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# Potential of Epiphytic, Endophytic and Rizospheric Bacteria from Various Gogo Rice Fields in Kulawi District, Sigi Regency, Indonesia

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**Abstract:** Microbes in leaves (flosphere) and roots (rhizosphere) of gogo rice plants have the potential that can be used as a source of biological fertilizers such as $N_2$ fastening microbes, microbial boosters plants grow or phosphate solvent microbial. The purpose of this research is to analyze the potential of the phosphosphere and rhizosphere microbial from various upland rice sources in Central Sulawesi, Indonesia. This type of research is experimental research. Leaf samples (source of filospheric bacteria) and soil (source of rhizosphere root) taken from gogo rice plants. The stages of research are microbial analysis molecularly, test the ability of microbes to inhibit $N^2$, test the ability of solvent isolates P, motality test, pathogenicity test of hypersensity, test for the pathogenicity of hemolysis. Identification of 9 types of bacteria which were isolated from the filosphere microbes: epiphytes and endophytes and rhizosphere 10 isolates each microscopic tests show differences in color, shape, elevation, edge, texture and size. of each isolate. Cell morphology tests differ for each isolate. The average bacteria has the ability to tether N, dissolve P is Motil, not pathogenic to plants but all isolates can cause pathogens in humans and animals. Gogo rice plants have microbe potential in the philosphere and rhizosphere, so that, they can be used as biological fertilizers.

**Key words:** Filospheric microbes (epiphytes, endophytes), rhizosphere microbes, gogo rice plants, biological, fertilizers, bacteria

## INTRODUCTION

The decline in rice production, especially, gogo rice can be anticipated by way of managing farms that maintain environmental sustainability. Plant management must be carried out by taking into account all aspects that balance agroecosystems such as biodiversity, the life of microorganisms that have an important role in the cultivation of plants (Marlina *et al*., 2014; Ortiz-Castro *et al*., 2009). Efforts to maintain the balance of the agro-ecosystem, namely the application of the use of biological agents is one of the technological packages in addressing environmentally friendly and sustainable farming systems (Lachacz *et al*., 2017).

Microorganisms contained in soil are important for plant life which can improve soil properties (Wang *et al*., 2017). In addition, soil microorganisms, especially, roots have a very important role in stimulating plant growth (Katznelson *et al*., 1948). The potential habitat as a source of raw material for biofertilizers is the ecosystem of the leaf surface (filosphere) and rooting area (rhizosphere). The results showed that the population of microorganisms with various forms of associations (consortia) in the filosphere ecosystem could benefit plants through the supply of nitrogen, reduction of excess photosynthate distribution, provision of natural growth regulators and protection against ultraviolet rays and pathogenic attacks (Bodenhausen *et al*., 2014).

Exploration carried out in the filosphere area (leaves) and rhizosphere area (roots) in gogo rice plants to obtain the type of microbes that have potential that can be used as sources of biological fertilizers such as $N^2$ fastening microbes, microbial boosters plants growth or phosphate solvent microbial microbes as well as enemies natural. Increased soil fertility is the use of biological agents. Biological agents are microbes given to increase nutrient uptake by plants from the soil or air.

Epiphytic and endophytic bacteria can be isolated from the surface and sterile plant tissue or extracted from internal plant tissue. Specifically, bacteria enter the tissues through tissues that germinate, root, stomata or damaged tissue (Zinniel *et al*., 2002).

The diversity of bacteria can be seen from various perspectives such as morphology, physiology and

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genetics. Each different habitat provides a different diversity (Amanda *et al*., 2010). Examples of habitats that are often inhabited by bacteria are leaves. Each plant has a different leaf, both in terms of its shape, size and exudates issued. According to Vorholt (2012) that this difference causes the bacteria that inhabit it to be different, although, in certain plants the same bacterial population is found (Vorholt, 2012).

This study aims to analyze the microbial potential of the filosphere and rhizosphere of various gogo rice sources, so that, it can be applied by farmers in increasing agricultural production.

## MATERIALS AND METHODS

**Type of research:** This research is an experimental research conducted at the MIPA Biology Laboratory of Tadulako University and at the Center for Biodiversity and Biotechnology (ICBB) Laboratory Situ Gede, Bogor. This research was conducted from February to September 2018.

**Research material:** Leaf samples (source of filospheric bacteria) and soil (sources of rhizosphere leaf) taken from gogo rice plants are equipped with data on site height and soil pH, soil texture, content of organic matter, microbial climate, temperature and humidity based on methods (Steel and Torrie, 1980).

**Data collecting method**

**Microbial analysis molecularly:** Filospheric bacteria (epiphytes, endophytes) and rhizosphere have been extracted and the DNA extract results amplified by PCR technique with primers forward 16F26 5'-AGA GTT TGA TCM TGG CTC AG-3', Reverse R1492 5'-TAC GGY TAC CTT GTT ACG ACT T-3'.

Primary forward amplify the initial stage and primary reverse amplify the final stage (Huber *et al*., 2002). The amplification process was carried out as follows: initial denaturation for 3 min at 95°C, denaturation at 5°C for 30 sec, annealing at 48°C for 30 sec and lengthening of the chain at 72°C for 1.5 min. After 30 cycles end, it is added with the elongation of the chain at the end at 72°C for 10 min.

PCR products are electrophoresed on 0.8% agarose gel, each DNA chain sample is 0.2 ug/lanen, each sample volume of 1 uL/lanen with additional dye ethidium bromide uses TAE buffer (Tris-acetate-EDTA).

**Test the ability of microbes to tether $N^2$:** Test the ability of isolates to tether $N^2$ qualitatively by growing isolates on NFb media (Nitrogen Free bromthymol) with compositions per liter: 5.0 g malic acid, 0.5 g $K_2HPO_4$, $MgSO_4.7H_2O$ 0.2 g, 0.1 g NaCl, $CaCl_2$. The $2H_2O$ 0.02 g., 2 mL minor element solution ($CuSO_4.5H_2O$ 0.4 g, $ZnSO_4.7H_2O$ 0.12 g, $H_3BO_3$ 1.13 g, $Na_2MoO_4$ 1.0 g, $MnSO_4.H_2O$ 1.5 g/L of distilled water), bromthymol blue solution of 0.5% in 2 N KOH 2 mL, FeEDTA 1.64% 4 mL, vitamin 1 mL (Biotin 10 mg and Pyriodoxol-HCl 20 mg are treated to 100 mL with solvent distilled water) and so, 1.75 g. The pH of the solution is adjusted to 6.8 by KOH. 10 mL of pure culture solution was suspended in 90 mL of sterile physiological saline solution (0.85% NaCl in distilled water), then, diluted to $10^9$. The 1 mL of the solution was added to the NFb medium and incubated for 1 week.

The implementation was repeated three times. After 1 week, the color changes from green to blue and forms a white pellicle below the surface. The tethering of $N^2$ by microbes will cause an increase in the pH of the media because it forms $NH_4^+$. After a few days the pH increased by more than 1.0 units.

**Test the ability of solvent isolates P:** Test the ability of isolates to dissolve P was carried out using solid Pikovskaya medium, containing 5 g glucose, 5 g $Ca_3$ ($PO_4$) 2, 0.5 g $(NH_4)_2SO_4$, 2 g KCl, 0.1 g $MgSO_4.7H_2O$, 0.001 g $MnSO_4$, 0.001 $FeSO_4$, 0.5 g yeast extract, 20 g agar and 1000 mL distilled water. The 1 mL of solution from $10^{\bullet 4}$, $10^{\bullet 5}$ and $10^{\bullet 7}$ dilutions spread into a petri dish. Then, the Pikovskay agar media is poured into a petri dish with a pour plate method, then, left to freeze and incubated at room temperature (37°C) for 1-3 days. The presence of a clear zone around the bacteria indicates the ability of phosphate dissolving bacterial isolates.

**Motility test; Prior *et al*. (2013):** Semi-solid motility medium made with composition per 100 mL: beef extract 0.3 g, peptone 1 g, 0.5 g NaCl, 0.4 g Agar, 100 mL distilled water. The pH of the solution is adjusted 7.3. Then, heated and put in a test tube as much as 4-5 mL, then sterilized at 121°C for 15 min. Test was conducted by inserting isolate culture using an ose needle inserted right in the middle of the media to the bottom of the media. Then, incubated for 24 h at 35°C.

**Pathogenicity test for hypersensitivity:** Filosphere and rhizosphere microbial consortium were tested for hypersensitivity reactions in tobacco plants (*Nicotiana tabacum* L.) which were ±3 months old (Atkinson *et al*., 1985). This test is carried out to find out which consortium is potentially pathogenic. Each consortium is propagated in the Yeast Dextrose Carbonate (YDC) media for 48 h. Then, the microbial consortium is suspended in sterile distilled water until the solution looks cloudy ($10^8$-$10^9$ Spk/mL). Furthermore, the inoculum was injected into leaf tissue through secondary leaf bone. The control

treatment was made by means of leaves only inoculated with sterile distilled water. Observation on the appearance of hypersensitivity was carried out after 3×24 h.

**Pathogenicity test for hemolysis:** Hemolysis testing is used to test bacteria that have hemolytic activity or potentially pathogenic to humans and animals. Pure bacterial isolates are grown on blood agar media and then incubated for 18-24 h at 37°C. The zones produced by the isolates grown indicate that the isolates are potentially pathogenic (Lay, 1994).

**Data analysis:** Processing and analysis of sequenced data using the MEGA 6 (Molecular Evolutionary Genetic Analysis) program (Tamura *et al*., 2011). The results of the analysis that have been sequenced are determined by the identification of the species using the BLAST (Basic Local Alignment Search Tool) process which compares with the DNA sequence database in the National Center of Biotechnoloy Information (NCBI) genbank.

## RESULTS AND DISCUSSION

**Identification of phylospheric microbes (epiphytes, endophytes) and rizosphere Amplification 16s rRNA:** The results of 16s rRNA gene amplification using PCR (Polymerase Chain Reaction) against isolates in various upland rice plants are shown in Fig. 1.

Molecular identification of microbes is molecularly identified 9 types of bacteria with accession No. namely: *Bacillus cereus* strain VBE12 (S8EPa) MG027633.1, *Lysinibacillus fusiformis* strain BGSLP40 (S8EPb) KP192018.1, *Bacillus tropicus* strain MCCC1A01406 (S9ENa) KT380683.1, *Bacillus thuringiensis* strain AUSGS4 (S9ENb) 16S JX997827.1. *Bacillus cereus* strain VBE16 (S9ENc) MG6027671.1, *Bacillus anthracis* strain P0093Karwar (S9Ra) MG782855.1, *Baciluus anthracis* strain ES-9 (S9Rb) KY649402.1., *Baciluus cereus* strain RNS_01 (S9Rc) KT380683.1., *Bacillus anthracis* strain CP DE15 (S9Rd) MH304428.1 (Table 1).

**Test the ability of the microbes in the filosphere and rhizosphere of various upland rice sources:** Table 2 shows that microbial species of *Bacillus cereus* strain

Table 1: Result of Basic Lokal Alignment Search Tool (BLAST)

| Isolate codes | Species | Strain | Accession |
|---|---|---|---|
| S8EPa | *Bacillus cereus* | VBE12 | MG027633.1 |
| S8EPb | *Lysinibacillus fusiformis* | BGSLP40 | KP192018.1 |
| S9ENa | *Bacillus tropicus* | MCCC1A01406 | KT380683.1 |
| S9ENb | *Bacillus thuringiensis* | AUSGS4 | JX997827.1 |
| S9ENc | *Bacillus cereus* | VBE16 | MG6027671.1 |
| S9Ra | *Bacillus anthracis* | P0093Karwar | MG782855.1 |
| S9Rb | *Baciluus anthracis* | ES-9 | KY649402.1 |
| S9Rc | *Baciluus cereus* | RNS_01 | KT380683.1 |
| S9Rd | *Bacillus anthracis* | CP DE15 | MH304428.1 |

Table 2: Results of testing the quality of the phylospheric bacteria (epiphytes, endophytes) and rhizosphere

| Species | A | B | C | D | E |
|---|---|---|---|---|---|
| *Bacillus cereus* strain VBE12 | + | + | + | - | + |
| *Lysinibacillus fusiformis* strain BGSLP40 | + | - | - | - | + |
| *Bacillus tropicus* strain MCCC1A01406 | + | + | + | - | + |
| *Bacillus thuringiensis* strain AUSGS4 | + | + | + | - | + |
| *Bacillus cereus* strain VBE16 | + | + | + | - | + |
| *Bacillus anthracis* strain P0093Karwar | + | + | + | - | + |
| *Baciluus anthracis* strain ES-9 | + | + | + | - | + |
| *Baciluus cereus* strain RNS_01 | + | + | + | - | + |
| *Bacillus anthracis* strain CP DE15 | + | + | + | - | + |

A = N-tethering test on NFb media (Nitrogen Free bromthymol); B = Test to dissolve phosphate on solid Pikovskaya media; C = Motility test of semi-solid; beef extract; D = Pathogenicity test of hypersensitivity in tobacco plants; E = Hemolysis test on blood agar base

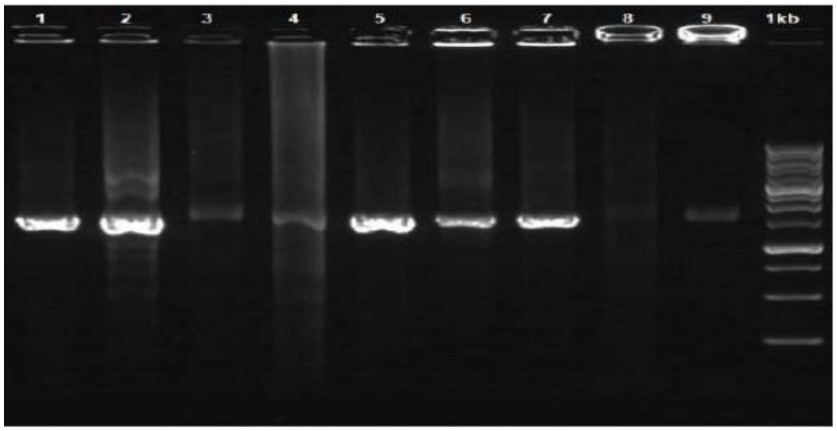


Fig. 1: Results of amplification of PCR products in various bacterial isolates; Condition: 0.8% gel agarose, amount of DNA ladder loaded per lanen: 0,2 ug each, volume of sampelloaded per lanen: 1 uL each. 1 kb DNA Ladder (bp): 250, 500, 750, 1000, 1500, 2000, 2500, 4000, 5000, 6000, 8000, 10000 1 kb DNA Ladder (ng/0.2 ug); 18,12, 9, 37, 7, 11, 11, 11, 11. S8EPa: 1), S8EPb; 2), S9ENa; 3), S9ENb; 4), S9ENc; 5), S9Ra; 6), S9Rb; 7), S9Rc and 8) dan S9Rd (9)

VBE12, *Bacillus tropicus* strain MCCC1A01406, *Bacillus thuringiensis* strain AUSGS4, *Bacillus cereus* strain VBE16, *Bacillus anthracis* strain P0093 Karwar, *Baciluus anthracis* strain ES-9, *Baciluus cereus* strain RNS_01 and *Bacillus anthracis* strain CP DE15 can tethering N to NFb media (Nitrogen Free Bromthymol), dissolving phosphate in the media on Pikovskaya, positive semi-solid motility of beef extract and positive hemolysis in blood agar base.

The electrophoresis results show that the sample amplified in the 1500 bp fragment, this corresponds to the type of DNA target. The amplified DNA is then, prepared for the nucleotide sequence determination using DNA sequencer. This is in line with the opinion of Dale and Park (2010) that PCR products are then sequenced and their nucleotide sequences are then compared with known and determined bacteria data.

The results of the epiphytic microbial kinship test showed that the two types of bacteria had different kinship, although, they were in the same source where *Bacillus cereus* strain VBE12 is an isolated bacterium in the same branch as *Amphibacillus tropicus* strain MCCC1A01406 both had 100% 16s rRNA sequence homology. Whereas the BGSLP40 strain of *Lysinibacillus fusiformis* bacteria isolated from epiphytic microbes was in the same branch as DSM2898 strain *Lysinibacillus fusiformis* bacteria with 100% 16s rRNA sequence homology (Fig. 2). The 1000 times the repeat bootstrap value is used to test the accuracy of the filogram (Tamura *et al*., 2011).

Endophytic microbes which have had three types of bacteria that have been isolated but all three types of bacteria each have different kinship. *Amphibacillus tropicus* strain MCCC 1A01406 bacteria are relatives with the *Bacillus proteolyticus* strain MCCC 1A00365 bacteria with 90% 16s rRNA sequence homology. *Bacillus thuringiensis* bacteria AUSGS4 strain has relatives with *Bacillus anthracis* strains CP DE15 rhizosphere bacteria with 100% 16s rRNA sequence homology. *Bacillus cereus* strain VBE16 bacteria are relatives with *Bacillus anthrax* strain P0093 Karwar with a 100% 16s rRNA homology sequence.

Rhizosphere microbes have four types of bacteria that have been isolated and the four types of bacteria have different kinship. *Bacillus anthracis* strain ES-9 has a kinship before with *Bacillus nitratireducens* strain MCCC 1A00732 and *Bacillus cereus* strain ATCC 14579 and homogeneous 16s rRNA sequence homology. *Bacillus cereus* strain RNS_01 (S9Rc) bacteria have a relationship with the *Bacillus paramycoides* strain MCCC 1A04098 with a 91% 16s rRNA sequence homology. While *Bacillus anthrcis* strain P0093Karwar (S9Ra) has a 100% 16s rRNA sequence homology, *Bacillus anthrax* strain CP DE15 with 93% 16s rRNA sequence homology.

Nine isolates are identified molecularly above the percentage of homology or their identities are at 91-100%. Based on Drancourt *et al*. (2000), species can be said to be identical to the reference species, if the percentage of similarity (homology) is at 99-100% (Drancourt *et al*., 2000). Based on the results of the phylogenetic tree design, it shows that the presence of types of bacteria that has different source does not have a close relationship. According to Ludwig and Reynolds (1988) that two different isolates in the same branch indicate the similarity of species. While *Bacillus cereus* strain VBE16 bacteria originating from endophytes and *Bacillus anthrcis* strain P0093Karwar bacteria is separated from other bacteria even though initially the nine bacteria have the same root but this is due to different evolution (Fig. 2).

The results showed that nine isolates that had been isolated and tested qualitatively on NFb media (Nitrogen Free bromthymol) were bacteria that had the ability to tether N. Bacteria isolated from the philosphere namely on the leaf surface were categorized as epiphytic bacteria in leaf tissue of endophytic bacteria and rhizosphere bacterial root area where all three endophytic microbial sources are able to tether air nitrogen, both through non-symbiosis (free-living nitrogen-fixing bacteria) and symbiosis (rootnodulating bacteria).

The ability of bacteria in tethering N is an advantage in increasing the need for plants where all plants need N uptake in high amounts. So that, in the presence of bacteria that have N-tethering ability can reduce N administration. In one research conducted in Brazil, it was shown that some sugarcane varieties can obtain large amounts of nitrogen through biological fixation (Urquiaga *et al*., 1992). Eady (1992) added that other facultative anaerobic species including *Citrobacter*, *Enterobacter*, *Erwinia* and *Klebsiella* can establish associations with grass and some strains able to fix the nitrogen (Eady, 1992).

The test of the ability of isolates to dissolve P was carried out using solid Pikovskaya medium. Qualitative testing of abilities can be seen with a clear zone around the colony. The results of the research showed that nine isolates that had been isolated had one isolate that was unable to dissolve P, namely *Lysinibacillus fusiformis* BGSLP40 strain bacteria thought to be influenced by its growing environment. This was in line with Fankem *et al*. (2006), bacterial activity in dissolving P in solid media and liquid is not absolutely the same. In addition, the *Lysinibacillus fusiformis* BGSLP40 strain cannot utilize

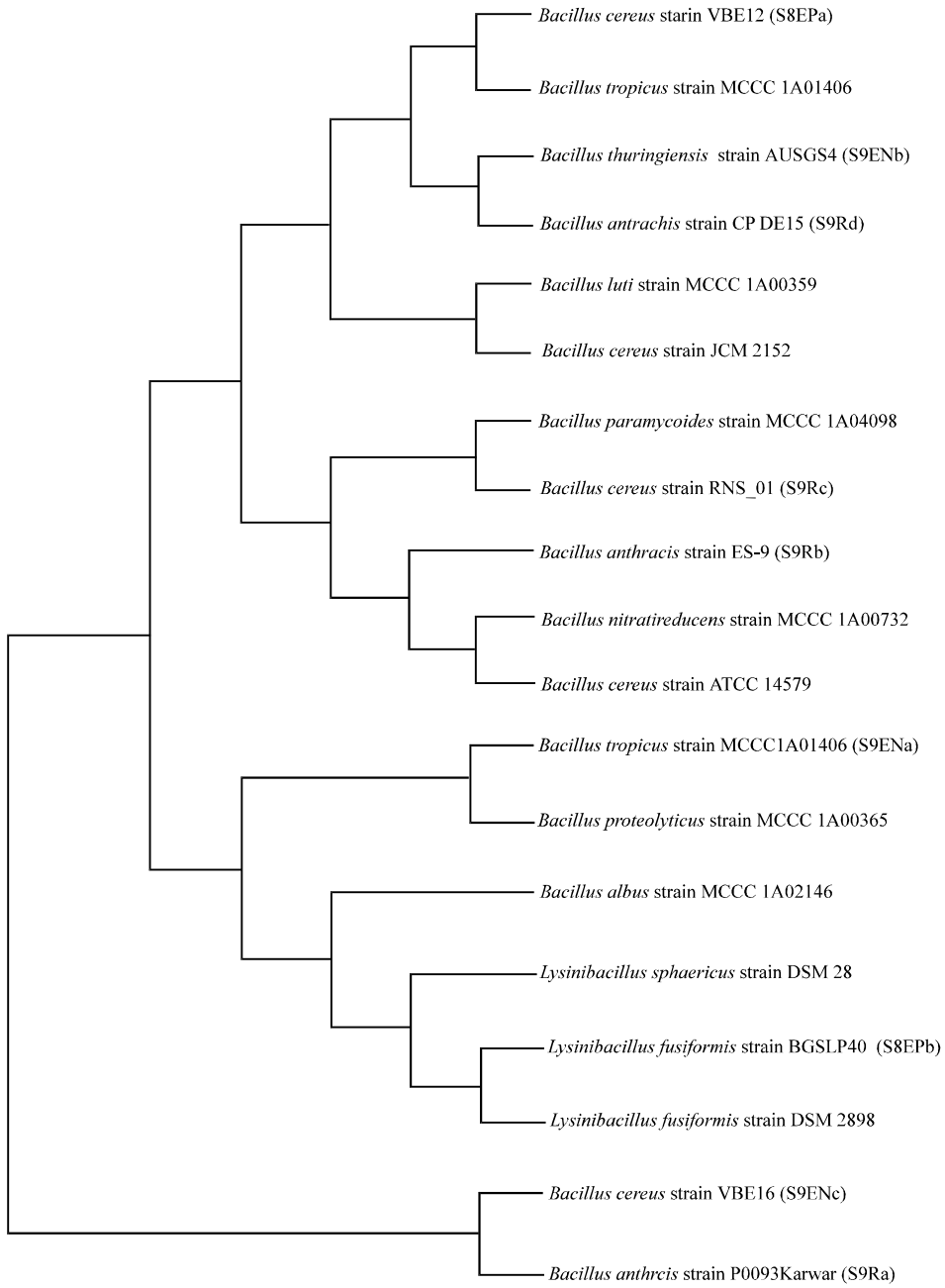


Fig. 2: Phylogenetic trees of various bacterial isolates

$KH_2PO_4$. While the phosphate solvent bacterial colonies can maintain the clear zone because these bacteria have the ability to utilize $K_2HP0_4$ as a source of phosfats while the non-phospholytic amino acid-forming bacteria lack a clear zone because they are unable to break down $K_2HPO_4$ compounds (Suryadi, 2007).

Other bacteria showing their ability to dissolve P Lestari *et al*. (2011) say that the formation of a clear zone indicates that bacteria can produce extracellular enzymes namely phosphatase and/or organic acids. According to Sylvia *et al*. (2005), this group of enzymes catalyzes the reaction of hydrolytic mineralization enzymically by releasing dissolved P.

The percentage of the amount of P that is dissolved differently in each bacterium can be estimated by the presence of different population numbers in each bacterium, besides that, the ability to adapt to the new environment is also a limiting part of the ability of bacteria

to dissolve phosphate. Widawati and Suliasih State that the activity of phosphate solvent bacteria and bacteria which cannot dissolve depends on temperature, humidity, pH, food supply and environmental conditions during its growth.

Nine types of bacteria have been identified and motility tests have been carried out to determine bacterial characterization. Nine types of bacteria tested in semi-solids were carried out by inserting isolate cultures using ose needles inserted right in the middle of the media to the bottom of the media. The research results showed that eight types of bacteria that were positively motile were indicated by the growth of spreading colonies. According to Waluyo, motile positive isolates were shown by the spread of isolates throughout the media. Motilic bacteria can make rapid movements due to the presence of flagella and also the presence of brown motion factors.

Testing of Hypersensitive Response (HR) on tobacco plants is carried out to determine whether a bacterial isolate is pathogenic or not. Isolates are infiltrated in tobacco leaves and show symptoms of necrosis within 24 h, the isolates have pathogenic potential. Hypersensitivity symptoms can be seen, if the infiltrated part of the bacterial suppression occurs necrosis within 1-4 days (Klement *et al*., 1990).

The results showed that all isolates that had been isolated were not pathogenic necrosis bacteria that did not occur in bacteria that did not have pathogenic potential. The rapid death of cells in and around cells infected by pathogens was a hypersensitive reaction and this was associated with a resistance response of plants.

Hemolysis level test on media cultivation media agar dara has shown positive traits. This indicates that the isolated bacteria are bacteria that have the potential to cause pathogens in humans and animals. According to Akhdiya *et al*. (2018) that hemolytic isolates have the potential to become pathogens for mammals. The cause of bacteria can be hemolysis can be suspected of the accumulation of pesticide residues on one agricultural area. According to Niti *et al*. (2013) that in the long term it can affect the health and productivity of the land. Pesticide residues that seep into groundwater and enter into aquatic bodies cause problems for the life of aquatic organisms (Smital *et al*., 2004).

The implications and recommendations of this research are that all types of bacteria can be used as biological fertilizers produced from upland rice plants and use of 9 types of bacteria for the cultivation of gogo rice plants. The limitations of the study are the need for testing 9 bacteria in different environments.

## CONCLUSION

Molecularly 9 types of filospheric bacteria (epiphytes, endophytes) and rhizosphere have been identified from gogo rice plants. Gogo rice plants have microbe potential in the philosphere and rhizosphere, so that, it can be used as biological fertilizers.

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