rhizobium Siman 1 (4), Rhizobium Mlarak 3 (4), Rhizobium Siman 2 (4). In the Rhizobium Mlarak 2 treatment, the number of branches was less when compared to the control plants. Although according to the analysis of variance, there is no significant difference. Previous studies have shown that control plants provide an average number of branches more than plants inoculated by Rhizobium sp[[86]](#footnote-86). Subsequent research also explained that Rhizobium inoculation did not provide a significantly different number of branches[[87]](#footnote-87).

The number of branches produced also affects the height of the plant itself. A large number of branches will make plant nutrition not focus on plant height growth but focus more on branch growth and the rate of plant height will decrease. However, as can be seen from the average plant height and number of branches, the relationship between plant height and the number of branches is not contradictory to the research that has been conducted. This means that the number of plant branches does not affect the height of the soybean plant.

Although it was not significantly different, several plants were inoculated with bacteria giving a higher number of branches. In addition to producing auxin hormones, Rhizobium bacteria also produce cytokinins to spur plant growth[[88]](#footnote-88). One of the cytokinins role is to stimulate the growth of plant branches. In addition, the influence of plant genotype and environmental conditions also affects plant branching[[89]](#footnote-89).

## 4.9 The Canopy’s Fresh Weight

Canopy Fresh weight is the weight of the plant that still contains water. In the vegetative growth phase, the canopy wet weight includes the stems and leaves of the soybean plant. In general, the wet weight cannot describe the photosynthetic results produced by plants because there is still water content.

**Figure 12.** The Graph of canopy's fresh weight

Canopy wet weight of plants inoculated by Rhizobium sp bacteria generally had higher than control plants (14.12 g), Rhizobium Mlarak 2 (14.74 gr), Rhizobium Siman 1 (16.24 gr), Rhizobium Mlarak 1 (17,7 gr) 74 gr), Rhizobium Siman 2 (16.24 gr), and Rhizobium Mlarak 3 (21, 84 gr). The inoculation results showed that Rhizobium Mlarak 3 gave the highest yield on the canopy wet weight of soybean with a value of 21.84 grams. However, after being analyzed by Analysis of Variance (ANOVA) the wet weight of canopy was not significantly different for each treatment with an F value of 0.45 and an F table of 5%.

Based on previous research, the wet weight of plants is more influenced by temperature, humidity and water content in plant cells. Therefore, the accumulation of photosynthetic products in plants cannot be determined based on the wet weight of the plant. Previous studies have also shown differences in soybean watering intervals on plant wet weight[[90]](#footnote-90). Based on the results of these studies, that the local *Rhizobium sp* has less effect on plant wet weight.

The amount of watering affects the wet weight of the plant more. The more water that is absorbed, the more water content in the plant. Also, protoplasm content has an important role to bind water and CO2 in plants. If the ability of the protoplasm in plants increases, the water content that is bound will increase[[91]](#footnote-91).

The intensity of light shining on plants also affects the plants water content. The combination of low water content and high irradiation intensity has been shown to reduce water content in leaves. This is caused by decreased humidity and increased temperature in plants which causes the plant tissue to transpire rapidly. So that the water content in the plant decreases quickly. Meanwhile, shaded plants increase soil moisture and avoid high temperatures[[92]](#footnote-92).

Also, N nutrient uptake affects plant wet weight.  A. Vulgaris plants given the N fertilizer dose of 1.86 grams gave the best plant wet weight. However, giving more than that N dose gave lower plant wet weight. this can be caused by the fertilizer given is not fully absorbed by the plant[[93]](#footnote-93).

Generally, it was found that the plant's wet weight was strongly influenced by the abiotic environmental conditions (soil water content, humidity, temperature, and sunlight intensity). Meanwhile, Rhizobium isolates inoculated on soybean plants had more impact on plant N uptake. However, the impact of N uptake affects the dry weight of the plant after removing its moisture.

## 4.10 The Canopy’s Dry Weight

The canopy dry weight is the wet weight of the plant after removing the moisture content in it[[94]](#footnote-94) and is the original weight of the plant from photosynthesis. Dry weight is an indicator that determines a plant's growth ability. Dry weight is the accumulation of photosynthate products in the form of protein, carbohydrates, and lipids[[95]](#footnote-95). Accumulation of photosynthesis is generally stored in stems, fruit, seeds, or pods. For the dry weight of the canopy, the highest average was found in soybean plants inoculated with Rhizobium Mlarak 3 isolate. The results of the analysis of variance showed that the results were not significantly different for each treatment. Based on previous research, the inoculation of Rhizobium bacteria was not significantly different when analyzed of variance[[96]](#footnote-96).

**Figure 13.** The Canopy's Dry Weight

However, the average dry weight of each treatment showed different results. Soybean plants inoculated with bacteria had a higher dry weight than the control. The sorted dry weight of plant canopy from smallest to largest is as follows. Soybean plants with control treatment (5.07 g), Rhizobium Mlarak 2 (5.46 g), Rhizobium Siman 1 (5.90 g), Rhizobium Siman 1 (5.90 g), Rhizobium Mlarak 1 (5.90 g ), Rhizobium Siman 2 (6.77 g), and Rhizobium Mlarak 3 (6.86 g).

From the observation of plant crown dry weight, it was found that the Rhizobium inoculated in soybean was not effective in increasing the dry weight of the plant. Many factors affect the effectiveness of *Rhizobium sp* bacteria in increasing plant dry weight, one of which is the N content and pH in the soil. The presence of available nitrogen content in the soil in the form of nitrate will inhibit the nitrogenase enzyme for nitrogen-fixing because there is no free nitrogen nutrient that will be converted into N available for plants. As for soil pH, Rhizobium growth is less suitable for soil conditions that have low pH or acidic.[[97]](#footnote-97).

Vegetative growth is strongly influenced by nitrogen, one of which is the dry weight. Rhizobium can provide N nutrients to plants by forming nodules. Rhizobium will fix free N into N available can be used for plant growth[[98]](#footnote-98). The nutrient N in plants plays a role in the formation of chlorophyll, nucleic acids, and amino acids that are building blocks of protein[[99]](#footnote-99). If the chlorophyll content is high, the photosynthesis process will run more optimal. The result of photosynthesis will be accumulated into dry weight.

## 4.11 The Number of Root Nodules

The average number of nodules in each treatment had different amounts. The highest average number of root nodules was found in soybean plants inoculated with Rhizobium Siman 1 bacteria with 27.7 root nodules. Meanwhile, the control treatment only had an average number of 9 nodules. However, after being analyzed by analysis of variance (Analysis of Variance), there was no difference in the number of nodules between treatments.

**Figure 14.** The number of root nodules

Based on the average number of nodules, soybean plants inoculated with Rhizobium bacteria were higher than the control without using Rhizobium. This happens because there is the addition of bacteria from bacterial inoculants that infect the roots of soybean plants. However, not all soybean plants have a lot of nodules in line with the increase in plant N uptake. As previously mentioned, an effective nodule is a root nodule that produce a red color when splitted. Meanwhile, root nodules that are not effective will turn green and brown when cleaved.

The cause of the ineffectiveness of root nodules can be due to the aging of root nodules. This aging will cause the bacteria and leghemoglobin to degrade so that they cannot fix free N completely. The formation of root nodules and plant N uptake were also influenced by the N concentration in the soil. Previous studies have shown that increasing the concentration of N in the soil will inhibit the formation of root nodules and the ability of N fixation. N buildup in plants will reduce carbohydrates in plants. When plants lack carbohydrates, the supply of carbohydrates for bacteria as an energy source will decrease. The reduced carbohydrate supply will reduce the effectiveness of Rhizobium to fix N in root nodules[[100]](#footnote-100).

Research conducted on Inceptisol soil in soybean and maize fields found that *Bradyrhizobium sp* bacteria were more abundant in soybean fields than corn. This proves that the soybean plant is the right host for the growth of Bradyrhizobium and Rhizobium sp. These bacteria were symbiosis with the roots of the soybean plant and were characterized by the formation of root nodules. This symbiosis adds N plants due to Rhizobium's ability to fix N in the air[[101]](#footnote-101).

The addition of Rhizobium to soybean cultivation, although it did not significantly increase the number of nodules, could still provide higher vegetative growth than soybean plants in control treatment. Apart from the addition of Rhizobium, organic matter also has an important role in providing a growing environment for Rhizobium by providing energy, the stability of soil aggregates, and improving soil chemistry. A Good soil conditions will encourage Rhizobium bacteria to form nodules.

## 4.12 The Total N Plants

Nitrogen is an element that plays an important role in increasing crop yields. Every plant fertilizer that is traded must have an N content in it. However, the use of N fertilizers can easily be carried away by groundwater flow. Nitrogen carried by water can pollute the surrounding environment, especially if carried by river flows[[102]](#footnote-102). Therefore, using Rhizobium bacteria can reduce environmental pollution caused by chemical fertilizers.

Rhizobium will increase plant N uptake. Calculation of plant total nitrogen using the Kjehdal method shows a different average for each treatment. The average N total plants from the lowest to the highest were as follows: Control (27.6 mg), Rhizobium Mlarak 1 (33.3), Rhizobium Siman 2 (3,6,7 mg) and Rhizobium Mlarak 2 (38 mg), Rhizobium Siman 1 (42.3 mg) and Rhizobium Mlarak 3 (45.8 mg).

The plant nitrogen content data that has been obtained was analyzed by Analysis of Variance (ANOVA). The analysis results show that each treatment does not give significantly different results with F count 1.17 and F Table 5% of 2.62. When viewed from the average N of soybean plants, Rhizobium Mlarak 3 gave the highest plant N compared to other treatments.

**Figure 15.** The Graph of Plant N Upatke

One of the factors causing Rhizobium's low ability to fix nitrogen is the availability of Nitrate in the soil. If the nitrate in the soil is available, the ability of Rhizobium sp bacteria to fix free nitrogen is reduced, due to inhibition of the nitrogenase gene[[103]](#footnote-103). N fertilizers added to the soil can also reduce nitrogenase activity in root nodules[[104]](#footnote-104). Also, planting polyculture between soybeans and corn can increase the ability of nitrogenase to fix nitrogen. Nitrogen absorption by root nodules depend on the level of leghemoglobin contained in it. Leghemoglobin plays a role in the absorption of oxygen to bacteroids. Nitrate in the soil when absorbed by root nodules will be reduced to nitrite and will eventually form Nitrogen Monoxide (NO) compounds. NO compounds present in root nodules will prevent leghemoglobin from binding the O2 and reduce N2 fixation in root nodules. This causes the ineffectiveness of the root nodules to absorb nitrogen[[105]](#footnote-105).

The ineffectiveness of Rhizobium inoculants in increasing vegetative growth and plant N uptake could be due to differences in plant hosts. As it is known that each type of Rhizobium bacteria has a specific plant host[[106]](#footnote-106). This difference can be caused due to differences in the exudate content produced by each plant[[107]](#footnote-107). Soybean plants themselves produce exudates in the form of isoflavones, saponins, glucose, pinitol, arabinose, galactose, sucrose and oligosaccharides[[108]](#footnote-108). The bacteria isolated were bacteria that came from the roots of the gepak hijau variety. Meanwhile, the soybean plant used in this study was the Detam 4 variety.

Several Rhizobium have their respective types of host plants, the soybean plant is famous for the bacteria Rhizobium japonicum and Bradyrhizobium japonicum, Alfalfa and (Rhizobium meliloti) plants, clover plants (Rhizobium trifoli), legumes (Rhizobium leguminosarum), and M. pruriens (Rhizobium Phaseoli). Rhizobium will be more effective in tethering N if applied following the host plant[[109]](#footnote-109).

A large number of nodules did not increase the plant's N uptake. This can be happen due to the ineffectiveness of plant root nodules in fixing nitrogen. Previous studies also showed that the addition of Rhizobium did not affect increasing N uptake significantly[[110]](#footnote-110). The absence of adding organic matter to the soil is also one of the causes of the ineffectiveness of Rhizobium, as was done in the study that there was no addition of organic matter so that it is possible that the growth of Rhizobium bacteria is not as good as when given with compost.

# CHAPTER V: CONCLUSION AND SUGGESTIONS

**5.1 Conclusion**

Rhizobium Mlarak 3 isolate was the best isolate compared to other isolates with the highest growth rate of 72 X 1011 in YEMA media. Rhizobium Mlarak 3 colonies are yellowish-white, slightly translucent, round, convex surface, flat edges, the form of coccus, and gram-negative cells. Although the local application of Ponorogo Rhizobium had not significantly increased the total plant N uptake, Rhizobium Mlarak 3 provided the highest average plant total N uptake (45.8 mg) compared to controls and other isolates.

**5.2 Suggestions**

The suggestion from this research is that it is necessary to conduct a quantitative test of the ammonia content in the liquid culture of the local Ponorogo Rhizobium bacteria and its effect on the growth of soybean plants. Analysis of soil nutrient content at the beginning and end of planting soybeans also needs to be done to determine the effect on soybean growth.

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# APPENDIX

**Appendix 1: Research Flowchart**

Root sampling

**Research parameters**

- Bacterial Growth

- Bacterial discoloration

- Color of Gram Bacteria

- Bacterial Growth Curve

- Nirogen Fixation By Bacteria

- Plant Root nodules

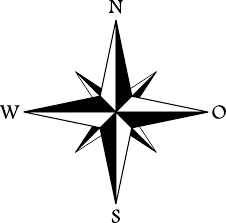
Media making

Inoculation of *Rhizobium sp* bacteria into soybean plants.

Growth and Characterization of *Rhizobium sp*

*Rhizobium sp* bacteria isolation

Sterilization media and equipment

**Appendix 2: Plan of Research Design**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| RS1 (1) | K (1) | RS 3(1) | RM1 (1) | RM2 (1) | RS2 (1) |
| K (2) | RS1 (2) | RM2 (2) | RS2 (2) | RS3 (2) | RM1 (2) |
| RM1 (3) | RS3 (3) | RS2 (3) | RS1 (3) | K (3) | RM2 (3) |
| RM1 (4) | RM2 (4) | RS2 (4) | RS3 (4) | RS1 (4) | K (4) |
| R2S (5) | RM2 (5) | K (5) | RS3 (5) | RM1 (5) | RS1 (5) |

Information:

K : Control without Rhizobium inoculation

RM : Rhizobium Mlarak

RS : Rhizobium Siman

( ) : Numbers in parentheses represent the replication of the treatment.

|  |  |  |  |
| --- | --- | --- | --- |
| **Week After Inoculation** | **F** | **F Tabel** | |
| **1%** | **5%** |
| 1 | 0,263tn | 2,62 | 3,9 |
| 2 | 0,32 tn |
| 3 | 0,421 tn |
| 4 | 0,402 tn |
| 5 | 0,94 tn |

**Appendix 3: *Analysis of Variance* (ANOVA) of soybean plant height**

**Appendix 4: *Analysis of Variance* (ANOVA) of soybean plant height**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Source** | **df** | **SS** | **MS** | **F** | **F Crit** | |
| **5%** | **1%** |
| Treatment | 5 | 51,45942 | 10,29188 | 0,751 | 2,62 | 3,9 |
| Error | 24 | 328,793 | 13,69971 |  |  |  |
| Total | 29 | 380,2524 |  |  |  |  |

**Appendix 5: *Analysis of Variance* (ANOVA) of number of branches**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Source** | **df** | **SS** | **MS** | **F** | **F Crit** | |
| **5%** | **1%** |
| Treatment | 5 | 3,741667 | 0,748333 | 0,885 | 2,62 | 3,9 |
| Error | 24 | 20,3 | 0,845833 |  |  |  |
| Total | 29 | 24,04167 |  |  |  |  |

**Appendix 6: *Analysis of Variance* (ANOVA) of canopy fresh weight**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Source** | **df** | **SS** | **MS** | **F** | **F Crit** | |
| **5%** | **1%** |
| Treatment | 5 | 168,6284 | 33,72567 | 0,456 | 2,62 | 3,9 |
| Error | 24 | 1773,991 | 73,91629 |  |  |  |
| Total | 29 | 1942,619 |  |  |  |  |

**Appendix 7: *Analysis of Variance* (ANOVA) of canopy dry weight**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Source** | **df** | **SS** | **MS** | **F** | **F Crit** | |
| **5%** | **1%** |
| Treatment | 5 | 23,68 | 4,74 | 0,695 | 2,62 | 3,9 |
| Error | 24 | 163,59 | 6,82 |  |  |  |
| Total | 29 | 187,27 |  |  |  |  |

**Appendix 8: *Analysis of Variance* (ANOVA) of number of root nudules**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Source** | **df** | **SS** | **MS** | **F** | **F Crit** | |
| **5%** | **1%** |
| Treatment | 5 | 1239,0 | 247,8 | 1,118 | 2,62 | 3,9 |
| Error | 24 | 5319,7 | 221,7 |  |  |  |
| Total | 29 | 6558,7 |  |  |  |  |

**Appendix 9: *Analysis of Variance* (ANOVA) of total plant nitrogen**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Source** | **df** | **SS** | **MS** | **F** | **F Crit** | |
| **5%** | **1%** |
| Treatment | 5 | 0,17 | 0,034 | 1,172 | 2,62 | 3,9 |
| Error | 24 | 0,69 | 0,029 |  |  |  |
| Total | 29 | 0,85 |  |  |  |  |

**Appendix 10: Number of bacterial colonies on YEMA Media**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Isolate** | **Day** | | | | |
| **0** | **1** | **2** | **3** | **4** |
| Rhizobium Mlarak 1 (1011) | 0 | 15,1 | 21,67 | 21,86 | 21,86 |
| Rhizobium Mlarak 2 (1011) | 0 | 10,5 | 21,33 | 23 | 23 |
| Rhizobium Mlarak 3 (1011) | 0 | 34,7 | 58,4 | 72 | 72 |

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Isolate** | **Day** | | | | |
| **0** | **1** | **2** | **3** | **4** |
| Rhizobium Siman 1 (109) | 0 | 1,66 | 1,67 | 5,8 | 10,7 |
| Rhizobium Siman 2 (109) | 0 | 0 | 0 | 4,6 | 48 |

**Appendix 10: Number of bacterial colonies on YEMA Media**

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Isolate** | **Time (Hour)** | | | | | | | | | | | | |
| **0** | **2** | **4** | **6** | **8** | **10** | **12** | **14** | **16** | **18** | **20** | **22** | **24** | |
| R.Mlarak 1 | 0,081 | 0,377 | 0,454 | 0,928 | 1,37 | 1,564 | 1,762 | 1,847 | 1,873 | 1,979 | 1,94 | 1,99 | 1,92 | |
| R.Mlarak 2 | 0,051 | 0,689 | 0,543 | 0,437 | 1,219 | 1,456 | 1,706 | 1,821 | 1,845 | 1,981 | 1,969 | 1,994 | 1,96 | |
| R.Mlarak 3 | 0,039 | 0,394 | 0,271 | 0,382 | 1,053 | 1,348 | 1,629 | 1,806 | 1,87 | 2,002 | 1,934 | 2,045 | 2,008 | |
| R.Siman 1 | 0,092 | 0,532 | 1,046 | 0,6 | 0,677 | 0,794 | 1,345 | 1,686 | 1,706 | 1,918 | 1,888 | 2,018 | 2,024 | |
| R.Siman 2 | 0,052 | 0,324 | 0,542 | 1,05 | 0,974 | 1,061 | 1,238 | 1,68 | 1,767 | 1,892 | 1,838 | 1,991 | 2,050 | |

# DOCUMENTATION

## Laboratory Research

|  |  |  |
| --- | --- | --- |
| **Num** | **Activities** | **Photos** |
| 1. | Rhizobium bacteria isolation |  |
| 2. | Colony counting of bacteria using *Colony Counter* |  |
| 3. | Propagation of bacteria in YEM broth with the help of a *water bath* |  |
| 4. | Measurement of the bacteria absorbance using a spectrometer |  |
| 5. | Heating samples of soybean leaves for N plants analysis in a fume hood. |  |
| 6. | The color resulting from N plants analysis. |  |
| 7. | The color change of the liquid YEM media containing bacteria after adding Nessler's reagent. |  |

## Field Research

|  |  |  |
| --- | --- | --- |
| **Num** | **Activities** | **Photos** |
| 1. | Soybean Plants var. Detam 4 |  |
| 2. | Comparison of Plant Height |  |
| 3. | The flowering soybean plants are ready to be analyzed for N plants. |  |
| 4. | Plant Root Nodules |  |

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