Identification of Endophytic and Rhizosphere Bacteria in Maize (*Zea mays* L.) in Limapuluh Kota Region, West Sumatra, Indonesia

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**Abstract.** The variety of microorganisms that are very abundant began to be used for the benefit of sustainable agriculture. A group of non-pathogenic bacteria has been reported to control disease-causing pathogens and improve plant growth. The purpose of this study is (a) to identify and characterize endophytic bacteria and corn rhizosphere (b) to study endophytic bacteria and rhizosphere which have the potential to spur plant growth. The experiment was carried out at the Payakumbuh State of Agricultural Polytechnic Laboratory, Limapuluh Kota Region, West Sumatra, and LIPI Bogor. The sampling method was carried out by random sampling at four locations. Endophytic bacteria taken from the roots of maize and rhizosphere bacteria originated from the soil around the roots. Bacterial isolation was carried out by the pour and the streak plate method. Identification of bacterial characteristics based on the form of colonies, edges, surfaces, rough surface roughness, surface color, pigment color, bacterial body density, and halo zone colonies. Six species of bacteria have been identified using molecular analysis based on 16S rRNA gene fragments. Bacteria are dominated by the Bacillus genera compared to the Pseudomonas genera. The ability of phosphate dissolving bacteria indicates that endophytic bacteria and the rhizosphere act as growth stimulants.

1. Introduction

The variety of microorganisms that are very abundant began to use for the benefit of sustainable agriculture. Microorganisms for stimulating growth and increase crop production have been studied. A group of non-pathogenic bacteria has been reported to control disease-causing pathogens and improve plant growth. Endophytic bacteria are bacteria that found in plant tissues and do not harm the host plants [1]. Endophytic bacteria live in certain periods and are able to form colonies in plant tissues [2]. Endophytic bacteria can increase plant growth and yield, press pathogens, dissolve phosphates, and contribute nitrogen which can be assimilated into plants [3].

Endophytic bacteria are bacteria that found in the tissues of the roots, stems and leaves of plants. Root tissue is more colonized by endophytic bacteria than stems and leaves [4]. Some endophytic bacteria utilize auxin phytohormones to interact with their host plants as part of a bacterial colonization strategy [5]. Endophytic bacteria produce special metabolites which can increase plant resistance to pathogenic attacks [6].

Rhizosphere bacteria are a complex and dynamic part of the soil ecological system and play an important role in the nutrient cycle of nitrogen and phosphorus. Rhizosphere bacteria are commonly found in layers of soil around plant roots. The number of bacteria that grow and develop in the rhizosphere, due to the many nutrients secreted by plants to the rhizosphere region [6,7]. The most
common genera of bacteria found in the rhizosphere are Pseudomonas, Bacillus, Arthrobacter, Rhizobia, Agrobacterium, Azotobacter, Mycobacterium, Flavobacter, Cellulomonas, and Micrococcus [7].

The Bacillus genera are one of the bacterial biological control agents because many Bacillus species can maintain plant health. Antibiotics produced by bacteria are effective in controlling plant pathogens and their diseases [8]. The same opinion by [9] that the role of Bacillus sp is to inhibit the growth of pathogenic microorganisms.

The purpose of this study was (a) to know the type and population of bacteria found in the roots of maize plants in different locations, (b) to know the type and population of bacteria found in the maize rhizosphere of different plants and (c) to identify endophytic bacterial isolates and rhizosphere in maize plants.

2. Materials and Methods

2.1. Preparation of Bacterial

Sources of bacteria were taken from 4 locations of maize plantations in Limapuluh Kota Region namely Gurun, Taram, Kandang Lamo, and Pulutan. Bacteria isolated from endophytic in maize roots and maize rhizosphere. The isolation and identification of bacteria were carried out at the Payakumbuh State Agricultural Polytechnic Laboratory of Food Crops, Limapuluh Kota Region, West Sumatra, Indonesia and continued with identification using molecular analysis based on 16S rRNA fragments at LIPI Bogor.

Isolation was carried out from root samples of maize plants from healthy plants with characteristics of green left, plants not dwarfed, and plants aged 2 months. The next sample is from the soil around the rhizosphere of maize. The growing media are NA, NB, and Pikovskaya's, litmus paper, wrapping, alcohol, distilled water, and chemicals for molecular identification of bacteria. The tools used are beaker glass, Erlenmeyer, petri dish, test tube, one use hose needle, Bunsen lamp, analytical scales, oven, and laminar airflow.

2.2. Preparation of Cultivation Medium

Media for the growth of bacteria were used Nutrient agar media, and for the growth of certain bacteria used selective media, phosphate solvent bacteria used Picovskaya solid, to calculate the population of bacteria used by nutrient Broth.

2.3. Bacterial Isolation

2.3.1. Isolation of Endophytic Bacteria

Healthy maize plants were taken from four locations in Limapuluh Kota Region. Cut the root part of the corn plant and wash it with running water to remove the attached soil, then dry it on filter paper. Root surface sterilization by soaking in 70% alcohol for 2 minutes and 5.2% Na-hypochlorite solution for 5 minutes, then rinsed with sterile distilled water 2 times and dried on sterile filter paper. After drying, weigh 1 g and gently grind the roots in a sterile mortal, then put in Erlenmeyer and add sterile distilled water to 10 ml. Stir by turning Erlenmeyer. Take 1 ml of extract using a micropipette, put it in the tube and add 9 ml sterile distilled water. In the same way, do it until dilution 10-7. Then take 1 ml solution and grown on Petri dish containing NA media. See the development of bacteria on the third day. Bacteria that grow are separated and purified. Pure isolates of endophytic bacteria are characterized by their morphology based on shape, edge, surface, and color.

2.3.2. Isolation of Rhizosphere Bacteria

The isolated soil was taken from a layer of soil around the same roots of corn plants. The soil around the roots of the corn plant dried and taken as much as 1 g, then put into Erlenmeyer and add sterile equates to 10 ml. Then dilution carried out to 10-7. Then take 1 ml of solution and grown on Petri dish containing NA media. Look at the development of bacteria on the third day. Bacteria that grow are separated and purified. Pure isolates of endophytic bacteria are characterized by their morphology based on shape, edge, surface, and color.
2.4. Identification of Bacterial Isolates

The identification of breast milk bacteria from 4 rhizosphere samples and 4 root samples from corn plants on selective media. The media that used are NA and Pikovskaya's. Bacterial isolation was carried out by the pour cup method and the scratch plate. Identification of characteristics of pure bacterial isolates based on colony shape, the edge of the colony, surface, smooth rough surface, surface color, pigment color, and density of the bacterial body. Followed by identification using molecular analysis based on 16S rRNA gene fragments. Extraction of bacterial genomic DNA was carried out using the GES method [10]. Amplification of the 16S rDNA fragment was done using GoTaq (Promega) with a pair of general 27F primer (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [11]. Then, sequencing data is processed with the Bioedit program [12]. Isolates were identified using the EzTaxon server [13] based on the 16S rRNA gene sequence data.

Use one of pure culture was transferred into a PCR tube and added 50 mL of nuclease-free water and boiled for 10 minutes [14]. The resulting solution would be used as a DNA template for PCR. For PCR mixture solution consisted of 16S 0.5 µl Forward Primary reagent, 0.5 µl Reverse 16S Primer, 2X 12.5 µl GoTaq Green Master Mix, 1 µl template DNA, and 10.5 µl Nuclease-free water.

The PCR mixture was inserted into the PCR machine by setting: initial denaturation of 950C for 30 seconds, followed by 30 cycles consisting of denaturation at 950C for 30 seconds, annealing at 500C for 30 seconds and elongation of strands at 720C for 90 seconds. After 30 cycles end, followed by lengthening the final strand at a temperature of 720C for 10 minutes. The PCR results were then sequenced with the Sanger method to find out the sequence of DNA strands and matching using the Basic Local Alignment Search Tool (BLAST) [15].

2.5. Calculation of Bacterial Populations

Bacterial population on NB media was calculated using a serial dilution method from dilution 10-1, 10-2, 10-3 until a dilution of 10-6. Each dilution was piped 0.1 ml and put into NB media, then incubated for 3 x 24 hours. Bacterial density = Colony count x 1 / (Dilution factor) x (CFU / ml)

3. Result and Discussion

3.1. Phylogenetic Analysis

Phylogenetic tree based on the 16S rRNA gene of endophytic and rhizosphere bacteria. The application of 16S rDNA fragments was carried out using GoTaq (Promega) with primary 27F primers (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) [11]. Sequencing results processed with the Bioedit program and EzTaxon server can be seen in Fig. 1.

Figure 1. Phylogenetic tree of bacteria based on the 16S rRNA gene. Evolutionary analyses were conducted in MEG 6.

Endophytic bacteria is one of the symbiotic bacteria which in their lifetime are in plant tissues and always interact with plants. Maize plants can contain several endophytic bacteria that are capable of producing secondary metabolites that similar to their host plants due to the genetic transfer of the host plant. Ref. [6] endophytic bacteria of tea parasites cultured in vitro can produce flavonoids.

3.2. Characteristics of Bacteria
Table 1 shows the bacteria found in the roots of maize plants have the potential as biological control agents. This can be seen from the character of bacterial isolates that can produce fluorine zones. Ref. [17] reported that the Gramineae family has the ability as an antagonistic bacterium against plant pathogens. Some bacteria from the Bacillus genera are able to suppress plant diseases caused by pathogenic bacteria, systemic viruses, and leaf spot fungi [8]. Many Bacillus sp bacteria include endophytic bacteria that live and are associated with plant root without causing disease in these plants.

| Isolate Code | Colony form | Edge | Elevation | Surface | Color | Concentration | Florescent | Gram |
|--------------|-------------|------|-----------|---------|-------|---------------|------------|------|
| AJ1g         | circular    | entire | flat      | soft    | white | dry           | 2 mm       | -    |
| AJ2b         | circular    | wave  | raised    | soft    | white | mucus        | 1 mm       | +    |
| AJ2c         | circular    | wave  | raised    | soft    | white | mushy        | 2 mm       | +    |
| AJ2e         | circular    | serrate | flat    | rough  | Non-translucent | dry        | 2 mm       | +    |
| TJ1b         | circular    | mycoides | flat  | waxy   | Non-translucent | dry        | 1 mm       | +    |
| TJ2g         | circular    | wave  | flat      | soft    | white | mushy        | 1 mm       | +    |
| TJ3a         | circular    | wave  | raised    | soft    | white | mushy        | 1 mm       | +    |
| TJ4a         | circular    | entire | flat      | soft    | white | dry           | 2 mm       | -    |
| TJ4b         | circular    | entire | flat      | soft    | white | dry           | 2 mm       | -    |

Table 1. Characteristics of bacteria from four locations in Limapuluh Kota Region

3.3. Species of Endophytic and Rhizosphere Bacteria

Identification of endophytic bacteria was isolated from the roots of maize plants that taken from four location sources, namely Gurun, Taram, Kandang Lamo, and Pulutan. A sources of bacteria, species, and the number of endophytic and rhizosphere bacteria colonies identified can be seen in Tables 2.

| Source of bacteria | Species of bacteria          | Number of colonies (cfu/ml) |
|--------------------|------------------------------|----------------------------|
| Gurun              | a. *Pseudomonas azotoformans* | 8.9 x 10^7                 |
|                    | b. *Bacillus paramycoides*   | 8.7 x 10^7                 |
| Taram              | a. *Bacillus paramycoides*   | 8.6 x 10^7                 |
|                    | b. *Bacillus licheniformis*  | 7.8 x 10^7                 |
|                    | c. *Bacillus pacificus*      | 9.6 x 10^7                 |
|                    | d. *Bacillus aerophilus*     | 8.1 x 10^7                 |
| Kandang Lamo       | a. *Bacillus paramycoides*   | 8.8 x 10^7                 |
|                    | b. *Bacillus licheniformis*  | 8.0 x 10^7                 |
| Pulutan            | a. *Pseudomonas azotoformans*| 8.5 x 10^7                 |
|                    | a. *Pseudomonas azotoformans*| 8.4 x 10^7                 |

Table 2. Species of endophytic bacteria from four locations in Limapuluh Kota Region

Table 2 shows the species of identified endophytic bacteria in each location, because of the different types of phosphates and organic matter at the location source. The root of the corn plant is dominated by bacteria from the Bacillus genera. [16] states that plant endophytes, especially plant roots can interact with several microbes, so that the interaction results are the main determinant of phytoremediation. Table 2 of the Bacillus genus dominates the rhizosphere of maize. The Bacillus genus has the ability to convert insoluble phosphate to available form for plants by removing organic acids [18].

*Pseudomonas azotoformans* bacteria are phosphate solvent bacteria which play a role in increasing P nutrient availability in the soil. The discovery of P-solvent bacteria in plant endophytes is probably the result of association with plant tissue. *Pseudomonas azotoformans* is a gram-negative
bacterium with stem cell form [19]. The area of halo zone around the bacterial colony shows the ability of bacteria to qualitatively dissolve phosphate. The wider the halo zone the greater the ability of bacteria to dissolve phosphate [20].

In Bacillus paramycoides isolates showing clear zones around bacterial colonies, this indicates that this bacterium can dissolve phosphate [21]. According to Ref. [19] Bacillus paramycoides are gram-positive bacteria with basil cell forms. Previously Bacillus paramycoides with the same strain as the findings [21] were isolated from the South China Sea sediment by [22].

Ref. [23] reported that two strains of Bacillus licheniformis were able to produce α-amylase enzymes with optimum activity at pH 7 and temperature 370C. This enzyme shows 100% stability at pH 7–9. Bacillus licheniformis is one of the bacteria found in the commercial seed in the process of biodegradation of paint wastewater treatment [24]. This bacterium produces the Raw Starch Breaker Amylum (APPM) enzyme, which breaks down the starch substrate on cassava skin into liquid sugar [25].

Bacillus pacificus strain EB422T was found in maize plantations flowed with wall paint waste and cement stirring. The growth of maize plants that are exposed to the waste stream experiences a rather stunted plant growth and small cob yields [21]. Previous findings by [25] Bacillus pacificus bacteria isolated from Pacific Ocean sediments and analyzed based on 16S rRNA gene sequences including Bacillus cereus group.

Bacillus aerophilus strain 28K (T) was found in the same place as Bacillus pacificus strain EB422T, that is, from the rhizosphere of corn plants in the area of paint and cement waste [21]. Bacillus aerophilus 28K (T) was also successfully isolated from cryogenic tubes used to collect air samples at an altitude of 28 km [26]. Bacillus aerophilus has similarities with Bacillus aerius strains of 24K (T) and Bacillus stratospheric strains 41KF2a and has similarities with Bacillus licheniformis of 98-99% [26].

The presence of endophytic bacteria is affected by host life conditions [27]. In this case a lot of the bacteria found in the rhizosphere of the plant, because this area is an area rich in nutrients such as amino acids and sugar as a source of nitrogen and carbon which is a food source for the life of bacteria. Some endophytic microbes provide resistance to biotic and abiotic pressure on host plants [3].

The species of bacteria in the rhizosphere depends on the type of soil, age, and type of plant. In the rhizosphere, some bacteria will form a community in a positive interaction with the host plant to determine plant growth [26]. The interaction between microbes and plants can only be done by microbial symbiosis [27]. [17] reported that the Gramineae family has the ability to increase population and microbial activity in roots.

The species of bacteria in maize endophytes are dominated by the genera Bacillus and Pseudomonas but are dominated by the Bacillus genera. Some Bacillus genera include P and K solvent bacteria, producers of growth regulators, and pathogenic suppressants. Types of bacteria in maize endophytes include Bacillus paramycoides, Bacillus licheniformis, Bacillus pacificus, and Pseudomonas azotoformans. Fluorescence found in bacterial isolates shows the ability of the genus Pseudomonas and Bacillus in dissolving phosphate. Reinforced by [28] that the genus Bacillus has the ability to dissolve phosphate high so that it has the potential to improve cultivated plants that have phosphate deficiency.

The number of bacteria in the rhizosphere is more than maize endophytes. This is due to the availability of organic material in the rooting zone (rhizosphere) more so that it can meet the nutritional need of bacteria. Compounds inorganic materials are used by bacteria for cell formation, nucleic acid formation, and energy sources for metabolic processes [29]. The interesting thing in one host plant is that there are several species of bacteria. Ref. [3] state that the result of the molecular analysis shows that plant defense responses limit bacterial populations in plants. The diversity of endophytic microorganisms is influenced by the chemical composition of the soil and host resistance to disease [30]. Ref. [31] concluded that the number of microbial populations was influenced by soil type, age, and plant conditions.

In this study, 9 isolates of Bacillus sp were isolated based on stem shape and identified based on morphological characteristics of bacterial isolates. Identification results show the clear zones (halo zone) around bacterial colonies which indicate Bacillus can dissolve phosphate. To ascertain the species, the nucleotide bases were tested at 16S rDNA with a base similarity between 99.0–99.86%.
In the rhizosphere of corn plants more bacterial species were found even though there were still two genera Bacillus and Pseudomonas.

Endophytic bacteria can stimulate plant defense responses [32]. Plant defenses depend on the type of bacteria found in plant tissue. [33] stated that the genus Bacillus was used as a Biocontrol agent because it produced antimicrobial substances in the form of bacteriocins. The bacteriocin mechanism kills the target cell by inserting it on the target membrane and resulting in unstable cell membrane function [33].

The Bacillus genus is rod-shaped, gram-positive, aerobic and facultative anaerobic bacteria. This genus has the ability to produce the enzyme catalase, cytrase, and urease. [34] stated that this bacterial genus can be used as a Biofertilizer.

The results of the isolation of endophytic bacteria from the maize plant roots were found 36 bacterial isolates which then discerned produced 4 types of bacteria, whereas the results of bacterial isolation from a layer of soil around the rhizosphere of maize were found 39 bacterial isolates which after discerning produce 5 types of bacteria. The bacteria found are from the genera Bacillus and Pseudomonas, but are predominantly from the Bacillus genera. Bacterial isolates from the Bacillus genera can be used as biofertilizers, biopesticides, and decomposers.

4. Conclusion
The isolates of endophytic bacteria on maize plant roots were found in 4 types of bacteria, and isolates of bacteria from rhizosphere of corn was found in 5 types of bacteria. The bacteria found are from the genera Bacillus and Pseudomonas, but are predominant from the Bacillus genera. Bacterial isolates from the Bacillus genera can be used as biofertilizers, biopesticides, and decomposers.

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