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# EXPLORATION AND MORPHOLOGICAL CHARACTERIZATION OF PHOSPHATE-SOLUBILIZING AND NITROGEN-FIXING BACTERIA IN SALINE SOIL

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**SUMMARY**

Phosphate-solubilizing and nitrogen-fixing bacteria are crucial in increasing soil fertility and restoring soil properties damaged by salinity and other abiotic environmental factors. The presented study aims to explore and identify the morphological characteristics of phosphate-solubilizing and nitrogen-fixing bacteria in saline soil. This study took place from August until November 2022 in the field and laboratory of the Faculty of Agriculture, Universitas Muhammadiyah Sumatera Utara, Medan, Indonesia. Isolation of potential microbes proceeded to characterize phosphate-solubilizing and nitrogen-fixing bacteria. Sampling began with the soil planted with rice and palm oil at the sampling location. Morphological parameters observed were color, form, margin, surface, and elevation of pure colonies. The result showed that exploring various species in saline soil revealed 19 colonies and 14 cells of phosphate-solubilizing bacteria and 16 colonies and 14 cells of nitrogen-fixing bacteria. The pure colonies of the phosphate-solubilizing and nitrogen-fixing bacteria showed differences in the morphological characteristics, i.e., color, form, margin, surface, and elevation. The potential microbes obtained sought to increase soil fertility and crop production.

**Keywords:** Phosphate-solubilizing bacteria, nitrogen-fixing bacteria, morphological characteristics, saline soil

**Key findings:** Exploration and identification of morphological characteristics is a must action to analyze potential microbes in depth to improve the quality of saline soils to increase crop productivity.

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## INTRODUCTION

Salinity often degrades the soil system resulting in lower yields. Microbial communities respond well to salinized soils by keeping them functional under severe salt stress, increasing soil fertility, and restoring negatively impacted soil qualities; hence, the phosphorus-solubilizing and nitrogen-fixing bacteria are essential. These microorganisms can help promote sustainable agriculture by minimizing the overuse of commercial fertilizers.

Phosphorus (P) is the second-most crucial nutrient for plant growth (Maharajan *et al.,* 2018; Al-Tamimi and Farhood, 2022). The P plays a vital role in most elements of energy metabolism, nucleic acid and protein synthesis, and kinase control, which authenticates its remarkable position in the ecosystem (Nesme *et al.,* 2018). Nearly 0.05% (w/w) is effective.

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However, crop plants can only use 0.1% of the available P, making it a limiting element for plant growth (Lambers and Plaxton, 2018). Though, the proportion of the P depends on the rate of diffusion. The smaller the proportion, the slower it moves, so more required P will provide sufficient supply. For P, the soil's buffering capacity depends on the proportion in the solution, which determines the soil's capacity to sorb P (Njoyim *et al.,* 2016). A significant amount of P used as chemical fertilizer exits the plant-soil system due to interaction with $Ca^{2+}$ in calcareous soils and $Al^{3+}$ and $Fe^{3+}$ in acidic soils (Shafi *et al.*, 2020), resulting in almost 80% of administered P rendered unavailable to plants (Salvagiotti *et al.,* 2017).

Phosphate-solubilizing bacteria are non-pathogenic and play an imperative role as plant growth-promoting bacteria in the soil. These bacteria also produce vitamins and phytohormones to improve plant root growth (Van-der-Heijden and Schlaeppi, 2015) and enhancement of nutrient uptake (Glick, 1995; Anand *et al.,* 2016). Such organisms are also called phosphate-solubilizing microorganisms (PSMs). Several ideas explain the mechanism of inorganic phosphate solubilization. The main process comprises creating substances that dissolve minerals, such as, organic acids, siderophores, protons, hydroxyl ions, and $CO_2$ (Sharma *et al.,* 2013). For releasing P, the carboxyl and hydroxyl ions bound the cations, and pH the lowering generates the organic acids (Seshachala and Tallapragada, 2012) and the organic acids in the periplasmic region (Zhao *et al.,* 2014). Therefore, an environment-friendly approach (using biological soil amendments as an alternative to chemicals) is preferable, using phosphorus biofertilizers to manage the infertile soil, which eventually improves the yield and food production (Babalola and Glick, 2012).

Identifying phosphate-solubilizing bacteria can be morphological because various microbial species have distinctive morphological parameters. Microbes also have a fairly consistent size and shape under suitable conditions. Therefore, knowing the morphological structure of microbes is vital, as it provides better knowledge about their physiology, pathogenic mechanisms, antigenic characteristics, and species identification. Additionally, microbial morphology can help in disease diagnosis and prevention of microbial infection. Bacterial microbes are more complex and highly variable, with four basic shapes, i.e., spiral (spirochete), spherical (cocci), rod-shaped (bacilli), and arc-shaped (vibrio) (Li *et al.,* 2020).

Non-symbiotic nitrogen-fixing bacteria used as a biofertilizer have greatly advanced in agriculture (Pereg *et al.,* 2018). Soil fertility also gains influence from alterations in the major species involved in the nutrient cycle (Anshori *et al*., 2019; De-Beeck *et al.,* 2021). Microorganisms are very flexible and can easily change their community composition and diversity indices quickly to adapt to changing environmental conditions (Du *et al.,* 2022). Therefore, it is crucial to comprehend the mechanisms governing microbial communities below the ground and their spatial variation to sustain biodiversity beneath the paddy ecosystem in the current farming system (Luo *et al*., 2020). Biological fertilizers promote soil quality and long-term sustainability and produce environment-friendly goods (Shridhar, 2012). Nitrogen (N) is also one of the most abundant elements in nature, with two types of nitrogen fixation, i.e., symbiotic and non-symbiotic (Mus *et al.,* 2016).

In the rhizosphere, the microbial populations were crucial for determining the health and production of plants (Philippot *et al.,* 2013). Similarly, mycorrhizal fungi and nitrogen-fixing bacteria are responsible for 40%–50% of the nitrogen and up to 75% of the phosphorus absorbed by plants annually (Udvardi and Poole, 2013). Therefore, altering the rhizosphere microbiome may enhance crucial crop productivity (Turner *et al.,* 2013).

Bulk soil communities serve as a source for rhizosphere communities; the temporal variability of bulk soil microbial communities is a well-known phenomenon (Lauber *et al.,* 2013), causing alterations in the rhizosphere microbiome (Zarraonaindia *et al.,* 2015). In this situation, hypotheses emerged that plants actively attract beneficial bacteria by altering their root chemistry in response to pathogen and insect attacks, a mechanism known as a "cry for assistance" (Bakker *et al.,* 2018). Low molecular-weight organic acid (LMWOA) exudation into the rhizosphere has also occurred as a plant survival tactic to enhance nutrient uptake in metal-contaminated soils. For instance, the roots of soybean and maize secrete citrate with a strong metal chelating capability in acidic soils with high aluminum (Al) concentrations, reducing the uptake of harmful $Al^{3+}$ by the plants (Liang *et al.*, 2013) and defense phytohormones (Lebeis *et al.,* 2015). Roots also modify the pH and oxygen concentrations in the soil surrounding the root, creating a

unique soil microhabitat (Hacquard *et al.,* 2015).

Identification of microbes in simple terms can proceed through morphological observations. Simple observations by looking at morphological characters can provide an overview of macroscopic and microscopic morphological variations (Mukamto *et al.,* 2015). The macroscopic morphological observation observed colony morphology for isolation and purification, including colony size, form, elevation, margin, texture, color, and smell (Kreger-Van Rij, 1987; Widiastutik and Alami, 2013).

Therefore, exploration and identification are crucial to determine the morphological characteristics of phosphate-solubilizing and nitrogen-fixing bacteria in saline soil. The presented study aims to isolate and characterize the various species of phosphate-solubilizing and nitrogen-fixing bacteria found in saline soils.

## MATERIALS AND METHODS

This study transpired from August until November 2022 in the field and laboratory of the Faculty of Agriculture, Universitas Muhammadiyah Sumatera Utara, Medan, Indonesia. The material used in this research were soil microbial populations obtained from sorghum rhizosphere, saline soil, microbes growth medium (Martin Agar, NFM, Pikovskaya, Alexandrop), Potatoes Dextrose Agar, malic acid, $K_2HPO_4$, $MgSO_4.7H_2O$, NaCl, $CaCl_2.2H_2O$, BTB indicator, B vitamin solution, micronutrient solution, agar, $CuSO_4$, $ZnSO_4$, $H_3BO_3$, $Na_2MO_4.2H_2O$, $MnSO_4$, Fe-EDTA, KOH, $Ca_3(PO_4)_2$, KCl, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$, $(NH_4)2SO_4$, glucose, yeast extract, aquadest, $FeCl_3$, $CaCO_3$, Feldspar, and other supporting materials.

### Isolation of phosphate-solubilizing and nitrogen-fixing bacteria

Isolation of potential microbes ensued to characterize phosphate-solubilizing and nitrogen-fixing bacteria. Sampling used the soil planted with rice and palm oil at the sampling location. In saline soil, the soil samples taken were from the plant root zone with a depth of 10-15 cm using sterile plastic. Samples from each point were then put into sterile plastic, made a composite, and brought to the laboratory, with the degree of soil acidity measured using a pH meter. The collected soil samples were from five locations per sampling location, undergoing subsequent homogenization, with 10 g removed, dissolved in 90 ml of physiological NaCl, and vortexed for 60 min ($10^{-1}$ dilution). Using physiological NaCl, the findings of the $10^{-1}$ soil suspension got diluted up to $10^{-6}$. Following the $10^{-4}$ to $10^{-6}$ dilutions, cultivation of the findings progressed on Nutrient Agar (NA) media, and then incubated for 24 h at room temperature. Population observations were on each microbe after three to five days of incubation. Calculation of the microbial population in each field used the counting cup method (Barus *et al.,* 2017). The pour plate method was able to measure the growth of bacteria. In the cup counting method using the pour plate method, 1 ml of sample (all series of dilutions) was poured into a sterile petri dish, receiving 10 ml of PCA media into the petri dish. Leveling the media continued by shaking the petri dish and incubating at 20 °C for 24–48 h (Soesetyaningsih and Azizah, 2020). The TPC (Total Plate Count) value calculations came after counting the colonies expanding in the 25–250 colony range, with observed morphological parameters focusing on color, form, margin, surface, and elevation of pure colonies.

### Microbial Diversity Index

Calculations for the microbial diversity index employed the following formula:

$$H' = -\sum Pi \ln (Pi), \text{ where } Pi = (ni/N)$$

Where:
H' = Shannon-Wiener diversity index
ni = The number of individuals of the type-i
N = Number of individuals of all types

The criteria for the diversity index value of Shannon – Wiener (H') were:

- H'< 1 (low diversity)
- 1< H'< 3 (medium diversity)
- H'> 3 (high diversity)

## RESULTS AND DISCUSSION

### Phosphate-solubilizing and nitrogen-fixing bacteria in saline soils

Conducting an estimation of soil bacterial population took place since the study used only one medium that would enable only a few of the physiological groups of the bacterial population to develop. Estimation of soil

bacterial population using NA medium showed that in saline soil, the highest number of the populace were phosphate-solubilizing bacteria, having too many to count (> 250) colony forming unit (CFU) per mL of soil (Table 1). The reason was the high content of soil microbial populations obtained from sorghum rhizosphere in saline soils. The quantity and activity of phosphate-solubilizing fungi gained influence from the soil's organic matter concentration as a source of energy and carbon (Deni *et al.,* 2021). Besides, the research results by Munar *et al.* (2020) reported that sound treatment affected the growth of phosphate-solubilizing microbes and all microbes that were not pathogenic to plants.

The medium microbial diversity index emerged in the sorghum rhizosphere of PD (1.220606 CFU/ml), followed by SA (1.209368 CFU/ml) (Table 2). PD rhizosphere harbored a higher microbial diversity index than the SA and the others. It could be due to the high salt tolerance of the PD-producing root exudates, which helps create a better rhizospheric position that can better support microorganisms compared with other biotypes. Salt-tolerant microbes survive in soils of medium to high salinity and play a vital role in cycling C, N, and P in saline-alkaline soils (Egamberdieva *et al.,* 2019). The P and Zn solubilizer populations (Kamran *et al.,* 2017; Wan *et al.,* 2020), as well as, the ammonifying, nitrifying, and denitrifying bacteria are all strongly related to the N, P, and Zn transformation and supply in soil (Pajares and Bohannan, 2016).

**Phosphate-solubilizing bacteria colony morphologies**

Various species of phosphate-solubilizing bacteria appeared in saline soils. However, based on classical microscopic techniques of color, form, shape, surface, and elevation of pure colonies, isolation of 19 colonies and 14 cells came from saline soils (Table 3). Reports also stated that organisms capable of doing phosphate solubilization also provide a clear zone around the colony by which phosphate-solubilizing microorganisms are authenticated (Damor and Goswarni, 2016). Most colonies ably grew within 2–6 days of incubation at 30 ºC. The observed bacterial species underwent further evaluation for their Gram reaction and shape. However, characteristically, all the isolates were catalase-positive, 78% were Gram-positive, 22% were Gram-negative, and all were rod- and round-shaped.

The colony morphologies of phosphate-solubilizing Gram-positive and Gram-negative bacteria were also different from each other (Figure 1). Gram-positive bacteria have peptidoglycan cell walls that react with iodine and crystal violet, creating a combination forceful for safranin to penetrate. Inversely, the Gram-negative bacteria have double membrane systems, i.e., a plasma membrane and a permeable membrane that surrounds the plasma membrane, a very thin cell wall that allows the main paint pigment to dissolve, and the cell wall colored by safranin.

**Table 1.** Total of soil microbial populations obtained from sorghum rhizosphere in saline soils.

| No. | Sample code | Bacteria (CFU/ml) | Fungus (CFU/ml) | Phosphate-solubilizing bacteria (CFU/ml) | Phosphate-solubilizing fungus (CFU/ml) |
|---|---|---|---|---|---|
| 1 | PD Number | 42 x$10^4$ | TFTC | 6 x$10^4$ | 1 x$10^4$ |
| 2 | PD Super 1 | 114 x$10^4$ | TFTC | 16 x$10^4$ | - |
| 3 | PD Super 2 | 196 x$10^4$ | - | TMTC | - |
| 4 | PD Suri 3 | 235 x$10^4$ | - | TMTC | - |
| 5 | PD Suri 4 | TFTC | TFTC | 8 x$10^4$ | 2 x$10^4$ |
| 6 | PD Soper 6 | 1.78 x$10^4$ | - | TMTC | - |
| 7 | PD Soper 7 | TFTC | TFTC | 5 x$10^4$ | - |
| 8 | PD Soper 9 | TFTC | - | 8 x$10^4$ | - |
| 9 | SA Numbu | 179 x$10^4$ | - | - | - |
| 10 | SA Super 1 | 98 x$10^4$ | TFTC | - | - |
| 11 | SA Super 2 | TSUD | - | 2 x$10^4$ | - |
| 12 | SA Suri3 | TSUD | - | 8 x$10^4$ | - |
| 13 | SA Suri 4 | 1,59 x$10^4$ | - | 3 x$10^4$ | - |
| 14 | SA Soper 6 | 154 x$10^4$ | - | TMTC | - |
| 15 | SA Soper 7 | TFTC | - | 6 x$10^4$ | - |
| 16 | SA Soper 9 | 148 x$10^4$ | TFTC | 65 x$10^4$ | - |

Note: TFTC= Too Few to Count (< 25 colonies), TMTC = Too Many to Count (> 250 colonies).

**Table 2.** Microbial diversity index obtained from sorghum rhizosphere in saline soils.

| No. | Sample Code | Microbial diversity index (CFU/ml) | Criteria |
|---|---|---|---|
| 1 | PD Numbu | 0.923962 | Low |
| 2 | PD Super 1 | 0.654475 | Low |
| 3 | PD Super 2 | 0.288958 | Low |
| 4 | PD Suri 3 | 1.030675 | Medium |
| 5 | PD Suri 4 | 1.220606 | Medium |
| 6 | PD Soper 6 | 0.76077 | Low |
| 7 | PD Soper 7 | 1.03991 | Medium |
| 8 | PD soper 9 | 0.940448 | Low |
| 9 | SA Numbu | 1.209368 | Medium |
| 10 | SA Super 1 | 0.531713 | Low |
| 11 | SA Super 2 | 1.102593 | Medium |
| 12 | SA Suri 3 | 0.693147 | Low |
| 13 | SA Suri 4 | 1.01957 | Medium |
| 14 | SA Soper 6 | 1.022022 | Medium |
| 15 | SA Soper 7 | 0.59827 | Low |
| 16 | SA soper 9 | 1.125526 | Medium |

Note: PD = land planted with rice; SA = land planted with palm oil.

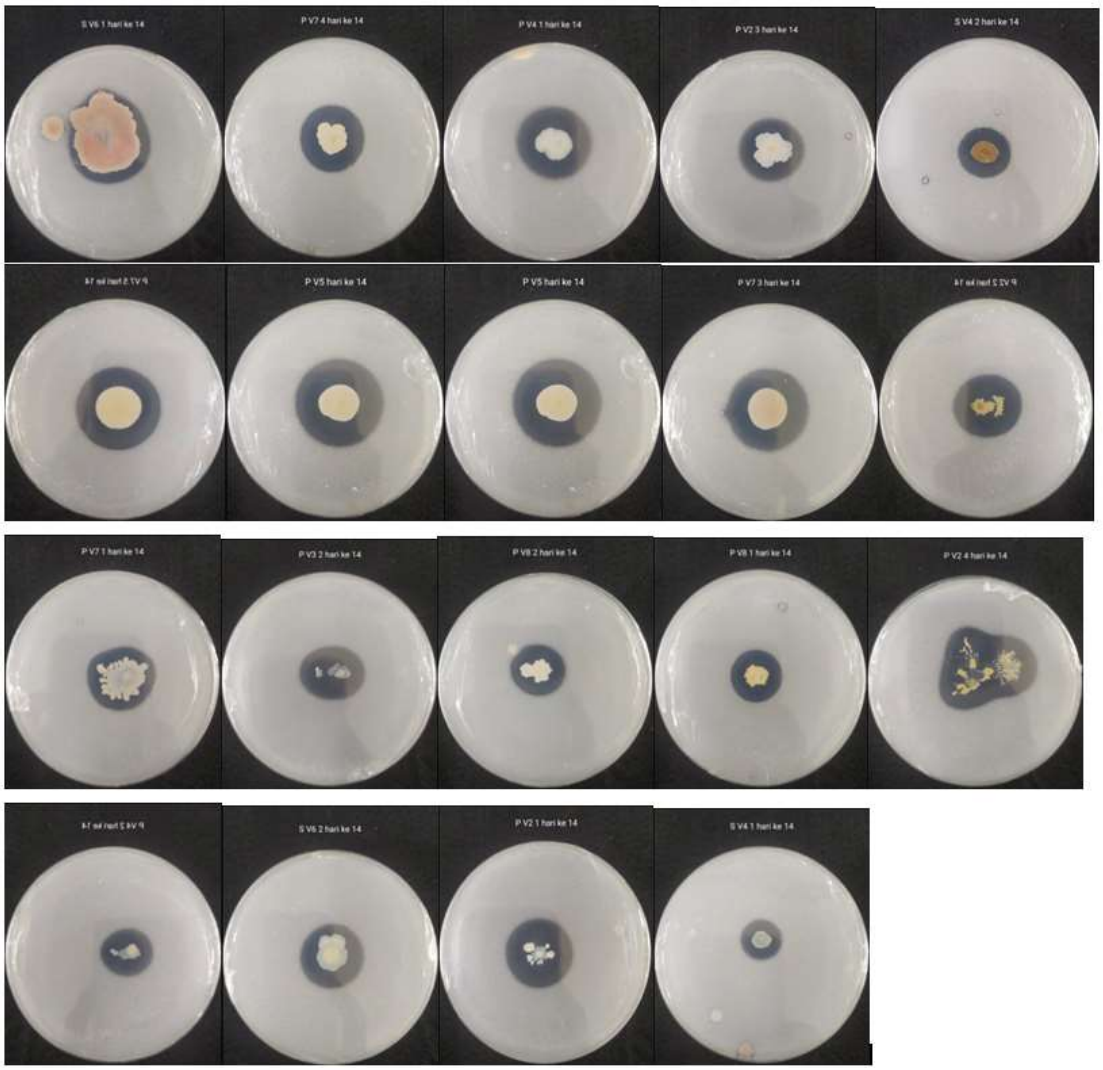

**Figure 1.** Pure colonies of phosphate-solubilizing bacteria collected from sorghum rhizosphere in saline soils.

**Table 3.** Colony morphologies and identities of phosphate-solubilizing bacteria strains.

| Sample ID | Characterization | | | | | | |
|---|---|---|---|---|---|---|---|
| | Colony | | | | | Cell | |
| | Form | Margin | Elevation | Color | Surface | Gram | Cell shape |
| S V4 2 | Circular | Entire | Crateriform | Brown | rough | | |
| P V4 1 | Circular | Entire | Crateriform | White cream | slippery | + | rods |
| S V6 1 | Irregular | Curled | Crateriform | Orange | rough | - | rods |
| P V2 3 | Irregular | Undulate | Flat | White cream | slippery | + | round |
| P V7 4 | Irregular | Undulate | Crateriform | Cream | rough | + | rough |
| P V2 2 | Irregular | Curled | Flat | Brown | rough | - | rods |
| P V7 5 | Circular | Entire | Flat | Yellow cream | slippery | + | round |
| P V7 3 | Circular | Entire | Flat | Cream | slippery | + | round |
| P V5 | Circular | Entire | Flat | White cream | slippery | + | round |
| P V7 2 | Circular | Entire | Flat | White cream | slippery | - | round |
| P V2 4 | Filamentous | Filiform | Flat | Yellow | rough | + | rods |
| P V8 1 | Irregular | Undulate | Umbonate | Cream | rough | + | rods |
| P V8 2 | Irregular | Undulate | Raised | White milk | slippery | + | rods |
| P V3 2 | Irregular | Undulate | Flat | Cream | slippery | + | rods |
| P V7 1 | Irregular | Lobate | Umbonate | Brown | slippery | + | round |
| S V4 1 | Irregular | Curled | Flat | Cream | slippery | | |
| P V2 1 | Irregular | Lobate | Raised | White cream | slippery | | |
| P V4 2 | Irregular | Undulate | Flat | Cream | slippery | | |
| S V6 2 | Irregular | Undulate | Raise | Yellow cream | rough | | |

Note: Isolates collected from sorghum rhizosphere in saline soils.

The quantity of living cells is among the quality parameters within the biofertilizer regulation in international standards for many countries (Malusá and Vassilev, 2014). The standard has different groups of microorganisms (rhizobia, for fast or slow-growing species; N-fixing bacteria; phosphate-solubilizing bacteria [PSB]), classified based on the ability to act on organic and inorganic phosphates. Plants require added mechanisms to obtain Pi (P inorganic) under low P conditions, as the roots have entrance to a somewhat small fraction of total soil P. After applying chemical fertilizers, plants can use tiny amounts of phosphorus because of the phosphorus complexities of developing fragile soil structures.

Conducting a hypersensitivity test found out whether the bacterial isolates that had been tested for antagonistic activity reacted positively or not. Out of the 21 bacterial isolates tested of phosphate-solubilizing bacteria, five of each bacterial isolates reacted positively on day two and day four, respectively (Table 4).

Hypersensitivity reactions are rapid forms of programmed cell death associated with plant defense, which can be morphologically characterized by the formation of necrotic lesions around the pathogen entry sites. Hypersensitive reactions are induced by fungi, oomycetes, bacteria, and viruses (Balint-Kurti, 2019). The study's comprehension of HR also got hampered by the fact that cell death often occurs in plants and has a wide range of underlying reasons. The word "HR" has been used and referred to a wide variety of occurrences, many of which have one thing in common: a macroscopic appearance that suggests cell death. The term 'hypersensitive-like cell death' or similar is often used simply when cell death is observed without any explicit link to R-genes, the defense response, and disease resistance (Kumar and Kirti, 2015; Na *et al.,* 2015; Chen *et al.,* 2016; Wei *et al.,* 2016).

The isolation resulted in 19 phosphate-solubilizing isolates with a clear zone for the said bacteria (Figure 2). After growing those bacteria on a nutrient-agar medium, producing a clear zone around their colony gave the ability to dissolve phosphate. Additionally, Paul and Sinha (2017) demonstrated that a clear zone develops when the pH of a medium decreases due to phosphatase enzyme activities. These microorganisms also produce organic acids that can lower the pH and interact with P-binding substances, including $Al^{3+}$, $Fe^{3+}$, $Ca^{2+}$, and $Mg^{2+}$, to form organic chelates that allow the release of free phosphate ions ($PO^4$) (Ranjan *et al.,* 2013).

**Table 4.** Hypersensitivity test results of phosphate-solubilizing bacteria.

| No. | Isolate | Isolate code | Observation Day | |
|---|---|---|---|---|
| | | | 2 | 4 |
| 1 | P V3 2 | P1 | - | - |
| 2 | P V2 1 | P2 | - | - |
| 3 | P V2 4 | P3 | + | + |
| 4 | P V4 1 | P4 | - | - |
| 5 | P V7 5 | P5 | - | - |
| 6 | P V4 2 | P6 | - | - |
| 7 | P V8 2 | P7 | - | - |
| 8 | P V7 2 | P8 | - | - |
| 9 | P V7 3 | P9 | - | - |
| 10 | P V6 | P10 | - | - |
| 11 | P V7 1 | P11 | - | - |
| 12 | P V5 | P12 | - | - |
| 13 | P V8 1 | P13 | + | + |
| 14 | P V2 2 | P14 | + | + |
| 15 | P V2 3 | P15 | - | - |
| 16 | P V7 4 | P16 | + | + |
| 17 | P V3 4 | P17 | - | - |
| 18 | S V6 2 | P18 | - | - |
| 19 | S V6 1 | P19 | + | + |
| 20 | S V4 1 | P20 | - | - |
| 21 | S V4 2 | P21 | - | - |

Note: + = pathogen, = non-pathogen, V1 = Numbu; V2 = Super 1; V3 = Super 2; V4 = Suri 3; V5 = Suri 4; V6 = Soper 6; V7 = Soper 7; V8 = Soper 9, P = land planted with rice; S = land planted with palm oil.

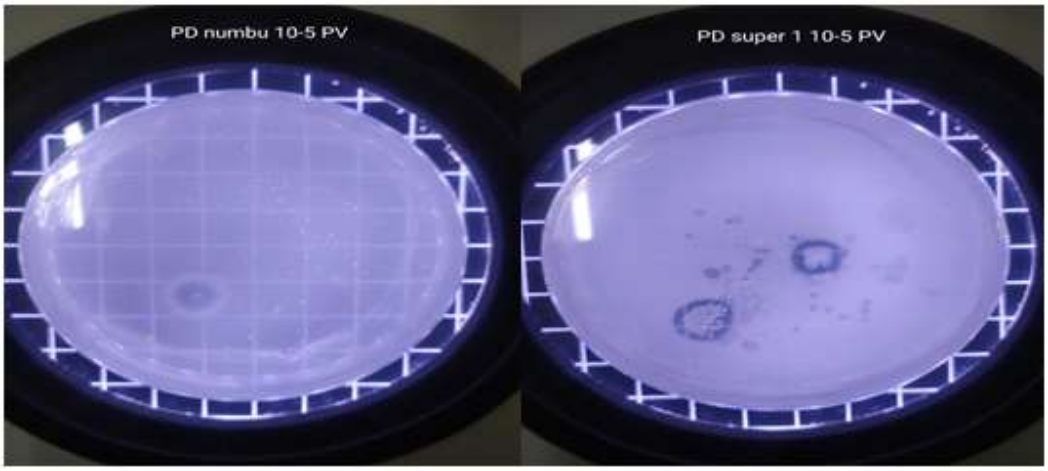


**Figure 2.** Phosphate-solubilizing isolates on a Nutrient agar medium; a) There is no clear zone of phosphate-solubilizing bacteria, b) There is a clear zone for phosphate-solubilizing bacteria.

**Nitrogen-fixing bacteria colony morphologies**

In saline soils, various species of nitrogen-fixing bacteria showed, with 16 colonies and 14 cells isolated based on classic macroscopic techniques of color, form, margin, surface, and elevation of pure colonies (Table 5). The gathered bacteria could fix nitrogen in a non-symbiotic way and grow on N-free media. The nitrogen-fixing bacteria carry them out because their cells include an enzyme called nitrogenase, made up of two mutually supportive components, i.e., Fe and Mo-Fe protein (Geddes *et al.,* 2015).

Most colonies grew within 2–6 days of incubation at 30 °C. These bacterial species underwent further investigation for their Gram reaction and cell shape. However, characteristically, all the bacterial isolates were catalase-positive; 92% were Gram-positive, 8% were Gram-negative, and rod- and round-cell shaped.

**Table 5.** Colony morphologies and identities of nitrogen-fixing bacteria strains.

| Sample ID | Characterization | | | | | |
|---|---|---|---|---|---|---|
| | Colony | | | | Cell | |
| | Margin | Elevation | Color | Surface | Gram | Cell shape |
| SA Super 1 | Entire | Flat | Pink | Slippery | + | long |
| SA Super 2 | Entire | Raised | Pink | Slippery | + | round |
| SA Suri 3 | Entire | Flat | Cream | Slippery | + | rods |
| SA Suri 4 | Entire | Raised | Pink | Slippery | + | rods |
| SA Soper 6 | Undulate | Raised | Pink | Slippery | + | rods |
| SA Soper 7 | Entire | Convex | Red | Slippery | + | rods |
| SA Soper 9 | Undulate | Raised | Red | Slippery | + | rods |
| SA Numbu | Entire | Flat | red browny | Slippery | | |
| PD Super 1 | Undulate | Raised | Red | Slippery | + | rods |
| PD Super 2 | Undulate | Raised | Pink | Slippery | + | rods |
| PD Super 3 | Undulate | Raised | Red | Slippery | + | rods |
| PD Super 4 | Undulate | Raised | Red | Slippery | + | rods |
| PD Super 6 | Undulate | Convex | Red | Slippery | + | round |
| PD Super 7 | Undulate | Raised | Pink | Slippery | + | rods |
| PD Super 9 | Curled | Flat | Pink | Slippery | - | rods |
| PD Numbu | Entire | Flat | Cream | Slippery | | |

Note: Isolates collected from sorghum rhizosphere in saline soils.

The morphological characteristics of the 20 collected bacteria differed from each other (Figure 3). The genus and species recognition of these bacterial isolates still requires further analysis. Based on the Gram test, six isolates were Gram-positive and 14 were Gram-negative. The structure of the cell wall was the cause of this variation. Gram-positive bacteria have peptidoglycan-based cell walls, whereas, Gram-negative bacteria have lipid-based cell walls (Sudewi *et al.,* 2020).

This justifies the growth to encourage sustainable agriculture and agroforestry and limit the widespread use of inorganic N fertilizers (Araujo *et al.,* 2012; Shah and Wu, 2019). Through the exploitation of biological nitrogen fixation (BNF), reducing the use of N fertilizers in agriculture can occur, and consequently, their detrimental effects on the environment. In reality, BNF is a naturally occurring process that converts atmospheric nitrogen ($N_2$) into a straightforward soluble harmless form ($NH^{4+}$ mainly), which plant cells use to synthesize a variety of biomolecules. Nitrogen fixation is one of the major sources of nitrogen for plants and a key step in distributing this nutrient in the ecosystem (Bottomley and Myrold, 2015).

The hypersensitivity test results revealed that only one nitrogen-fixing bacteria in the positive control (+) showed symptoms of necrosis after two days of incubation, and the rest tested negative (-) (Table 6). The possibility of plant disease prevents the use of isolates having positive hypersensitivity reactions in subsequent testing. All the higher plants also exhibit the hypersensitive defense response, characterized by a fast cell death at the site of disease entry. It typically results in disease resistance, but under certain circumstances, it can also lead to pathogen susceptibility and growth retardation throughout evolution and speciation (Balint-Kurti, 2019).

Testing the growth of 16 isolates was in test tubes containing a semi-solid medium of nitrogen-free bromothymol blue (NFB). The isolates formed a circle, mist-like ring below the surface of the medium after incubation at room temperature (Figure 4). The nitrogen-fixing bacteria's synthesis of nitrogenase caused the ring to form. Similar findings from Baldani *et al.* (2014) and Kusumawati *et al.* (2017) reported that the appearance of a circular fog beneath the surface of a semi-solid NFB medium indicated the synthesis of nitrogenase by nitrogen-fixing bacteria. The nitrogenase production also indicated that the bacteria that fix nitrogen were utterly mobile. The nitrogen fixation process attained impact from the nitrogenase enzyme activities managed by the cellular respiration activity of bacteria that is linked to the oxygen in the environment (Merlo *et al.,* 2014). The fixed nitrogen will collect on the surface of the medium as a result of the nitrogen-fixing process by the nitrogenase enzyme reaching a balance point with the contribution of ATP (Susilowati and Setyowati, 2016; Inomura *et al.,* 2018).

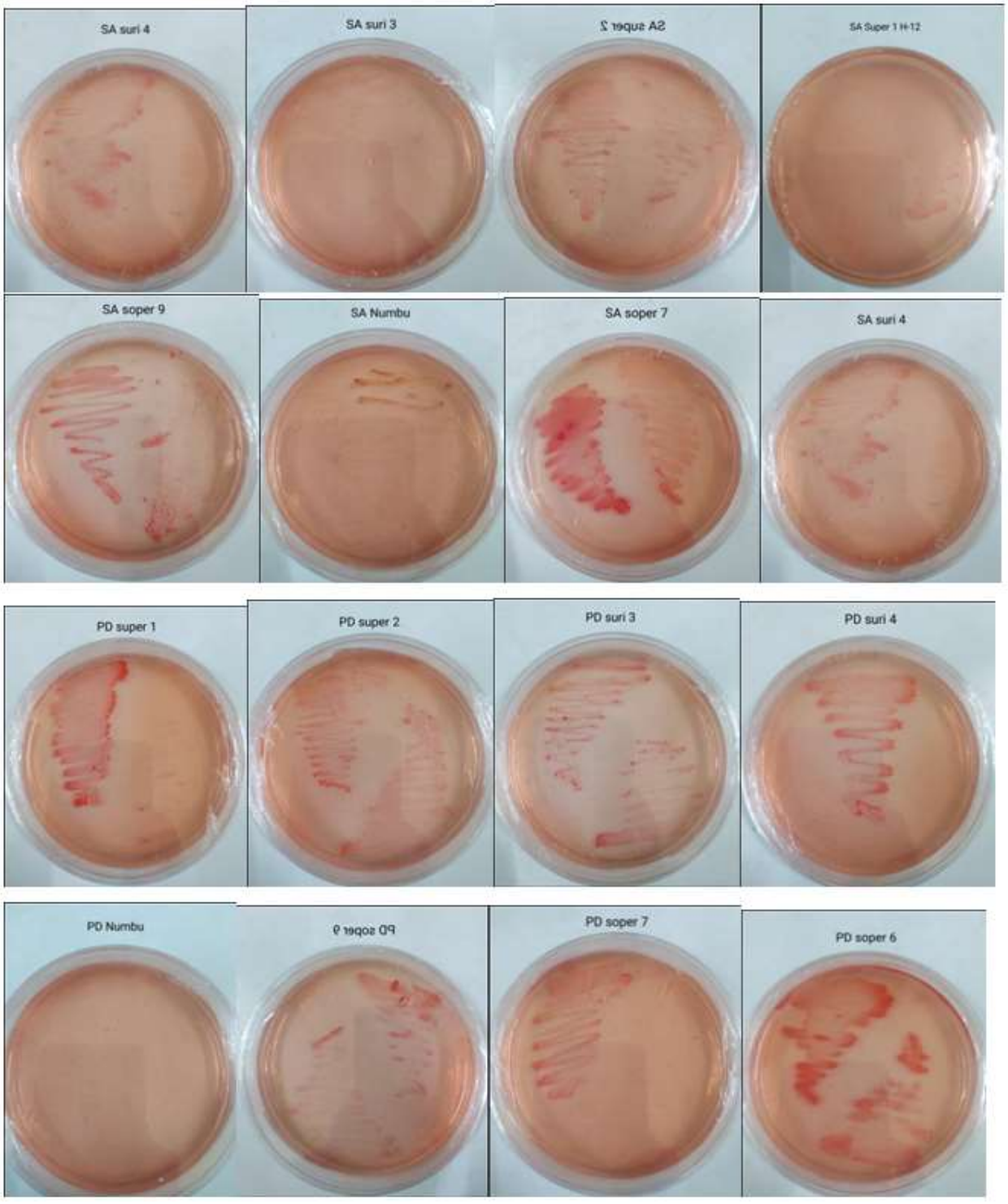


**Figure 3.** Pure colonies of nitrogen-fixing bacteria collected from sorghum rhizosphere in saline soils.

**Table 6.** Hypersensitivity test results of nitrogen-fixing bacteria.

| No. | Isolate | Isolate code | Observation Day | |
|---|---|---|---|---|
| | | | 2 | 4 |
| 1 | SA Super 2 | N1 | - | - |
| 2 | SA Super 1 | N2 | - | - |
| 3 | SA Soper 9 | N3 | - | - |
| 4 | SA Soper 6 | N4 | - | - |
| 5 | SA Numbu | N5 | - | - |
| 6 | SA Soper 7 | N6 | - | - |
| 7 | SA Suri 3 | N7 | - | - |
| 8 | SA Suri 4 | N8 | + | + |
| 9 | PD Soper 6 | N9 | - | - |
| 10 | PD Suri 4 | N10 | - | - |
| 11 | PD Suri 7 | N11 | - | - |
| 12 | PD Numbu | N12 | - | - |
| 13 | PD Soper 9 | N13 | - | - |
| 14 | PD Suri 3 | N14 | - | - |
| 15 | PD Super 1 | N15 | - | - |
| 16 | PD Super 2 | N16 | - | - |

Note: + = pathogen, = non-pathogen, V1 = Numbu; V2 = Super 1; V3 = Super 2; V4 = Suri 3; V5 = Suri 4; V6 = Soper 6; V7 = Soper 7; V8 = Soper 9, PD = land planted with rice; SA = land planted with palm oil.

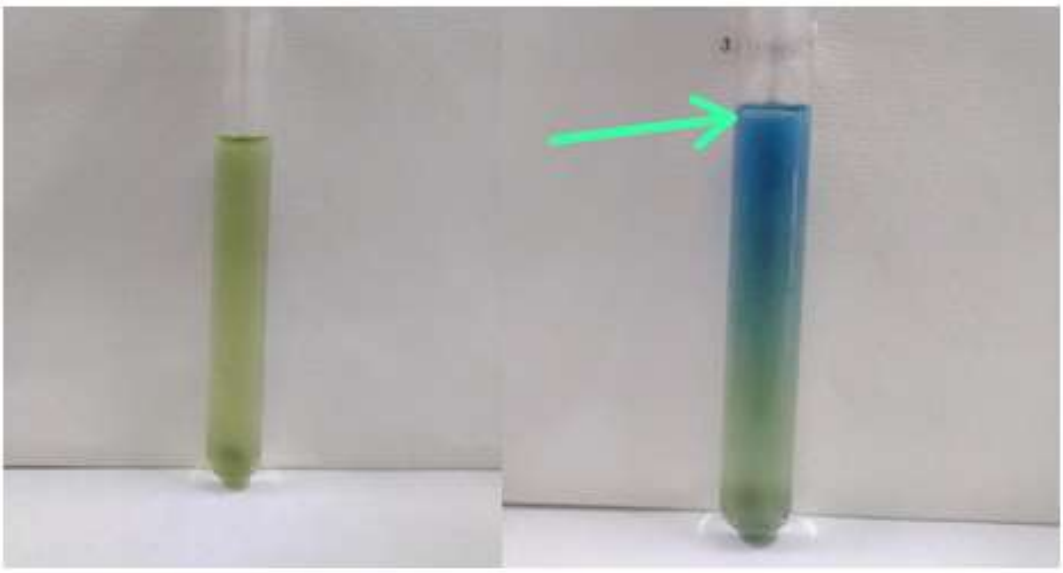

**Figure 4.** The growth of isolates was tested in test tubes containing a semi-solid medium of nitrogen-free bromothymol blue (NFB); a) Color of NFB semisolid media before isolation of nitrogen-fixing bacteria, b) The color of NFB semi-solid media after isolation and the follicles formed indicated the presence of nitrogen-fixing bacteria.

Tobacco leaves received injection with four bacterial isolates; no necrosis sign occurred on the leaves until five days of treatment (Figure 5 a-d). However, Figure 3b shows necrotic changes, where brown patches appeared surrounding the injected area of the leaf. The four isolates also responded similarly to sterile aquadest and demonstrated a lack of resistance to pathogens (Figure 5a). The four isolate testing produced a hypersensitivity-negative and environmentally beneficial outcome. According to Hanif and Susanti (2017), hypersensitivity reactions are rapid cell death, which is localized after inoculation with bacteria. Hypersensitivity reactions were the rapid forms of programmed cell death associated with the plant defense system, with morphological characterization by the formation of necrotic lesions around the pathogen entry sites (Balint-Kurti, 2019).

**Figure 5.** Hypersensitivity test on tobacco leaves; a) before treatment, b) tobacco leaves injected with bacteria, c) leaves injected with phosphate-solubilizing bacteria, d) leaves injected with nitrogen-fixing bacteria.

In conclusion, the phosphate-solubilizing bacteria evaluated in this study met the requirements for use as eco-friendly biofertilizers for crops. Bashan *et al.* (2014) also found that when properly formulated, ecologically acceptable bio-inoculants for biofertilizers do not cause necrotic symptoms in plants. However, according to Lynn *et al.* (2013), biofertilizers made from a combination of different bacterial strains were more successful at promoting plant development.

## CONCLUSIONS

Exploration of various species in saline soil found 19 colonies and 14 cells of phosphate-solubilizing bacteria and 16 colonies and 14 cells of nitrogen-fixing bacteria. The pure colonies of the phosphate-solubilizing and nitrogen-fixing bacteria showed differences in the morphological characteristics, i.e., color, form, margin, surface, and elevation.

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