Identification of plant growth promoting rhizobacteria in rhizosphere of bamboo thorns with gram methylene blue and lugol staining

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Abstract. Plant Growth Promoting Rhizobacteria is a group of microorganisms that can increase plant growth and yield through reactions that occur in the soil, however, the large number of microorganisms contained in the PGPR rhizosphere of bamboo thorns makes it difficult to know which bacteria are the most dominant and most active in influencing plants. Generally, gram staining of bacteria aims to facilitate the observation of bacterial morphology with the aid of a microscope. Bacteria are generally colorless and almost invisible due to the lack of contrast with the water in which they may be present. Staining using methylene blue and lugol is generally needed to see the bacteria clearly. This study aims to determine the effect of using the gram stain test method with methylene blue and lugol in identifying PGPR bacteria from rhizosphere of bamboo thorn. This research was conducted at the Laboratory of Biological Fertilizers and Potential Microbes, Faculty of Agriculture, Hasanuddin University in October-December 2019. The research methods included the manufacture of microorganism planting media, pouring media, planting bacteria using the scatter and scratch method, and the gram reaction test using methylene blue and lugol. The results obtained showed that the use of the gram test with methylene blue and lugol resulted in a blue color which indicated the dominance of the genus Pseudomonas, while the pink/purple color indicated the dominance of the genus Bacillus. Changes in the color of the bacteria to pink/dark purple, the bacteria were gram negative and belong to the Pseudomonas group and there was a change in blue, so the bacteria were gram-positive and were group of Bacillus. Therefore, the use of the gram test with methylene blue and lugol has an optimal effect in detecting microorganisms especially in identifying PGPR.

1. Introduction
Plant growth promoting rhizobacteria, more popularly known as Plant Growth Promoting Rhizobacteria (PGPR), is a group of beneficial bacteria that actively colonize the rhizosphere. PGPR plays an important role in increasing root development which has an impact on plant growth, crop yields and soil fertility [1]. Plants with well-developed roots will efficiently absorb nutrients so that plants are not susceptible to pathogens. In addition, the increase in plant growth by PGPR can occur through one or more mechanisms related to the functional character of PGPR and conditions in the rhizosphere environment [2]. PGPR is a biological microorganism that can increase plant growth and yield. The bacteria contained in PGPR can be classified based on their effect on plants and the way they interact with roots, PGPR can affect plants directly and indirectly [3]. The role of PGPR in
increasing plant growth and production is related to the ability to synthesize growth substances, such as indole acetic acid hormone (IAA) [4]. Bamboo plants are plants that can grow in several areas in Indonesia with a variety of functions and species. In Indonesia there are 60 species of bamboo plants of 200 species in Southeast Asia and can be found in areas that are free from standing water, ranging from lowlands to mountains. The high adaptability of bamboo makes this plant grow well in almost every type of soil [5]. Until now, there have been many potential antagonistic microbes from bamboo rhizosphere which have antagonistic power against soil borne disease through antagonistic mechanisms in the form of life competition, parasitism, antibiosis and induced systemic resistance. In addition to suppressing the development of pathogens, rhizosphere microbes can also increase plant growth through various mechanisms, including through the production of growth stimulant compounds such as phytohormones. In the soil there are many microbes that have the ability to dissolve phosphate and potassium, and produce phytohormones. These microbes can increase plant growth by producing indole acetic acid (IAA) phytohormones as nutrients for plants [6].

Research on the existence and diversity of bamboo rhizosphere microbes has been conducted by several previous researchers. According to [7] found antagonistic fungi such as Aspergillus, Penicillium, Trichoderma in the rhizosphere of healthy bamboo plants which were able to suppress the pathogens of Fusarium and Phytophthora. Research conducted by [8] showed that the inoculation of the fungi Paecilomyces sp and Chaetomium globosum from the rhizosphere of bamboo into the nursery soil had a significant effect on reducing the clubroot index and increasing the wet weight of broccoli plants. Research conducted by [9] in China on the rhizosphere of 6 bamboo species showed that the total population of fungi and bacteria as well as microbial activity on the rhizosphere of bamboo was very high and had a positive effect on plant growth.

Thorny bamboo (Bambusa blumeana) is a non-timber forest product that can be used for various purposes. This plant has great potential, namely having a fibrous root system with a very strong rhizome root that can maintain a hydrological system as a soil and water binder and roots that can prevent erosion and can absorb water up to 90% besides thorn bamboo is also a clump that can create the surrounding micro climate [10]. The root conditions of the rhizome fibers and the micro humidity conditions found in this type of bamboo result in a wide range of microorganisms that live and are in the rhizosphere of thorn bamboo, causing the soil around the bamboo roots to be quite fertile.

The number of microorganisms contained in the PGPR rhizosphere of bamboo thorns makes it difficult to know which types of bacteria are the most dominant and most active in influencing plant growth. This will also have an impact on the difficulty of knowing and selecting good and bad bacteria contained in the PGPR, so that it greatly affects plants. Therefore, the method that can be used to determine the type of bacteria contained is identification of microorganisms by biochemical test treatment. According to [11], a bacterial biochemical test is a method or treatment carried out to identify and determine a pure culture of isolated bacteria through its physiological properties. Biochemical processes are closely related to cellular metabolism, namely during chemical reactions carried out by cells that produce energy or use energy for the synthesis of cell components and for cellular activities, such as movement. One of the methods used in identifying microorganisms is the gram test using simple staining.

Gram staining is the determination of isolate characters based on differences in the cell wall structure of gram-positive and negative bacteria. This staining is a differentiator between other bacteria. The peptidoglycan layer contained in the cell wall layer of gram-positive bacteria is thicker when compared to gram-negative bacteria. According to [12], gram-positive bacteria have a special element, namely teichoic as much as 5% of the dry weight of the cell wall. This element has a function to maintain ion transport, cell wall integrity, replacement of choline by ethanolamine so that it is resistant to autolysis and maintains external permeability. The results of the isolation that have been obtained are then performed with gram staining. Gram staining was carried out on 2-3 days of bacterial culture grown on slant media. Methylene blue is a simple gram coloring medium that has the ability to detect bacteria, afterwards, an aromatic hydrocarbon compound that is poisonous and is a
cationic dye with a very strong adsorption power. In general, methylene blue is used as a dye for silk, wool, textiles, paper, office equipment and cosmetics. This compound is a dark green crystal. When dissolved, methylene blue in water or alcohol will give a blue solution. Methylene blue has a molecular weight of 319.86 gr/mol, with a melting point of 105 °C and a solubility of 4.36 x 104 mg/L [13]. In addition, lugol is a solution that can detect various types of indigenous bacteria in nature. Iodine lugol, also known as liquid iodine is a solution consisting of a compound of potassium iodide and iodine in water. It is a drug and disinfectant that is used for a number of specific uses in the medical world as well as in identifying microorganisms.

This study aims to determine the effect and benefits of using the gram methylene blue and lugol staining test method in identifying Plant Growth Promoting Rhizobacteria bacteria from the rhizosphere of bamboo thorns (Bambusa blumeana). This research will have an effect on the identification process of microorganisms more easily and quickly, especially in identifying plant growth promoting rhizobacteria.

2. Methods

This research was conducted at the Biofertilizer Laboratory and Potential Microbes, Department of Agricultural Cultivation, Faculty of Agriculture, Hasanuddin University, Makassar. This test took place from October to December 2019. The tools used were analytical scales, mortar, test tubes, tube racks, petri dishes, measuring cups, erlenmeyers, ose needles, drop pipettes, micro pipettes, matches, spatulas, autoclaves, ovens, hotplate, vortex, glass preparations, laminar air flow, storage boxes, cameras and writing instruments. The materials used are a solution of Plant Growth Promoting Rhizobacteria from the rhizosphere of bamboo thorns (Bambusa blumeana), 3% KOH, 70% alcohol, 96% alcohol, water, distilled water, deg glass, nutrient brooth (NB), agar, bunsen, tissu, plastic wrap, aluminum foil paper, paper, label paper.

The steps for implementing the test are as follows:

2.1. Preparation of microorganisms medium

Make a medium for growing microorganisms using Nutrient Agar (NA) media. NA preparation was carried out by weighing 8 grams of brooth nutrients and 20 grams of agar on analytical scales. Furthermore, the weighed material is put into a 1000 ml erlenmeyer and 500 ml distilled water is added and then stirred until the material is dissolved. Furthermore, erlenmeyer is heated using a hotplate while stirring and adding distilled water gradually until the volume of the solution reaches 1000 ml. When the solution is homogeneous, the erlenmeyer is covered with aluminum foil and then put into the autoclave to be wet sterilized for 15 minutes.

2.2. Media pouring

Before starting the pouring of the media, first do the laminar air flow sterilization by turning on the light and spraying 70% alcohol all over the inside of the laminar air flow then cleaning it using a tissue. Then the laminar air flow is covered with black plastic and the UV lamp is turned on for 30 minutes. After the UV sterilization process is complete, turn on the laminar air flow blower and enter all the tools and materials that will be used in pouring the media. Before inserting the tools and materials into the laminar air flow, first spray them with 70% alcohol. Light the bunsen with a lighter and open the NA medium that has been sterilized wet and has been at medium temperature. Furthermore, the NA media was poured over a petri dish about 15-20 ml, then covered and glued with plastic wrap and then labeled. The media is stored in a storage box and allowed to harden.

2.3. Source of microorganisms

The microorganisms that used from a fluid of PGPR which made a medium by the rhizosphere of bamboo thorns previously. Then, made a particular treatment by fermentation for the rhizosphere so that it was giving some groups of typical microorganisms.
2.4. Planting microorganisms

Planting microorganisms or bacteria is carried out again in laminar air flow, which is done by two methods, namely the scattering method and the scratching method. Each method is made in 5 petri dishes each. The material in the form of a bamboo rhizosphere PGPR solution that has been made and NA media is put into laminar air flow after spraying with 70% alcohol. The implementation of the scatter method was carried out by taking 1 ml of PGPR solution using a micro pipette then spread it over NA media and leveled using a spatula, while for the etching method it was carried out by taking an ose needle then carried out by sterilizing the bunsen and then immersing it in the PGPR solution and then scratching it regularly, zigzagging on the surface of NA medium in a petri dish. After planting the bacteria, the plates are stored in a storage box and checked and physically observed for bacterial growth every day.

2.5. The gram reaction test uses gram staining of methylene blue and lugol bacteria

The method of staining was done using methylene blue. First, the glass preparations are washed using 70% alcohol and then sterilized by annealing Bunsen. Then take the ose needle and then sterilize it and then dip it in 96% alcohol. Take one ose distilled water and place it on the glass preparation. Burn the ose needle back on the bunsen then let it air dry and used to pick up the bacteria then spread it on the glass and let it dry. Drop 1-2 drops of methylene blue on the glass preparation and let it dry. Next wash the glass preparations with distilled water then drop 1-2 drops of lugol solution on the glass and leave it for 1 minute then rinse with 70% alcohol for 30 seconds then dry. After drying, attach the deglass to the slide and then observe it under a microscope with magnification (40/0.65) or (160/0.17). If there is a change in the color of the bacteria to pink or dark purple, the bacteria is gram positive and is a Pseudomonas group and if there is a change in blue, the bacteria is gram negative and is a Bacillus group.

3. Results and discussion

3.1. Test for gram bacteria reaction using simple staining

In the following, this is a results of gram bacteria reaction using simple staining method which observed by under a microscope. There are two methods of the results, divided to spread and scratch method which are five results of observation respectively.
Generally, bacterial staining aims to facilitate the observation of bacterial morphology with the aid of a microscope. Bacteria are generally colorless and almost invisible due to the lack of contrast with the water in which they may be present. Staining is needed to see bacteria very clearly both for intracellular observations and overall morphology. Simple coloring is coloring that uses a single dye.

The single dyes that are usually used in simple coloring are Methylene Blue, Basic Fuchsin, and Crystal Violet. All of these dyes can work well on bacteria because they are alkaline and alkaline (the chromophore component is positively charged), while the cytoplasm of bacteria is basophilic (likes bases) so that there is an attraction between the chromophore components in the dye with bacterial

**Figure 1.** Microscopic observation results of bacteria under a microscope with magnification (40 / 0.65) after a simple staining method
cells, this causes the bacteria to absorb dye well. However, what is used in this test is staining with Methylene Blue.

Microscopic observations show that PGPR bamboo rhizosphere staining with methylene blue produces blue for the genus Pseudomonas and pink/purple for the genus Bacillus. If there is a change in the color of the bacteria to pink/dark purple, the bacteria is gram negative and is a Pseudomonas group and if there is a change in blue, the bacteria is gram positive and is a the Bacillus group.

In accordance with the statement of the Jambi Agricultural Training Agency (2010), which states that the PGPR content is dominated by Pseudomonas fluorescens and Bacillus polymixa. The benefit claims obtained are as a bioprotectant, biofertilizer and as a biostimulant. It is also in line with the research of [14], which states that several bacterial genera obtained from the rhizosphere of bamboo plants include: genus Bacillus, Pseudomonas, Enterobacter.

Gram positive bacteria will give a purple color when given a gram stain. This is because these bacteria have a lower lipid content, so that the bacterial cell wall will be easier to dehydrate due to alcohol treatment. Dehydrated cell walls cause the size of the pores of the cells to be small and the permeability is reduced so that the crystal purple dye which is the main dye cannot leave the cells and the cells will remain purple. Gram-negative bacteria will give a red color when given a gram stain. This is because the bacteria lose their color when staining the crystal violet when rinsing with alcohol, but are able to absorb the opposite color, namely the safranin stain. Gram-negative bacteria contain a higher percentage of lipids than that contained by gram-positive bacteria, the cell wall of gram-negative bacteria is thinner than that of positive garment cell walls [15].

4. Conclusion
The recent research shows that the use of the gram-methylene blue and lugol staining test method in identifying the Plant Growth Promoting Rhizobacteria bacteria from the rhizosphere of bamboo thorns (Bambusa blumeana), namely the scatter and scratch method testing from microscopic observations shows that PGPR bamboo rhizosphere is stained with methylene blue produces a blue color for the genus Pseudomonas and a pink/purple color for the genus Bacillus. So that the use of the simple gram staining test method has the optimum effect in detecting microorganisms, especially in identifying plant growth promoting rhizobacteria.

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