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Identification of acid-resistant PGPR potential as stem rot antagonists and biofertilizers from peatlands of Central Kalimantan

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Abstract

Sweet corn (*Zea mays* L. *saccharata*) is a commodity of economic value, and cultivation constraints include stem rot disease by *Fusarium sp.* and acidic peat soil. To control this disease, farmers use destructive methods or using chemical pesticides that hurt the environment. Therefore, environmentally friendly control is needed, including using Plant Growth Promoting Rhizobacteria (PGPR). PGPR is a bacterium that lives around plant roots or in plant root tissue and has the potential as a biostimulant, biofertilizer, and bioprotectant. Peatlands are acidic and poor in nutrients. Therefore, soil amendments are needed. Therefore, it is necessary to identify and characterize PGPR which is capable of acting as a biofertilizer, biostimulant, and biofertilizer. This study aims to obtain rhizobacteria isolates that act as biofertilizers, biostimulants, and bioprotectants and are resistant to acid soils and study the role of indigenous PGPR in controlling corn stem rot disease *in vitro*. The research used descriptive, quantitative, and qualitative methods. PGPR isolates were obtained from peatlands in Kelampangan Village, Central Kalimantan, Indonesia. The results showed that six rhizobacteria isolates functioned as bioprotectants and were tolerant of acidity in peat soils, namely *Pantoea stewartii*, *Burkholderia cepacia, Pseudomonas luteola, Bacillus cereus, Bacillus subtilis*, and *Brevibacillus laterosporus*, and four isolates were able to inhibit the development of the *Fusarium sp* is *Burkholderia cepacia, Bacillus cereus, Bacillus subtilis*, and *Brevibacillus laterosporus*. Two isolates were found to be able to solubilize phosphate so that they have potential as biofertilizers, such as *Burkholderia cepacia* and *Brevibacillus laterosporus*.

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Introduction

Sweet corn (*Zea mays* L. *saccharata*) is an agricultural commodity that has high economic value because it is used in various fields, both household scale and industrial/company scale and much developed Palangka Raya City. The increase in sweet corn production is limited by several obstacles including the attack of plant-disturbing organisms, one of which is the attack of pathogens that cause stem rot disease. Stem rot is the second major disease in maize after downy mildew (Pakki *et al*., 2019). This disease is caused by *Fusarium moniliforme* which is the dominant species that infects all parts of corn, including roots, stems, midribs, cobs, and seeds. As a result of this disease, corn plants will die.

This fungus has a high diversity and population and is influenced by environmental stress and the presence of insect pests that support the development of pathogens to be faster. Symptoms caused by rot at the base of the stem are called stem rot disease.

Stem rot disease is a disease caused by *F. graminearum*. Stem rot disease mostly attacks corn plants in Kalampangan Village. Applications using chemical pesticides such as streptomycin and copper-mancozeb for control (Lindsey *et al*., 2020) can cause contamination of surface water, and soil, decrease soil fertility, and is harmful to humans, wild animals, and others. The negative impact of using chemical pesticides needs to be minimized by using natural enemies or antagonist agents. Several antagonistic agents can control plant diseases, such as B. subtilis as an antagonist of *F. verticillioides* (Cavaglieri *et al*., 2005), controlling *F. graminearum, F. culmorum* (Grosu *et al*., 2015). Use of *Brevibacillus laterosporus* as biocontrol of potato common scab (PCS) on potato plants (Li *et al*., 2021). Proper microbial treatment and using the interaction of plant roots and soil microorganisms in reducing environmental stress with an environmentally friendly, sustainable approach to the use of beneficial microorganisms (Kurek *et al*., 2013; Etesami, Alikhani, and Hosseini 2015; Shahzad *et al*., 2013). Another solution offered is the use of PGPR. The selected rhizobacteria were included in the PGPR criteria as biofertilizers and bioprotectants. Solutions using rhizobacteria provide options for farmers and help meet food demands and achieve sustainable farming practices. Environmentally-friendly control is needed, taking into account the acidic nature of the peat soil (Bulgari *et al*., 2015; Chauhan *et al*., 2015).

The purpose of this study was to obtain isolates of rhizobacteria from the rhizosphere of sweetcorn plants which acted as biofertilizers and bioprotectants and were resistant to the acidity of peat soils and to examine the role of indigenous PGPR in controlling stem rot disease of corn plants in vitro.

Materials and methods

This research was conducted from December 2020 to November 2021. Sampling of the corn plant rhizosphere soil in Kelampangan Village, Sabangau District, Palangka Raya City in (S02°17'09.41"E114°01'10.64"). Isolation and characterization of rhizobacteria were carried out at the Phytopathology Laboratory, Soil Laboratory, and Agroindrustrial Technology Departemen Laboratory of Lambung Mangkurat University Banjarbaru and Banjarbaru Vetinier Center.

Condition of peatlands (chemistry, physics, and biology)

Based on the results of soil chemical analysis on peatlands that the peat soil used in the study with a pH range of 3.0-4 is included in the acid criteria, the results of soil analysis show that the content of N, P and K is low with an average bulk density of 0.29, permeability 2.03 (medium), and organic matter 0.153 (medium), PO 1.65, porosity 89.1 and initial bacterial density of 10^{10} CFU gr^{-1}. Sampling location soil in the corn plant rhizosphere for the study in Fig.1.

The materials used during the study were: nutrient agar (NA) media. Selective media for bacteria, nutrient broth (NB), potato dextrose agar (PDA) media, tryptic soy broth (TSB) medium, Tryptic Soy Agar (TSA) medium, Kings'B medium, glycine

medium, 70% alcohol, Zinc acetate. Aquades, picric acid, methanol, acetic acid, distilled water, potassium ferrocyanide, $FeCl_3$, potassium ferricyanide, HCl, Hathway reagent, sodium salicylate, $MgSO_47H_2O$, $FeSO_47H_2O$, $NaMoO_42H_2O$, $MnSO_45H_2O$, $CaCl_2$, K_2HPO_4, NaCl, glucose, KCl, $MgSO_4$, $MnSO_4$, $FeSO_4$, H_2SO_4, agar, $(NH_4)_2$ sprites yeast extract, and plastic wrap. Tools used: binocular microscope, haemocytometer, spectrophotometer, autoclave, laminar air flow, pH meter, thermometer, test tube, petri dish, oven, shaker, and centrifuge (Kurek *et al.*, 2013).

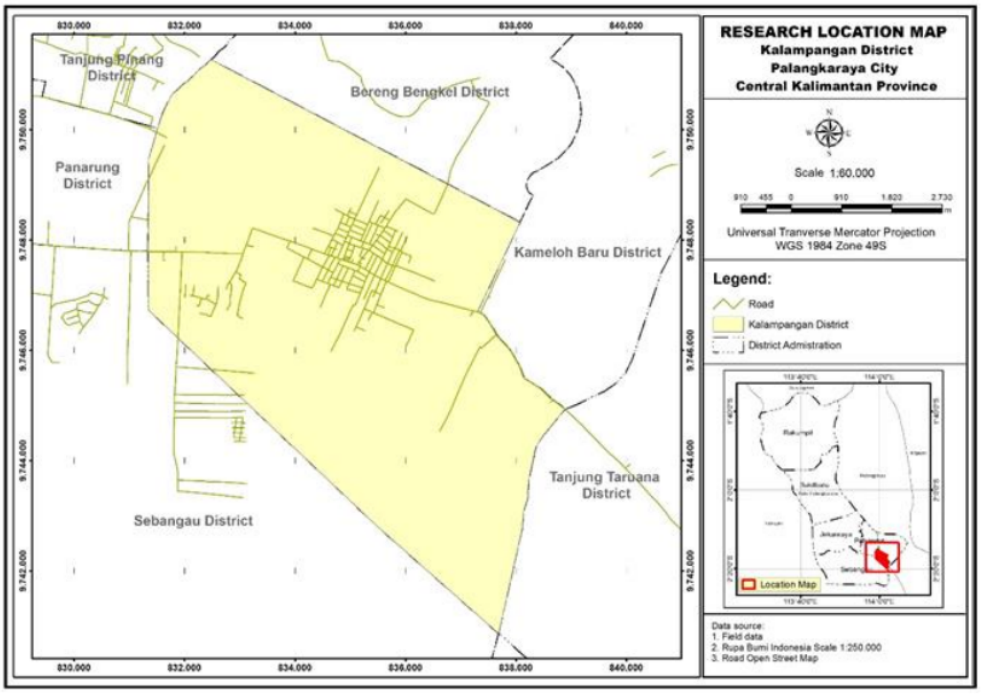

Fig. 1. The location of the corn plant rhizosphere sampling in Kalampangan Village, Sabangau District, Palangka Raya City.

Preparation and propagation of rhizobacteria isolates

The media used for the isolation of bacterial propagation were NA (nutrient agar), TSA and Kings'B Method (Sutariati *et al.*, 2016). Stages of isolation from the soil with graded dilutions up to 10^{-6}. Isolation is done by taking 0.1 mL of samples from dilutions 10^{-4}, 10^{-5}, and 10^{-6} then each of them is dripped on flattened media; the media used are NA, TSA and Kings'B which had been prepared in different (Ø 9cm) Petri dishes and then incubated for 24 hours. The results of bacterial isolation from the corn plant rhizosphere, purified by the line method (strict) (Sutariati *et al.*, 2014; Dewi and Advinda 2022; Sutariati *et al.*, 2016).

Testing the resistance of PGPR to the acidity of peat soil

Acidity resistance test, bacterial isolates were inoculated on NB media which had previously been added with citrate buffer so that it had a pH of 3, 4, and 5. Observations were made by observing acid-resistant bacteria, which will grow and cause the media to become cloudy (Kurnia, 2016). Changes in the turbidity of the media were not very visible, so bacteria were grown using the spread plate method on NA media, and observations were made after 24 hours.

Testing the ability to inhibit pathogens in vitro

Testing the inhibition of rhizobacteria as biocontrol

agents (antagonist test) against the fungus *Fusarium spp*. using the antagonist test on PDA media. Incubation was carried out for 1 week. Observations are seen from the percentage of inhibition of rhizosphere bacteria using the formula according to Wu *et al.* (2019).

$$\text{Percentage of inhibition} = \frac{r1 - r2}{r1} \times 100\%$$

Remarks

r_1: The radius of the pathogenic colony away from the antagonist

r_2: The radius of the pathogenic colony that approaches the antagonist

Phosphate solvent test

Testing the ability of bacteria to dissolve phosphate using Pikovskaya's agar test media with the addition of a phosphate source, namely tricalcium phosphate (TCP). The composition of the media used per liter consisted of glucose (10g), NaCl (0.2g), KCl (0.2g), $MgSO_4$ (0.1g), $MnSO_4$ (2.5mg), $FeSO_4$ (2,5mg), yeast extract (0.5g), $(NH_4)_2SO_4$ (0.5g), and agar (15g).

The media was sterilized by heating using an autoclave and after sterilization the pH of the media was adjusted to 7.2 with 5 N KOH. The test medium was poured into a petri dish (ф 9cm). Inoculation of the isolates with sterile toothpicks by dipping them in the suspension and placing them in the test medium was then incubated for 3 days. Qualitative and quantitative observations based on the calculation of the solubility index P, formed a halo zone around the point containing the bacterial suspension (Sutariati *et al*., 2014; Cui *et al.*, 2020).

Calculation of Phosphate Solubility Index (PSI) = $\frac{dk + dzb}{dk}$

Remarks

dk = Colony diameter.

dzb = Diameter of clear zone.

PSI measurements are carried out every 48 hours to 144 hours.

Nitrogen-fixing test

Testing the ability to fix N using media consisting of $MgSO_47H_2O$ (25 g), $FeSO_4$ $7H_2O$ (0.01g), $NaMoO_4$ $2H_2O$ (0.01g), $MnSO_45H2O$ (0.01 g), $CaCl_2$ (0.1 g), K_2HPO4 (5mL) and Agar (17 g-20 g). Pure isolates aged 48 hours were used in the scratch/strike method on the media, and incubated at room temperature for 4 days, then observed the growth of bacteria that grew. The indicator of isolates capable of fixing N was characterized by a change in the turbidity of the media (Sutariati *et al*., 2014).

HCN testing

The isolates were grown on glycine media in a Petri dish to the measurement of HCN production qualitatively. The lid of the petri dish is attached to a piece of filter paper that has been soaked in an indicator solution for detecting HCN compounds, namely 8g of sodium carbonate and 2g of picric acid, in 200 ml of distilled water. Bacterial isolates were scratched on the media and incubated for 5 days.

The color change on the filter paper from yellow to light brown, and dark brown indicates the presence of HCN production by bacteria (Chauhan *et al*., 2015; Kumar, Maurya, and Raghuwanshi, 2014).

Results and discussion

PGPR insulation is resistant to soil acidity

The results of the exploration and isolation of bacteria from the rhizosphere of corn plants on peat soil were screened for resistance to soil acidity at pH levels 3, 4, and 5 (Fig. 2) to obtain 17 isolates of rhizobacteria in Table 1.

The results obtained are *Bacillus spp, Pseudomonas spp, Pantoea stewartii, staphylococci,* and *Burkhoderia cepacia*.

Bacillus sp, including *B. subtilis*, is gram-positive bacteria that can dissolve phosphate. The gram-positive bacteria are more resistant and can adapt to soil acidity. Gram-positive bacteria have thicker peptidoglycan, making them able to survive in low pH (acidic) areas.

Table 1. Identification and characterization of rhizobacteria isolates.

Isolation Code	gram	Form	Color	Edge	Texture	Optical properties (translucent / opaque)	Size	Type	Physiological properties of fluorence
TB 1.1	+	Round	Yellow	Flat	Slippery	Not	5 mm	*Staphylococcus sp*	-
TB 1.2	+	Round	White	Flat	Slippery	Not	4 mm	*Bacillus sp*	yes
TB 1.3	+	Round	White	Uneven	Slippery	Not	3 mm	*Bacillus sp*	Not
TB 2.1	-	Round	White	Flat	Slippery	Not	2 mm	*Staphylococcus sp*	yes
TB 4.1	+	Round	White	Flat	Slippery	Not	1 mm	*Bacillus sp*	yes
TB 4.2	+	Notin order	White	Uneven	Slippery	Not	7 mm	*Bacillus sp*	yes
TB 4.3	+	Round	White	Flat	Slippery	Not	2 mm	*Pantoea stewartii*	yes
TB 4.5	+	Round	Yellow	Flat	Slippery	Not	2 mm	*Bacillus sp*	yes
TB 4.7	-	Round	Yellow	Flat	Slippery	Not	2 mm	*Burkholderia cepacia*	Not
KB 4.1	-	Round	Yellow	Flat	Slippery	see through	3 mm	*Pseudomonas sp*	yes
KB 4.2	+	Round	White	Flat	Slippery	Not	1 mm	*Pseudomonas sp*	yes
KB 4.3	-	Round	Yellow	Flat	Slippery	Not	3 mm	*Pesudomonas luteola*	yes
TSA 2.1	-	Round	White	Flat	Slippery	Not	4 mm	*Bacillus cereus*	Not
TSA 2.2	+	Round	White	Flat	Slippery	Not	6 mm	*Bacillus subtilis*	Not
TSA 3.1	+	Round	Yellow	Flat	Slippery	Not	6 mm	*Bacillus sp*	Not
TSA 4.1	+	Round	White	Flat	Slippery	Not	6 mm	*Bacillus sp*	Not
TSA 4.2	+	Round	White	Flat	Slippery	Not	5 mm	*Brevibacillus laterosporus*	Not

Note: + = exists, - = does not exist.

Table 2. Qualitative ability of bacteria.

Isolate	Antagonist test	Solvent P	N fixation	HCN	pH3	pH 4	pH5
TB 1.1	-	+	+	-	+	+	+
TB 1.2	-	+	+	-	+	+	+
TB 1.3	-	+	+	+	+	+	+
TB 2.1	-	+	-	+	+	+	+
TB 4.1	-	+	+	-	+	+	+
TB 4.2	-	+	-	+	+	+	+
TB 4.3	+	+	+	+	+	+	+
TB 4.5	-	+	+	-	+	+	+
TB 4.7	+	+	+	+++	+	+	+
KB 4.1.	-	+	+	-	+	+	+
KB 4.2	-	-	+	-	+	+	+
KB 4.3	+	+	-	-	+	+	+
TSA 2.1	+	-	+	-	+	+	+
TSA 2.2	+	-	+	-	+	+	+
TSA 3.1	-	+	-	-	+	+	+
TSA 4.1	-	+	-	-	+	+	+
TSA 4.2	+	-	+	+	+	+	+

Note: + = exists, - = does not exist.

These bacteria can stimulate plant growth and development and can act as bioprotectants because they produce antibiotics that can control several pathogens, including Fusarium. Easily applied in liquid or solid form because this type of bacteria is resistant to extreme conditions such as temperature, pH, mechanical.

Morphological characteristics of rhizobacterial isolates

Microscopic observations for identification and characteristics of PGPR isolates on shape, gram reaction, and bacterial colony morphology from corn plant rhizosphere showed 16 isolates with round shape, 1 isolate with irregular, smooth texture.

Isolates with flat edges 15 isolates and 2 isolates uneven. The colors of 11 isolates were white, and 6 were light yellow (cream), and the single colony size ranged from 1 mm to 7 mm. The isolates were gram-positive 10 isolates and 7 isolates were gram-negative in Table 1.

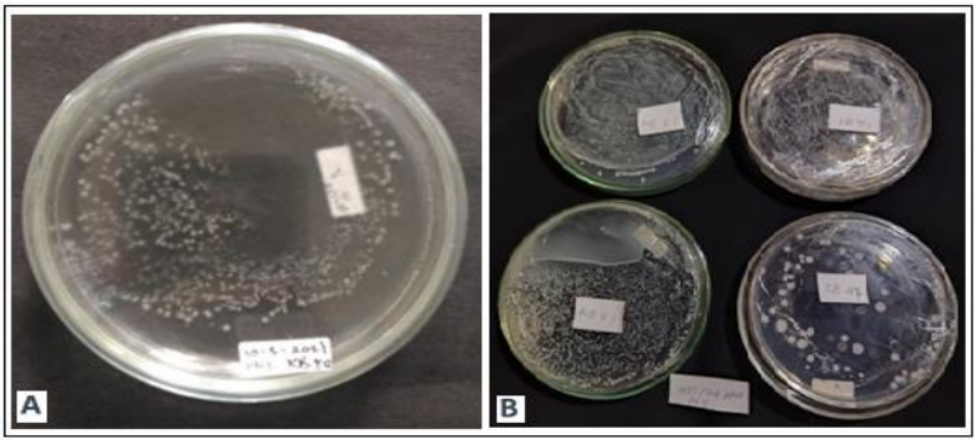

Fig. 2. Acid resistance test of isolates: (A) Isolate in pH 3; (B) Isolate in pH 4 and pH 5.

Ability as a bacterial antagonist (bioprotectant agent)

There were 17 isolates of acid-resistant bacteria obtained, then the antagonist test with the pathogen *Fusarium spp*. The characteristics of *Fusarium spp*. which have the potential to cause stem rot in corn plants, are shown in Fig. 3. Fusarium fungus is a fungus that attacks many safe species and plant parts and spreads to many countries (Wu *et al*., 2019; Bhatt *et al*., 2022; X. Wu *et al*., 2022).

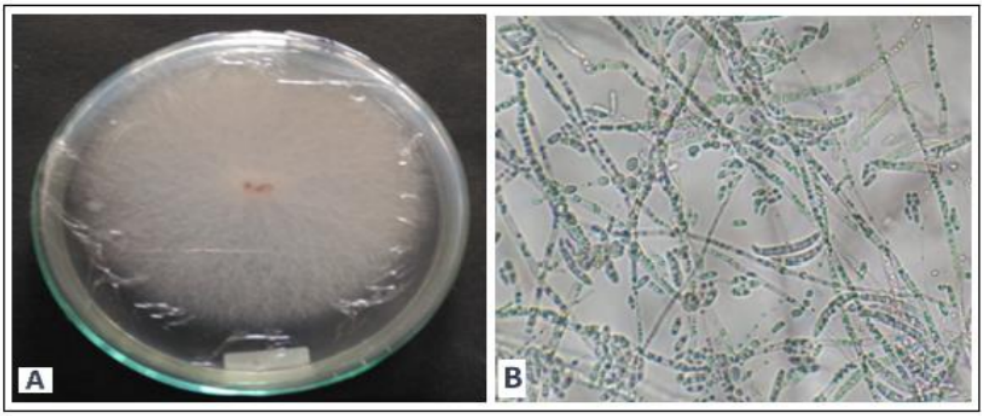

Fig. 3. The *Fusarium graminearum* causes stem rot disease; A. growth observed after 5 on PDA media; B. Development of *Fusarium sp* after 3 days on cube media.

Antagonistic test results of 17 bacterial isolates with the Fusarium pathogen obtained 5 isolates that have the potential to be antagonistic bacteria against stem rot disease, TB 4.7, KB 4.5, TSA 2.2, TSA 2.4, in Table 2 and Fig. 4. of the 5 isolates of rhizobacteria there are rhizobacteria. Bacteria as biofertilizer as well as phosphate solvent, there were 2 isolates is *Burkholderia cepacia* (TB.4.7.) and *Brevibacillus laterosporus* (TSA 4.2) (Shahzad *et al*., 2013).

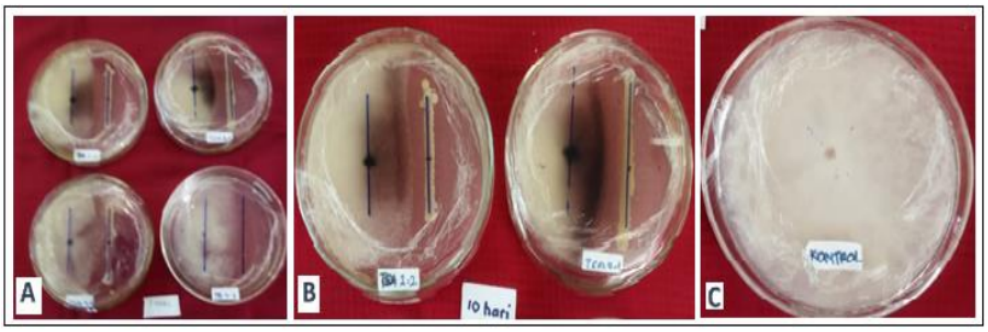

Fig. 4. Rhizobacteria antagonist test with *Fusarium sp*: A. Observation 7 days after inoculation (DAI); B. Observation 10 DAI; and C. Control (7 DAI).

There were five isolates that have the potential as biocontrol agents, rhizobacteria antagonists, able to withstand the development of Fusarium pathogens. *B. subtilis* isolates can withstand the development of *Fusarium spp* disease by 66.67%, *B. cereus* by 59.57%, *P. luteola* 44.23.%, *Burkholderia cepacia* 12.78% in Fig.7. Graph of quantitative antagonist test values.

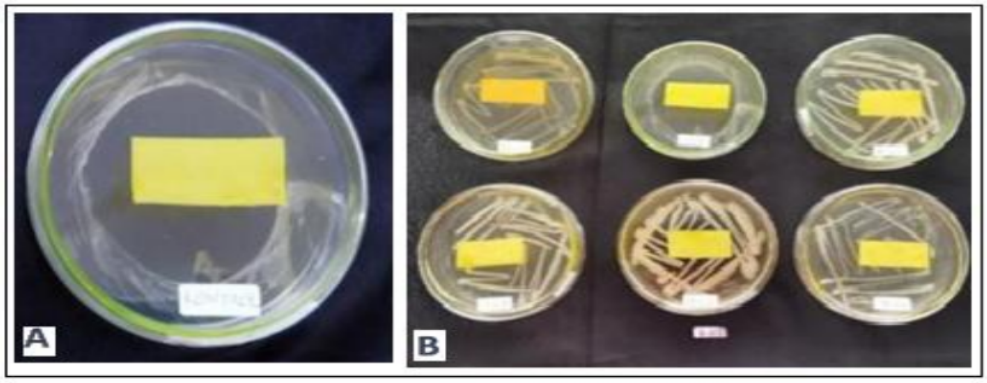

Fig. 5. HCN test on rhizobacteria, A. Control, B. Isolate producing HCN.

Bacillus subtilis and *B. cereus* can be used as biocontrol agents because they can suppress the development of stem rot disease, rotting of vegetables (S. Chen *et al.*, 2017; Y. Chen *et al.*, 2013; Savini, 2016). *Pseudomonas sp* is able to suppress the development of soil pathogens (David *et al.*, 2018). Bacteria that have the potential to fixation N are *Bukhoderia spp*, so they can be included in the PGPR criteria (Chauhan *et al.*, 2015). PGPR can produce one or more mechanisms, for example, biological control through competition, production of antibiotics or siderophores, induction of plant resistance, production of phytohormones, and increased availability of nutrients through tethering of N fixation and solubility of organic and inorganic phosphates (Glick, 2014). Bacillus was able to produce 12 main antibiotics including bacillomicin, bacilysin, etc., while *Pseudomonas spp* only produced 6 antibiotics (Al-Ajlani *et al.*, 2007).

HCN production capability

There were 6 isolates that produced HCN. Bacteria *Burkholderia cepacia* (isolate TB.4.7) showed orange indicator paper, meaning that the rhizobacteria produced the strongest HCN (Fig. 5). Isolate TB1.3, TB 2.1, TB 4.2, TB 4.3 and TSA4.2 produced HCN but were weak, slightly dark yellow from the control. The

ability of bacterial isolates to produce HCN was also determined by the availability of Fe. HCN compounds are produced by *Pseudomonas sp*. One of the secondary metabolites that are antimicrobial (Chauhan *et al.*, 2015; Kumar, Maurya, and Raghuwanshi, 2014; Dewi and Advinda, 2022).

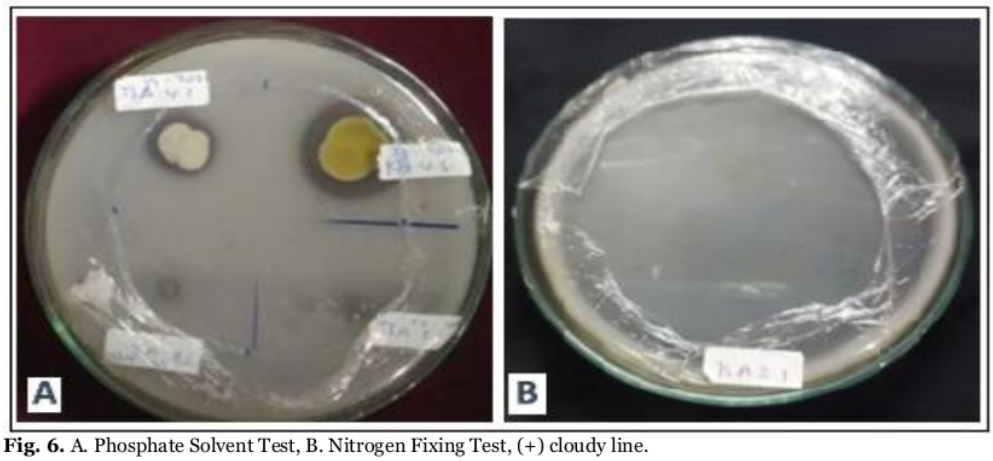

Fig. 6. A. Phosphate Solvent Test, B. Nitrogen Fixing Test, (+) cloudy line.

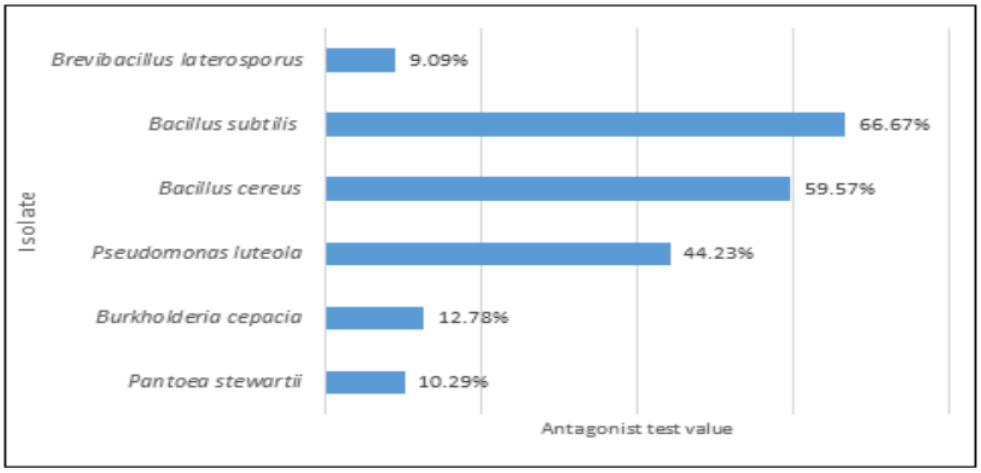

Fig. 7. Graph of quantitative antagonist test values.

Testing of phosphate solvent and nitrogen-fixing bacteria as biofertilizer agents

Exploration results obtained 13 isolates of phosphate solubilizing bacteria, in Fig. 6. Phosphate solvent biofertilizer is *Burkholderia stewartii* (isolate TB4.7.) and *Brevibacillus laterosporus* (TSA4.2). The results of the exploration obtained 11 isolates of N-fixing bacteria. Bacteria with the potential for N-fixing were *Bukhoderia sp*. There were 11 nitrogen-fixing isolates in Table 2, isolates TB 1.1, TB 1.3, TB 1.3., TB. 4.1, TB. 4.3, KB. 4.1, KB.4.2, TSA 2.1, TSA 2.2, TSA 4.2.). In Fig. 8. Graph of Phosphate solubility index value test according to Glick (1995), PGPR can produce one or more mechanisms, namely as antagonist agents, production of antibiotics or siderophores, induction of plant resistance, production of phytohormones, and increased availability of nutrients through tethering of N-fixation and solubility of organic and inorganic phosphates. There are several types of bacteria that can dissolve inorganic P, such as *B.*

thuringiensis and *Pantoea ananatis* as a source of P; these bacteria can dissolve inorganic P, thereby increasing the dissolved P concentration. This type of *P. luteola* has the ability as a phosphate solvent and increases plant growth (Glick, 2014; Liu *et al*., 2021). Bulkholderia species have the ability to compete and thrive in acid areas compared to alkaline areas, act as antifungals (antagonistic bacteria), can stimulate plant growth (Santi and Goenadi 2013; Chauhan *et al*., 2015; Tabassum *et al*., 2017; Kong *et al*., 2012; Santi, Goenadi, and Darmosarkoro, 2014).

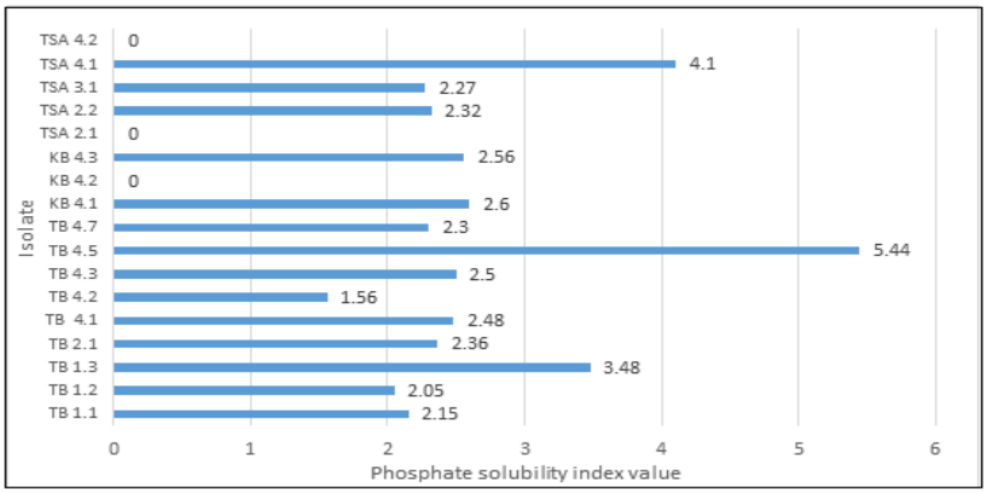

Fig. 8. Graph of Phosphate solubility index value test.

Bacteria *Pantoea sp* can fixation N, *Bulkhoderia sp* (isolate TB 4.7) and *Pseudomonas luteola* (KB 4.3) did not fixation N. *Pseudomonas sp* (KB 4.1 and KB 4.2) fixation N and *Bacillus sp* was positive for N-fixer.

Conclusion

There were 17 isolated from rhizobacteria resistant to soil acidity pH 3, 4, and 5. There were 6 isolates of indigenous PGPR that were bioprotectants from the rhizosphere of corn plants on peat soil, 6 isolates were obtained that had the potential as bioprotectants, *Pantoea stewartii, Burkholderia cepacia. Pseudomonas luteola, Bacillus cereus, Bacillus subtilis* and *Brevibacillus laterosporus*. PGPR isolates which have two functions as bioprotectant and biofertilizer (solvent P), are isolates of *Burkholderia cepacia*, and *Brevibacillus laterosporus*.

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