Gmail

**endang mugiastuti <endangmugiastuti@gmail.com>**

# [biodiv] Submission Acknowledgement

1 message

**Ahmad Dwi Setyawan** <smujo.id@gmail.com> Thu, Nov 14, 2019 at 12:06 AM
Reply-To: Ahmad Dwi Setyawan <editors@smujo.id>
To: endang mugiastuti <endangmugiastuti@gmail.com>

endang mugiastuti:

Thank you for submitting the manuscript, "Isolation and Characterization of The Endophytic Bacteria, And Their Potential As Maize Diseases Control" to Biodiversitas Journal of Biological Diversity. With the online journal management system that we are using, you will be able to track its progress through the editorial process by logging in to the journal web site:

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/4844
Username: endang_mugi

If you have any questions, please contact me. Thank you for considering this journal as a venue for your work.

Ahmad Dwi Setyawan

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Biodiversitas Journal of Biological Diversity

Notifications

# [biodiv] Editor Decision

2020-01-10 07:29 AM

endang mugiastuti:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control".

Our decision is: Revisions Required

Smujo Editors
editors@smujo.id

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Biodiversitas Journal of Biological Diversity

Notifications

# [biodiv] Editor Decision

2020-03-11 12:14 AM

endang mugiastuti:

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control".

Our decision is: Revisions Required

Smujo Editors
editors@smujo.id

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Reviewer A:
Recommendation: See Comments

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Biodiversitas Journal of Biological Diversity

Notifications

# [biodiv] Editor Decision

2020-04-07 05:40 AM

ENDANG MUGIASTUTI, SUPRAYOGI, NUR PRIHATININGSIH, LOEKAS SOESANTO :

We have reached a decision regarding your submission to Biodiversitas Journal of Biological Diversity, "Short Communication: Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control".

Our decision is to: Accept Submission

Smujo Editors
editors@smujo.id

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Biodiversitas Journal of Biological Diversity

Notifications

# [biodiv] Editor Decision

2020-04-07 05:54 AM

ENDANG MUGIASTUTI, SUPRAYOGI, NUR PRIHATININGSIH, LOEKAS SOESANTO :

The editing of your submission, "Short Communication: Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control," is complete. We are now sending it to production.

Submission URL: https://smujo.id/biodiv/authorDashboard/submission/4844

Smujo Editors
editors@smujo.id

________________________________________________________________________
Biodiversitas Journal of Biological Diversity

Naskah Submit

## COVERING LETTER

Dear **Editor-in-Chief,**

I herewith enclosed a research article,

**Title:**

Isolation and Characterization of The Endophytic Bacteria, And Their Potential As Maize Diseases Control

**Author(s) name:**

Endang Mugiastuti, Suprayogi, Nur Prihatiningsih and Loekas Soesanto

**Address**

(Fill in your institution's name and address, your personal cellular phone and email)

Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, HP. 08122826706,
email: endangmugiastuti@gmail.com

**For possibility publication on the journal:**

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*Biodiversitas*

**Novelty:**

(state your claimed novelty of the findings versus current knowledge)

Research to get biological control agents that can control 2 main diseases of maize. Biological agents are obtained from 2 altitudes, so it is expected that biological agents can be applied in various maize growing areas

**Statements:**

This manuscript has not been published and is not under consideration for publication to any other journal or any other type of publication (including web hosting) either by me or any of my co-authors.
Author(s) has been read and agree to the Ethical Guidelines.

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(Fill in names of five potential reviewers **that agree to review your manuscpt** and their **email** addresses. He/she should have Scopus ID and come from different institution with the authors; and from at least three different countries)

**Place and date:**

Purwokerto, august 2019

**Sincerely yours,**

(fill in your name, no need scanned autograph)

Endang Mugiastuti

# ISOLATION AND CHARACTERIZATION OF THE ENDOPHYTIC BACTERIA, AND THEIR POTENTIAL AS MAIZE DISEASES CONTROL

**ENDANG MUGIASTUTI[1♥], SUPRAYOGI[1], NUR PRIHATININGSIH[1] AND LOEKAS SOESANTO[1]**
Faculty of Agriculture, Jenderal Soedirman University, Purwokerto. Jl. Dr. Suparno, Karangwangkal, Purwokerto 53123, Central Java, Indonesia.
Tel./Fax. +62-281-638791, ♥email: endangmugiastuti@gmail.com.

Manuscript received: 1411 2019 (Date of abstract/manuscript submission). Revision accepted: .................... 20.

**Abstract.** The research aimed to isolate and characterize morphologically and biochemically the endophytic bacteria, and their potential to control maize diseases, especially sheat blight and bacterial wilt. The study was conducted at the Plant Protection Laboratory from April to August 2019. The study consisted of four stages: isolation and characterization of endophytic bacteria, the antagonism test of the endophytic bacterial to *R solani*, the antagonism test of the endophytic bacteria to *Pantoea* sp., and the mechanism test of the endophytic bacteria as biological control agents and plant growth-promoting bacteria. Based on the research, it has been successfully isolated, and characterized morphologically and biochemically characterized four endophytic bacteria isolates that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

**Key words:** *Bacillus* sp., Fluorescents Pseudomonads, *Pantoea* sp, *Rhizoctoni solani*

**Running title:** Isolation and characterization of the endophytic

## INTRODUCTION

Maize is a strategic food commodity in the world. In Indonesia, the government seeks to achieve self-sufficiency in maize through increasing production of sustainable maize. However, these efforts have faced several obstacles, one of them is the presence of plant diseases such as sheat blight, caused by *Rhizoctonia solani* Kuhn, and bacterial wilt caused by (*Pantoea* sp.). *R. solani* can infect up to the midrib of the cob (Djaenuddin et al. 2017), resulting in a decrease in the yield of up to 100%. (Muis 2007). *Pantoea* sp. can attack all stages of the plant causing wilting and leaf blight, and is known as Stewart's wilt (Pataky 2004; Ammar et al. 2014). The pathogens can cause 40-100% yield loss.

Over the past 3 decades, the concept of sustainable and environmentally friendly agriculture have been carried out by minimizing the use of chemicals, both synthetic fertilizers and synthetic pesticides. In the management of pests and plant diseases, biological control is developed by applying biological control agents including the endophytic bacteria (Shanti and Vittal 2013). Many endophytic bacteria can pass the endodermic barrier across from the root cortex to the vascular system, and subsequently develop as endophytes in stems, leaves, tubers, and other organs (Compant et al. 2005). The use of endophytic bacteria as biological agents has an advantage compared to rhizosphere bacteria because endophytic bacteria live and survive in the plant tissue during plant development, thus protecting the plants.

*Bacillus* sp. and fluorescents *Pseudomonads* are reported to be able to live as endophytes and are widely used as biological control agents for soil-borne and air-borne diseases. The endophytic bacteria could control plant diseases through several mechanisms including competition, hyperparasite, producing microbial inhibiting compounds (antibiotics, lysis enzymes, other physical or chemical disorders), enhancing plant resistance, and promoting plant growth (Compant et al 2005, Pal and McSpadden 2006; Rosenblueth and Martinez Romero 2006;).

Based on the mechanisms, the use of endophytic bacteria isolated from maize, both upland and lowland, suggested potentially alternative control for sheath blight (*R. solani*) and bacterial wilt (*Pantoea* sp). The research aimed to isolate and characterize morphologically and biochemically the endophytic bacterial as well as their potential to control pathogens that cause disease in maize especially *R. solani* and *Pantoea* sp.

## MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Protection, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia, from April to August 2019

**Isolation *R. solani***

*R. solani* was isolated from maize with sheath blight symptoms and there was sclerotium as a resistant structure from the pathogenic fungi in Banyumas. Samples were isolated on PDA medium to obtain pure *R. solani* isolates.

**Isolation *Pantoea* sp.**

*Pantoea* sp. isolated from diseased maize, which was taken from the maize growing area in Banyumas Regency. *Pantoea* sp. was isolated according to Coplin et al. 2012; Aini et al. 2013and Desi et al. 2014. Diseased leaves or stems were washed with running water, then dried with a tissue. Diseased samples were cut 1.5 x 5 cm, then sterilized with ethanol 70% and rinsed with sterile water 3 times. Furthermore, the sample was crushed with 5 ml of sterile distilled water using a sterile mortar. The bacterial suspension was streaked on nutrient agar and incubated 3-5 days. Bacterial colonies that exhibit the character of *Pantoea* sp. are yellow, shiny, slimy, flat or convex, then separated as pure cultures of. stewartii candidates. The culture was then tested by Gram Reaction (KOH test), Hugh-Leifson test, pigment production in YDC medium, oxidase test, hypersensitivity test, and pathogenicity test on maize.

**Isolation and characterization of endophytic bacteria**

Sampling for the isolation of endophytic bacteria was carried out in Banyumas and Purbalingga, Central Java, Indonesia, with purposive stratified random sampling. Samples were taken from two areas of altitude, i.e., low-moderate lands (0-500 m above sea level), and highlands (> 500 m above sea level) (Nuryanto et al., 2014). In each district, 2 locations were selected for the low-medium lands, and 1 location for the highlands. Age of maize plants was 20-30 days after planting, when the number of endophytic microbial populations that can be cultured is in the highest population (Cavaglieri et al. 2009).

The endophytic bacteria are isolated from the roots and stems of healthy maize plants. Roots and stems are washed, sterilized with 70% ethanol (1 minute), 20% natrium hypochlorite (5 minutes) and Ringer's thiosulfate solution (5 minutes). Separately, the roots and stems of 10 g each were crushed with 90 ml PBS on a sterile mortar. Subsequently, samples were plated on NA and Kings B media (Cavaglieri et al. 2009). To isolate *Bacillus* sp., the suspension is heated for 10 minutes at 80 ° C, before plating on NA. Bacterial isolates were further purified and characterized, such as morphological characteristics, gram properties, catalase tests, and hypersensitivity tests

**The antagonism test of endophytic bacterial to *R solani***

The antagonism test of endophytic bacteria on *R. solani* was carried out using the dual culture method. The level of inhibition of antagonist is calculated using the formula (Abidin et al., 2015).

$$I = \frac{C - T}{C} \times 100\%$$

I = The level of inhibition of antagonist (%)
C = The radius of pathogen colonies opposite antagonist
T = The radius of the colony of pathogens towards antagonist

**The antagonism test of endophytic bacterial to bacterial pathogens**

Antagonism testing was carried out using the double-layer test method (Santiago et al. 2015). Endophytic bacteria to be tested were grown on the NA medium, incubated at 28 C for 48 hours. In the upside-down position, 1 ml of chloroform was added to the cup lid and left for 2 hours. Next, add 5 mL so that 0.6% water containing 0.5 mL of P. stewartii bacterial suspension. The culture was re-incubated for 24 hours, and there were clear zones around the antagonistic bacterial colony. The antibiotic activity was assessed based on the diameter of the clear zone compared to the diameter of the colony. Characterization of the type of antibiosis can be divided into bactericidal and bacteriostatic types according to the method of Djatmiko et al. 2017.

The mechanism test of endophytic bacteria as controlling agents biological and plant growth-promoting microbial

The testing mechanism of endophytic bacteria is carried out for bacteria that have the potential in testing the antagonism of the fungus *R. solani* and *Pantoea* sp.

1). Protease Test

The activity of the ability of antagonistic bacteria to produce extracellular protease enzymes was tested using Skim Milk Agar (SMA) medium. Each bacterium to be tested was grown in a medium SMA and incubated at 28 C for 24-48 hours. The presence of clear zones around the colony shows that positive bacteria produce protease enzymes (Abed et al. 2016). The protease activity index is assessed based on the diameter of the clear zone compared to the diameter of the colony..

$$\text{Protease index} = \frac{(\text{clear zone diameter} - \text{colony diameter})}{\text{colony diameter}}$$

2.) Lipase test

Detection of the ability of bacteria to produce the enzyme lipase was done by growing the antagonistic bacteria on a medium containing 1% Tween 80. The presence of lipase enzyme activity was demonstrated by milky white sediment around the bacterial colony, after incubating at 28 C for 4-7 days. The lipolytic index was measured using a formula Djuric et al. (201).

$$\text{Lipolytic index} = \frac{(\text{milky white diameter} - \text{colony diameter})}{\text{colony diameter}}$$

3.) Uji fosfatase

Detection of the ability of bacteria to produce the enzyme phosphatase was done by growing bacterial isolates on Pikovkaya medium. After incubating for 7 days at 28 C, the presence of a clear zone around the bacterial colony shows that the bacteria has the ability to produce the phosphatase enzyme to dissolve phosphates. The solubility index is measured using a formula (Farooq and Bano 2013)

$$\text{Phosphatase Index} = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diamater}}$$

## RESULTS AND DISCUSSION

**Isolation and characterization of endophytic bacteria**

The results of the exploration, isolation, and characterization of endophytic bacteria obtained 23 isolates of endophytic bacteria, consisted of 9 isolates of the *fluorescent Pseudomonads* and 14 isolates of *Bacillus* sp. (Table 1). *Fluorescent Pseudomonads* colony on King's B is round, with a flat edge, and yellowish-white, to greenish-yellow, gram-negative, rod-shaped, non-spore and fluorescent under ultraviolet light. According to Arwiyanto et al. 2007), *P. fluorescens* have round, flat-edged, fluidal and release greenish-yellow colony in the King's B. Individual rod-shaped bacteria with a size (0.5-1.0) - (1.5-4.0) µm. The P. fluorescens isolates is gram-negative, which can form catalase, a positive oxidase, needed to grow aerobes.

*Bacillus* sp. has a spherical colony, cell rod-shaped, gram-positive, and endospores within cells. *Bacillus* sp. has the characteristics of a circular colony and punctiform (small round), variations in the entire margin and lobate, white dull, non-slimy, gram-positive, has endospores, flagellum and some are motile.

Based on its distribution, *fluorescent Pseudomonads* and *Bacillus* sp. found in all sampling locations, high or low-medium lands. This shows that *fluorescent Pseudomonads* and *Bacillus* sp spread and can live in various altitudes, both high and low-medium land. According to Bacon and Hilton 2002 and Ganeshan and Kumar 2005, *P. fluoresscens* and *Bacillus* sp, are species of bacteria with a wide range of life and are very adaptive in various environments. Both types of bacteria are also found in the roots or corn stalks. According to Ganeshan and Kumar 2005; Orole and Adejumo 2011; Costa et al. 2013, *Bacillus* sp, and *Pseudomonas* sp. including a group of endophytic bacteria have a wide range of life and more isolated in maize.

**Antagonism test between the endophytic bacteria against *R. solani***

Based on the results of in vitro tests (Table 2), 24 isolates of the endophytic bacteria were able to inhibit the growth of *R. solani*, with varying degrees of inhibition. The endophytic bacteria that have inhibition rates above 50%, i.e *Pseudomonas* Pf BK.A1 (51%), *Bacillus* sp. B.K.A1 (55.39%), *Bacillus* sp BK.A3 (51.52%), PP.A5 (50.66%), and PPD.B2 ( 50.8%). The effect of the endophytic bacteria in inhibiting the growth *of R. solani* is inversely proportional to the dry weight mycelium. The greater the percentage of inhibition of endophytic bacteria to the growth of *R. solani*, the smaller the dry weight mycelium (Table 2.)

The endophytic bacteria can inhibit the growth of R solani shown by the inhibition zone around the bacterial colony (Fig. 1). The endophytic bacteria have anti-pathogenic properties and can produce antibiotic compounds. The ability of the endophytic bacteria to control plant pathogens occurs through the mechanism of antibiosis, competition, lysis, inducing resistance and producing growth substances. Bacteria capable of producing secondary metabolites that can inhibit growth or damage pathogens (Hastuti et al. 2014). These compounds, including alkaloids, phenols, flavonoids, glycosides, and phytoalexin (Soesanto et al. 2010).

Fig.1. Antagonism test between the endophytic bacteria against *R. solani* (a) and *Pantoea* sp..

**Table 1. Isolation and characterization of endophytic bacteria**

| Land | Sampling location | sample | Gram test | Catalase test | oxidase test | Colony morphology | colony pigment | Fluorescence on KB Medium | Cell morphology | Endo spores | Isolat |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Highland | 1. Purbalingga, Pratin 7.13'33" LS, 109.17'21" BT, TT 1.190 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PP.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PP.A3 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. PP.A5 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PP. B4 |
| | 2.Banyumas, Baturaden 7.19"1" LS, 109.14'29" BT, TT 520 m dpl | Root | - | + | + | round | Greenish yellow | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BB.A2 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BB.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB.B4 |
| Medium-Lowland | 1.Banyumas, Sumbang7.21'54" LS, 109.17'33"BT, TT 200 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BS.A2 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BS.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS.A3 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS. B1 |
| | 2. Purbalingga, Bojongsari, 7.20'12" LS, 109.20'22" BT, TT 190 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | *Fluorescent Pseudomonads (Pf)* PB. A 4 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PB. B1 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB. B3 |
| | 3.Purbalingga, Padamara, 7.22'28" LS, 109.13'24" BT, TT 180 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | *Fluorescent Pseudomonads (Pf)* PPD A1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PPD. B1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PPD. B5 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B2 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B4 |
| | 4. Banyumas, Kembaran 7.23'47" LS, 109.17'9" BT, TT 110 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BK. A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.B3 |

**Table 2. Inhibition of endophytic bacteria against *R. solani***

| No | Isolate | Inhibition rate (%) | Dry weights Mycelium |
|---|---|---|---|
| 1 | Control | 0 | 0,093 |
| Endophytic bacteria from the root | | | |
| 2 | *Pseudomonas* Pf BB.A2 | 49,00 | 0,038 |
| 3 | *Pseudomonas* Pf BS.A 2 | 45,00 | 0,027 |
| 4 | *Pseudomonas* Pf BK.A1 | 51,00 | 0,017 |
| 5 | *Pseudomonas* Pf PPD.A1 | 10,33 | 0,059 |
| 6 | *Pseudomonas* Pf PP.A1 | 38,33 | 0,017 |
| 7 | *Pseudomonas* Pf PB.A4 | 18,00 | 0,037 |
| 8 | *Bacillus* sp.BB.A3 | 40,42 | 0,030 |
| 9 | *Bacillus* sp.BS.A1 | 48,73 | 0,016 |
| 10 | *Bacillus* sp. BSA3 | 37,42 | 0,039 |
| 11 | *Bacillus* sp. B.K.A1 | 55,39 | 0,002 |
| 12 | *Bacillus* sp. B.K.A3 | 51,52 | 0,003 |
| 13 | *Bacillus* sp.PP.A3 | 46,65 | 0,019 |
| 14 | *Bacillus* sp.PP.A5 | 50,66 | 0,009 |
| Endophytic bacteria from the stem | | | |
| 15 | *Pseudomonas* Pf PPD.B1 | 27,00 | 0,020 |
| 16 | *Pseudomonas* Pf PPD.B5 | 49,33 | 0,013 |
| 17 | *Pseudomonas* Pf PP.B4 | 65,67 | 0,004 |
| 18 | *Bacillus* sp. BB.B4 | 44,44 | 0,026 |
| 19 | *Bacillus* sp. BS.B1 | 49,74 | 0,012 |
| 20 | *Bacillus* sp. BK. B3 | 40,36 | 0,031 |
| 21 | *Bacillus* sp.PPD.B2 | 50,8 | 0,007 |
| 22 | *Bacillus* sp. PPD.B4 | 39,44 | 0,036 |
| 23 | *Bacillus* sp. PB.B1 | 37,29 | 0,047 |
| 24 | *Bacillus* sp. PB.B3 | 44,9 | 0,022 |

**The endophytic bacterial antagonism test against *Pantoea* sp.**

The results of antagonism between the endophytic bacteria and *Pantoea* sp. show varied results. The endophytic bacteria that can inhibit bacterial growth are indicated by the presence of clear zones around the endophytic bacterial colonies (Fig.1). From the nine isolate *Pseudomonas* sp. were tested, only three isolates were able to inhibit the growth of the *Pantoea* sp., i.e Pf BS.A2, Pf BK.A1 Pf PPD.B5. While the isolates Pf BB.A2, Pf PPD.A1, Pf PP.A1, Pf PPD.B1, and Pf PP.B4 are not able to inhibit the growth of pathogenic bacteria. Meanwhile, all isolate *Bacillus* sp tested (thirteen isolates) were able to inhibit the growth of *Pantoea* sp. (Table 3).

The presence of clear zones around endophytic bacterial colonies shows the ability of endophytic bacteria to produce antibiotics to inhibit the growth of *Pantoea* sp. *P. fluorescens* P60 can produce antibiotics that inhibit the growth of pathogens (Soesanto 2011). *Pseudomonas fluorescens* is reported to produce phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4 diacetyl phloroglucinol (DAPG), pyrrolnitrin (PRN) and or pyoluteorin (Plt) (Heydari and Pessarakli 2010). Nasrun and Burhanudin (2016) mention that *P. fluorescens* produce secondary metabolites, i.e. antimicrobial, cyanide acid and 2,4-diacetyl phloroglucinol phenazine, pyrrolnitrin, pyoluteorin antibiotics

The level of bacteria's ability to inhibit growth can be shown by the large diameter of the clear zone. The results showed that the antagonism index ranged from 1.67 - 8.17. Based on this index, most endophytic bacteria have a strong antagonism (index of antagonism> 4) (Davis and Stout 1971). Furthermore, bacterial isolates that showed antagonistic activity were tested for types of antagonism based on Djatmiko (2007). Based on the type of antagonistic activity, ten isolates the endophytic bacteria were bacteriostatic and nine isolates the endophytic bacteria were bactericidal. Bacteriostatic activity, growth inhibition is temporary, it is shown that regrowth of bacteria after being transferred to a new medium, which is free from the influence of antagonistic bacteria. Bactericidal activity, inhibition is permanent. Bacteria were unable to grow even though they are transferred to new medium

Table 3. Inhibition of endophytic bacteria against *Pantoea* sp.

| No | Isolate | Antagonism | Antagonism index | Antagonism catagory* | Antagonism activity |
|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | |
| 1 | *Pseudomonas* Pf BB.A2 | - | 0 | - | - |
| 2 | *Pseudomonas* Pf BS.A 2 | + | 4,91 | strong | bacteriostatic |
| 3 | *Pseudomonas* Pf BK.A1 | + | 4,42 | strong | bacteriostatic |
| 4 | *Pseudomonas* Pf PPD.A1 | - | 0 | - | - |
| 5 | *Pseudomonas* Pf PP.A1 | - | 0 | - | - |
| 6 | *Pseudomonas* Pf PB.A4 | + | 5,29 | strong | bactericidal |
| 7 | *Bacillus* sp.BB.A3 | + | 8,17 | strong | bacteriostatic |
| 8 | *Bacillus* sp.BS.A1 | + | 4,00 | strong | bacteriostatic |
| 9 | *Bacillus* sp. BSA3 | + | 5,07 | strong | bactericidal |
| 10 | *Bacillus* sp. B.K.A1 | + | 4,01 | strong | bakteriostatik |
| 11 | *Bacillus* sp. B.K.A3 | + | 4,91 | strong | bacteriostatic |
| 12 | *Bacillus* sp.PP.A3 | + | 6,63 | strong | bactericidal |
| 13 | *Bacillus* sp.PP.A5 | + | 6,56 | strong | bactericidal |
| Endophytic bacteria from the stem | | | | | |
| 14 | *Pseudomonas* PPD.B1 | - | 0 | - | - |
| 15 | *Pseudomonas* PPD.B5 | + | 5,86 | strong | bactericidal |
| 16 | *Pseudomonas* PP.B4 | - | 0 | - | - |
| 17 | *Bacillus* sp. BB.B4 | + | 7,80 | strong | bactericidal |
| 18 | *Bacillus* sp. BS.B1 | + | 6,22 | strong | bacteriostatic |
| 19 | *Bacillus* sp. BK. B3 | + | 5,33 | strong | bacteriostatic |
| 20 | *Bacillus* sp.PPD.B2 | + | 5,00 | strong | bacteriostatic |
| 21 | *Bacillus* sp. PPD.B4 | + | 8,75 | strong | bacteriostatic |
| 22 | *Bacillus* sp. PB.B1 | + | 1,67 | weak | bacteriostatic |
| 23 | *Bacillus* sp. PB.B3 | + | 5,67 | strong | bactericidal |

- Based on Davis and Stout, 1971

**Test the mechanism of endophytic bacteria as biological control agents and plant growth-promoting microbes**

The mechanism test is carried out on endophytic bacteria that have the potential to control the fungus *R. solani* and *Pantoea* sp., i.e. *Bacillus* sp. B.K.A1, *Bacillus* sp. B.K.A3, *Bacillus* sp. PP.A5, *Bacillus* sp. PPD.B2. The results of enzyme activity tests are as shown in Table 4. The production of compounds related to biocontrol of pathogens and/or promotion of plant growth in bacterial isolates was evaluated by measuring the production of antimicrobial compounds and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and phosphate solubilization. the isolates that have high protein and fat hydrolysis enzymes have the potential as biological control agents because proteins and fats are constituents of pathogen cells (Mota et al 2016).

The four isolates *Bacillus* sp. tested were able to produce protease, lipase and phosphatase enzymes, with varied activity indexes. All isolates of *Bacillus* sp. those tested had a high index of protease and lipase enzymes (> 3) (Table 4., Fig 2.). Protease and lipase enzymes, related to the ability of the endophytic bacteria as biological control agents. According to Anderson et al. 2014, the extracellular protease enzyme produced by P. fluorescens can inactivate antibiotic compounds produced by *Pantoea agglomerans.* The phosphate solubilization is related to the ability of endophytic bacteria as a plant growth promoter, providing phosphates for plants.

Table 4. Test results of proteases, lipases and phosphate solubilization**.**

| No | Isolate | Protease Test | | Lipase Test | | Phosphate solubilization | |
|---|---|---|---|---|---|---|---|
| | | activity | index | activity | index | activity | index |
| Endophytic bacteria from the root | | | | | | | |
| 1 | *Bacillus* sp. B.K.A1 | + | 3.75 | + | 3.23 | + | 1.17 |
| 2 | *Bacillus* sp. B.K.A3 | + | 3.20 | + | 3.73 | + | 1.27 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 3 | *Bacillus* sp.PP.A5 | + | 5.00 | + | 4.40 | + | 1.46 |
| Endophytic bacteria from the stem | | | | | | | |
| 4 | *Bacillus* sp.PPD.B2 | + | 3.00 | + | 3.90 | + | 2.60 |


Fig. 2. Hydrolysis enzyme activity, (a) protease , (b) lipase and (c) phosphate solubilization.

## CONCLUSION

Based on research carried out, it has been successfully isolated, morphologically and biochemically characterized four the endophytic bacteria that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

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Naskah Koreksi 1

# Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control

**Abstract.** The research aimed to isolate and characterize morphologically and biochemically the endophytic bacteria, and their potential to control maize diseases, especially sheat blight and bacterial wilt. The study was conducted at the Plant Protection Laboratory [LN1]from April to August 2019. The study consisted of four stages: isolation and characterization of endophytic bacteria, the antagonism test of the endophytic bacterial to *R solani,* the antagonism test of the endophytic bacteria to *Pantoea* sp., and the mechanism test of the endophytic bacteria as biological control agents and plant growth-promoting bacteria. Based on the research, it has been successfully isolated, and characterized morphologically and biochemically characterized four endophytic bacteria isolates that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization[LN2].

**Key words:** *Bacillus* sp., Fluorescents Pseudomonads, *Pantoea* sp, *Rhizoctoni solani*

**Running title:** Isolation and characterization of the endophytic

## INTRODUCTION

Maize is a strategic food commodity in the world. In Indonesia, the government seeks to achieve self-sufficiency in maize through increasing production of sustainable maize. However, these efforts have faced several obstacles, one of them is the presence of plant diseases such as sheat blight, caused by *Rhizoctonia solani* Kuhn, and bacterial wilt caused by (*Pantoea* sp.). *R. solani* can infect up to the midrib of the cob (Djaenuddin et al. 2017), resulting in a decrease in the yield of up to 100%. (Muis 2007). *Pantoea* sp. can attack all stages of the plant causing wilting and leaf blight, and is known as Stewart's wilt (Pataky 2004; Ammar et al. 2014). The pathogens can cause 40-100% yield loss.

Over the past 3 decades, the concept of sustainable and environmentally friendly agriculture have been carried out by minimizing the use of chemicals, both synthetic fertilizers and synthetic pesticides. In the management of pests and plant diseases, biological control is developed by applying biological control agents including the endophytic bacteria (Shanti and Vittal 2013). Many endophytic bacteria can pass the endodermic barrier across from the root cortex to the vascular system, and subsequently develop as endophytes in stems, leaves, tubers, and other organs (Compant et al. 2005). The use of endophytic bacteria as biological agents has an advantage compared to rhizosphere bacteria because endophytic bacteria live and survive in the plant tissue during plant development, thus protecting the plants.

*Bacillus* sp. and fluorescents *Pseudomonads* are reported to be able to live as endophytes and are widely used as biological control agents for soil-borne and air-borne diseases. The endophytic bacteria could control plant diseases through several mechanisms including competition, hyperparasite, producing microbial inhibiting compounds (antibiotics, lysis enzymes, other physical or chemical disorders), enhancing plant resistance, and promoting plant growth (Compant et al 2005, Pal and McSpadden 2006; Rosenblueth and Martinez Romero 2006;).

Based on the mechanisms, the use of endophytic bacteria isolated from maize, both upland and lowland, suggested potentially alternative control for sheath blight (*R. solani*) and bacterial wilt (*Pantoea* sp). The research aimed to isolate and characterize morphologically and biochemically the endophytic bacterial as well as their potential to control pathogens that cause disease in maize especially *R. solani* and *Pantoea* sp.

## MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Protection, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia, from April to August 2019

**Isolation *R. solani***

*R. solani* was isolated from maize with sheath blight symptoms and there was sclerotium as a resistant structure from the pathogenic fungi in Banyumas. Samples were isolated on PDA medium to obtain pure *R. solani* isolates.

**Isolation *Pantoea* sp.**

*Pantoea* sp. isolated from diseased maize, which was taken from the maize growing area in Banyumas Regency. *Pantoea* sp. was isolated according to Coplin et al. 2012; Aini et al. 2013 and Desi et al. 2014. Diseased leaves or stems were washed with running water, then dried with a tissue. Diseased samples were cut 1.5 x 5 cm, then sterilized with ethanol 70% and rinsed with sterile water 3 times. Furthermore, the sample was crushed with 5 ml of sterile distilled water using a sterile mortar. The bacterial suspension was streaked on nutrient agar and incubated 3-5 days. Bacterial colonies that exhibit the character of *Pantoea* sp. are yellow, shiny, slimy, flat or convex, then separated as pure cultures of stewartii candidates. The culture was then tested by Gram Reaction (KOH test), Hugh-Leifson test, pigment production in YDC medium, oxidase test, hypersensitivity test, and pathogenicity test on maize.

**Isolation and characterization of endophytic bacteria**

Sampling for the isolation of endophytic bacteria was carried out in Banyumas and Purbalingga, Central Java, Indonesia, with purposive stratified random sampling. Samples were taken from two areas of altitude, i.e., low-moderate lands (0-500 m above sea level), and highlands (> 500 m above sea level) (Nuryanto et al., 2014). In each district, 2 locations were selected for the low-medium lands, and 1 location for the highlands. Age of maize plants was 20-30 days after planting, when the number of endophytic microbial populations that can be cultured is in the highest population (Cavaglieri et al. 2009).

The endophytic bacteria are isolated from the roots and stems of healthy maize plants. Roots and stems are washed, sterilized with 70% ethanol (1 minute), 20% natrium hypochlorite (5 minutes) and Ringer's thiosulfate solution (5 minutes). Separately, the roots and stems of 10 g each were crushed with 90 ml PBS on a sterile mortar. Subsequently, samples were plated on NA and Kings B media (Cavaglieri et al. 2009). To isolate *Bacillus* sp., the suspension is heated for 10 minutes at 80 ° C, before plating on NA. Bacterial isolates were further purified and characterized, such as morphological characteristics, gram properties, catalase tests, and hypersensitivity tests

**The antagonism test of endophytic bacterial to *R solani***

The antagonism test of endophytic bacteria on *R. solani* was carried out using the dual culture method. The level of inhibition of antagonist is calculated using the formula (Abidin et al., 2015).

$$I = \frac{C - T}{C} \times 100\%$$

I = The level of inhibition of antagonist (%)
C = The radius of pathogen colonies opposite antagonist
T = The radius of the colony of pathogens towards antagonist

**The antagonism test of endophytic bacterial to bacterial pathogens**

Antagonism testing was carried out using the double-layer test method (Santiago et al. 2015). Endophytic bacteria to be tested were grown on the NA medium, incubated at 28 C for 48 hours. In the upside-down position, 1 ml of chloroform was added to the cup lid and left for 2 hours. Next, add 5 mL so that 0.6% water containing 0.5 mL of P. stewartii bacterial suspension. The culture was re-incubated for 24 hours, and there were clear zones around the antagonistic bacterial colony. The antibiotic activity was assessed based on the diameter of the clear zone compared to the diameter of the colony. Characterization of the type of antibiosis can be divided into bactericidal and bacteriostatic types according to the method of Djatmiko et al. (2017).

The mechanism test of endophytic bacteria as controlling agents biological and plant growth-promoting microbial

The testing mechanism of endophytic bacteria is carried out for bacteria that have the potential in testing the antagonism of the fungus *R. solani* and *Pantoea* sp.

*Protease Test*

The activity of the ability of antagonistic bacteria to produce extracellular protease enzymes was tested using Skim Milk Agar (SMA) medium. Each bacterium to be tested was grown in a medium SMA and incubated at 28 C for 24-48 hours. The presence of clear zones around the colony shows that positive bacteria produce protease enzymes (Abed et al. 2016). The protease activity index is assessed based on the diameter of the clear zone compared to the diameter of the colony..

$$\text{Protease index} = \frac{(\text{clear zone diameter} - \text{colony diameter})}{\text{colony diameter}}$$

*Lipase test*

Detection of the ability of bacteria to produce the enzyme lipase was done by growing the antagonistic bacteria on a medium containing 1% Tween 80. The presence of lipase enzyme activity was demonstrated by milky white sediment around the bacterial colony, after incubating at 28 C for 4-7 days. The lipolytic index was measured using a formula Djuric et al. (201).

$$\text{Lipolytic index} = \frac{\text{(milky white diameter - colony diameter)}}{\text{colony diameter}}$$

*Uji fosfatase*

Detection of the ability of bacteria to produce the enzyme phosphatase was done by growing bacterial isolates on Pikovkaya medium. After incubating for 7 days at 28 C, the presence of a clear zone around the bacterial colony shows that the bacteria has the ability to produce the phosphatase enzyme to dissolve phosphates. The solubility index is measured using a formula (Farooq and Bano 2013)

$$\text{Phosphatase Index} = \frac{\text{clear zone diameter – colony diameter}}{\text{colony diamater}}$$

## RESULTS AND DISCUSSION

**Isolation and characterization of endophytic bacteria**

The results of the exploration, isolation, and characterization of endophytic bacteria obtained 23 isolates of endophytic bacteria, consisted of 9 isolates of the *fluorescent Pseudomonads* and 14 isolates of *Bacillus* sp. (Table 1). *Fluorescent Pseudomonads* colony on King's B is round, with a flat edge, and yellowish-white, to greenish-yellow, gram-negative, rod-shaped, non-spore and fluorescent under ultraviolet light. According to Arwiyanto et al. (2007), *P. fluorescens* have round, flat-edged, fluidal and release greenish-yellow colony in the King's B. Individual rod-shaped bacteria with a size (0.5-1.0) - (1.5-4.0) µm. The *P. fluorescens* isolates is gram-negative, which can form catalase, a positive oxidase, needed to grow aerobes.

*Bacillus* sp. has a spherical colony, cell rod-shaped, gram-positive, and endospores within cells. *Bacillus* sp. has the characteristics of a circular colony and punctiform (small round), variations in the entire margin and lobate, white dull, non-slimy, gram-positive, has endospores, flagellum and some are motile.

Based on its distribution, *fluorescent Pseudomonads* and *Bacillus* sp. found in all sampling locations, high or low-medium lands. This shows that *fluorescent Pseudomonads* and *Bacillus* sp spread and can live in various altitudes, both high and low-medium land. According to Bacon and Hilton 2002 and Ganeshan and Kumar 2005, *P. fluoresscens* and *Bacillus* sp, are species of bacteria with a wide range of life and are very adaptive in various environments. Both types of bacteria are also found in the roots or corn stalks. According to Ganeshan and Kumar 2005; Orole and Adejumo 2011; Costa et al. 2013, *Bacillus* sp, and *Pseudomonas* sp. including a group of endophytic bacteria have a wide range of life and more isolated in maize.

**Antagonism test between the endophytic bacteria against *R. solani***

Based on the results of in vitro tests (Table 2), 24 isolates of the endophytic bacteria were able to inhibit the growth of *R. solani*, with varying degrees of inhibition. The endophytic bacteria that have inhibition rates above 50%, i.e *Pseudomonas* Pf BK.A1 (51%), *Bacillus* sp. B.K.A1 (55.39%), *Bacillus* sp BK.A3 (51.52%), PP.A5 (50.66%), and PPD.B2 ( 50.8%). The effect of the endophytic bacteria in inhibiting the growth *of R. solani* is inversely proportional to the dry weight mycelium. The greater the percentage of inhibition of endophytic bacteria to the growth of *R. solani*, the smaller the dry weight mycelium (Table 2.)

The endophytic bacteria can inhibit the growth of R solani shown by the inhibition zone around the bacterial colony (Fig. 1). The endophytic bacteria have anti-pathogenic properties and can produce antibiotic compounds. The ability of the endophytic bacteria to control plant pathogens occurs through the mechanism of antibiosis, competition, lysis, inducing resistance and producing growth substances. Bacteria capable of producing secondary metabolites that can inhibit growth or damage pathogens (Hastuti et al. 2014). These compounds, including alkaloids, phenols, flavonoids, glycosides, and phytoalexin (Soesanto et al. 2010).

**Table 1.** Isolation and characterization of endophytic bacteria

| Land | Sampling location | sample | Gram test | Catalase test | oxidase test | Colony morphology | colony pigment | Fluorescence on KB Medium | Cell morphology | Endo spores | Isolat |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Highland | 1. Purbalingga, Pratin 7.13'33" LS, 109.17'21" BT, TT 1.190 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PP.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PP.A3 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. PP.A5 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PP. B4 |
| | 2.Banyumas, Baturaden 7.19"1" LS, 109.14'29" BT, TT 520 m dpl | Root | - | + | + | round | Greenish yellow | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BB.A2 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BB.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB.B4 |
| Medium-Lowland | 1.Banyumas, Sumbang7.21'54" LS, 109.17'33"BT, TT 200 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BS.A2 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BS.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS.A3 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS. B1 |
| | 2. Purbalingga, Bojongsari, 7.20'12" LS, 109.20'22" BT, TT 190 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | *Fluorescent Pseudomonads (Pf)* PB. A 4 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PB. B1 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB. B3 |
| | 3.Purbalingga, Padamara, 7.22'28" LS, 109.13'24" BT, TT 180 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | *Fluorescent Pseudomonads (Pf)* PPD A1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PPD. B1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PPD. B5 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B2 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B4 |
| | 4. Banyumas, Kembaran 7.23'47" LS, 109.17'9" BT, TT 110 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BK. A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.B3 |

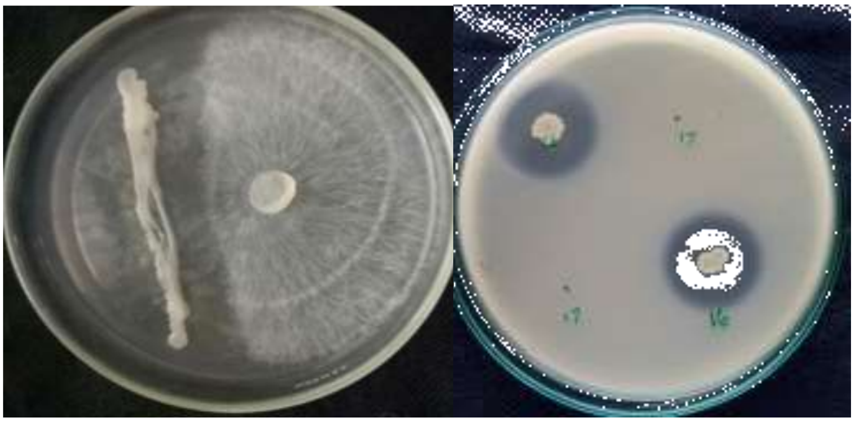

**Figure 1.** Antagonism test between the endophytic bacteria against *R. solani* (a) and *Pantoea* sp..

**Table 2.** Inhibition of endophytic bacteria against *R. solani*

| No | Isolate | Inhibition rate (%) | Dry weights Mycelium |
|---|---|---|---|
| 1 | Control | 0 | 0,093 |
| Endophytic bacteria from the root | | | |
| 2 | *Pseudomonas* Pf BB.A2 | 49,00 | 0,038 |
| 3 | *Pseudomonas* Pf BS.A 2 | 45,00 | 0,027 |
| 4 | *Pseudomonas* Pf BK.A1 | 51,00 | 0,017 |
| 5 | *Pseudomonas* Pf PPD.A1 | 10,33 | 0,059 |
| 6 | *Pseudomonas* Pf PP.A1 | 38,33 | 0,017 |
| 7 | *Pseudomonas* Pf PB.A4 | 18,00 | 0,037 |
| 8 | *Bacillus* sp.BB.A3 | 40,42 | 0,030 |
| 9 | *Bacillus* sp.BS.A1 | 48,73 | 0,016 |
| 10 | *Bacillus* sp. BSA3 | 37,42 | 0,039 |
| 11 | *Bacillus* sp. B.K.A1 | 55,39 | 0,002 |
| 12 | *Bacillus* sp. B.K.A3 | 51,52 | 0,003 |
| 13 | *Bacillus* sp.PP.A3 | 46,65 | 0,019 |
| 14 | *Bacillus* sp.PP.A5 | 50,66 | 0,009 |
| Endophytic bacteria from the stem | | | |
| 15 | *Pseudomonas* Pf PPD.B1 | 27,00 | 0,020 |
| 16 | *Pseudomonas* Pf PPD.B5 | 49,33 | 0,013 |
| 17 | *Pseudomonas* Pf PP.B4 | 65,67 | 0,004 |
| 18 | *Bacillus* sp. BB.B4 | 44,44 | 0,026 |
| 19 | *Bacillus* sp. BS.B1 | 49,74 | 0,012 |
| 20 | *Bacillus* sp. BK. B3 | 40,36 | 0,031 |
| 21 | *Bacillus* sp.PPD.B2 | 50,8 | 0,007 |
| 22 | *Bacillus* sp. PPD.B4 | 39,44 | 0,036 |
| 23 | *Bacillus* sp. PB.B1 | 37,29 | 0,047 |
| 24 | *Bacillus* sp. PB.B3 | 44,9 | 0,022 |

**The endophytic bacterial antagonism test against Pantoea sp.**

The results of antagonism between the endophytic bacteria and *Pantoea* sp. show varied results. The endophytic bacteria that can inhibit bacterial growth are indicated by the presence of clear zones around the endophytic bacterial colonies (Fig.1). From the nine isolate *Pseudomonas* sp. were tested, only three isolates were able to inhibit the growth of the *Pantoea* sp., i.e Pf BS.A2, Pf BK.A1 Pf PPD.B5. While the isolates Pf BB.A2, Pf PPD.A1, Pf PP.A1, Pf PPD.B1, and Pf PP.B4 are not able to inhibit the growth of pathogenic bacteria. Meanwhile, all isolate *Bacillus* sp tested (thirteen isolates) were able to inhibit the growth of *Pantoea* sp. (Table 3).

The presence of clear zones around endophytic bacterial colonies shows the ability of endophytic bacteria to produce antibiotics to inhibit the growth of *Pantoea* sp. *P. fluorescens* P60 can produce antibiotics that inhibit the growth of pathogens (Soesanto 2011). *Pseudomonas fluorescens* is reported to produce phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4 diacetyl phloroglucinol (DAPG), pyrrolnitrin (PRN) and or pyoluteorin (Plt) (Heydari and Pessarakli 2010). Nasrun and Burhanudin (2016) mention that *P. fluorescens* produce secondary metabolites, i.e. antimicrobial, cyanide acid and 2,4-diacetyl phloroglucinol phenazine, pyrrolnitrin, pyoluteorin antibiotics.

The level of bacteria's ability to inhibit growth can be shown by the large diameter of the clear zone. The results showed that the antagonism index ranged from 1.67 - 8.17. Based on this index, most endophytic bacteria have a strong antagonism (index of antagonism> 4) (Davis and Stout 1971). Furthermore, bacterial isolates that showed antagonistic activity were tested for types of antagonism based on Djatmiko (2007). Based on the type of antagonistic activity, ten isolates the endophytic bacteria were bacteriostatic and nine isolates the endophytic bacteria were bactericidal. Bacteriostatic activity, growth inhibition is temporary, it is shown that regrowth of bacteria after being transferred to a new medium, which is free from the influence of antagonistic bacteria. Bactericidal activity, inhibition is permanent. Bacteria were unable to grow even though they are transferred to new medium.

**Table 3.** Inhibition of endophytic bacteria against *Pantoea* sp.

| No | Isolate | Antagonism | Antagonism index | Antagonism catagory* | Antagonism activity |
|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | |
| 1 | *Pseudomonas* Pf BB.A2 | - | 0 | - | - |
| 2 | *Pseudomonas* Pf BS.A 2 | + | 4,91 | strong | bacteriostatic |
| 3 | *Pseudomonas* Pf BK.A1 | + | 4,42 | strong | bacteriostatic |
| 4 | *Pseudomonas* Pf PPD.A1 | - | 0 | - | - |
| 5 | *Pseudomonas* Pf PP.A1 | - | 0 | - | - |
| 6 | *Pseudomonas* Pf PB.A4 | + | 5,29 | strong | bactericidal |
| 7 | *Bacillus* sp.BB.A3 | + | 8,17 | strong | bacteriostatic |
| 8 | *Bacillus* sp.BS.A1 | + | 4,00 | strong | bacteriostatic |
| 9 | *Bacillus* sp. BSA3 | + | 5,07 | strong | bactericidal |
| 10 | *Bacillus* sp. B.K.A1 | + | 4,01 | strong | bakteriostatik |
| 11 | *Bacillus* sp. B.K.A3 | + | 4,91 | strong | bacteriostatic |
| 12 | *Bacillus* sp.PP.A3 | + | 6,63 | strong | bactericidal |
| 13 | *Bacillus* sp.PP.A5 | + | 6,56 | strong | bactericidal |
| Endophytic bacteria from the stem | | | | | |
| 14 | *Pseudomonas* PPD.B1 | - | 0 | - | - |
| 15 | *Pseudomonas* PPD.B5 | + | 5,86 | strong | bactericidal |
| 16 | *Pseudomonas* PP.B4 | - | 0 | - | - |
| 17 | *Bacillus* sp. BB.B4 | + | 7,80 | strong | bactericidal |
| 18 | *Bacillus* sp. BS.B1 | + | 6,22 | strong | bacteriostatic |
| 19 | *Bacillus* sp. BK. B3 | + | 5,33 | strong | bacteriostatic |
| 20 | *Bacillus* sp.PPD.B2 | + | 5,00 | strong | bacteriostatic |
| 21 | *Bacillus* sp. PPD.B4 | + | 8,75 | strong | bacteriostatic |
| 22 | *Bacillus* sp. PB.B1 | + | 1,67 | weak | bacteriostatic |
| 23 | *Bacillus* sp. PB.B3 | + | 5,67 | strong | bactericidal |

•Based on Davis and Stout, 1971

**Test the mechanism of endophytic bacteria as biological control agents and plant growth-promoting microbes**

The mechanism test is carried out on endophytic bacteria that have the potential to control the fungus *R. solani* and *Pantoea* sp., i.e. *Bacillus* sp. B.K.A1, *Bacillus* sp. B.K.A3, *Bacillus* sp. PP.A5, *Bacillus* sp. PPD.B2. The results of enzyme activity tests are as shown in Table 4. The production of compounds related to biocontrol of pathogens and/or promotion of plant growth in bacterial isolates was evaluated by measuring the production of antimicrobial compounds and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and phosphate solubilization. the isolates that have high protein and fat hydrolysis enzymes have the potential as biological control agents because proteins and fats are constituents of pathogen cells (Mota et al 2016).

The four isolates *Bacillus* sp. tested were able to produce protease, lipase and phosphatase enzymes, with varied activity indexes. All isolates of *Bacillus* sp. those tested had a high index of protease and lipase enzymes (> 3) (Table 4., Fig 2.). Protease and lipase enzymes, related to the ability of the endophytic bacteria as biological control agents. According to Anderson et al. (2014), the extracellular protease enzyme produced by P. fluorescens can inactivate antibiotic compounds produced by *Pantoea agglomerans.* The phosphate solubilization is related to the ability of endophytic bacteria as a plant growth promoter, providing phosphates for plants.

**Table 4.** Test results of proteases, lipases and phosphate solubilization.

| No | Isolate | Protease Test activity | Protease Test index | Lipase Test activity | Lipase Test index | Phosphate solubilization activity | Phosphate solubilization index |
|---|---|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | | | |
| 1 | *Bacillus* sp. B.K.A1 | + | 3.75 | + | 3.23 | + | 1.17 |
| 2 | *Bacillus* sp. B.K.A3 | + | 3.20 | + | 3.73 | + | 1.27 |
| 3 | *Bacillus* sp.PP.A5 | + | 5.00 | + | 4.40 | + | 1.46 |
| Endophytic bacteria from the stem | | | | | | | |
| 4 | *Bacillus* sp.PPD.B2 | + | 3.00 | + | 3.90 | + | 2.60 |

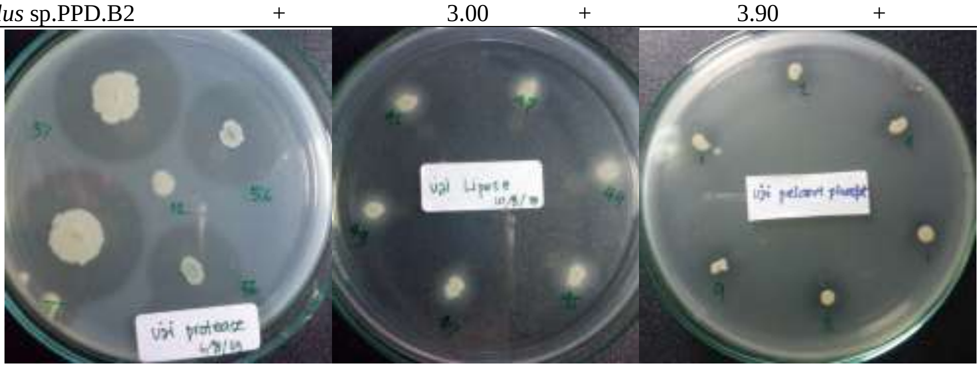

**Figure 2.** Hydrolysis enzyme activity, (a) protease , (b) lipase and (c) phosphate solubilization.

The author should expand the discussion by looking at previous published studies and compare with current findings.

## CONCLUSION

Based on research carried out, it has been successfully isolated, morphologically and biochemically characterized four the endophytic bacteria that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

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Naskah Perbaikan 1

# Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control

**Abstract.** The research aimed to isolate and characterize morphologically and biochemically the endophytic bacteria, and their potential to control maize diseases, especially sheat blight and bacterial wilt. The study was conducted at the Plant Protection Laboratory, Faculty of Agriculture, Jenderal Soedirman University, from April to August 2019. The study consisted of four stages: isolation and characterization of endophytic bacteria, the antagonism test of the endophytic bacterial to *R solani,* the antagonism test of the endophytic bacteria to *Pantoea* sp., and the mechanism test of the endophytic bacteria as biological control agents and plant growth-promoting bacteria. Based on the research, it has been successfully isolated, and characterized morphologically and biochemically characterized four endophytic bacteria isolates that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization[LN1].

**Key words:** *Bacillus* sp., Fluorescents Pseudomonads, *Pantoea* sp, *Rhizoctoni solani*

**Running title:** Isolation and characterization of the endophytic

## INTRODUCTION

Maize is a strategic food commodity in the world. In Indonesia, the government seeks to achieve self-sufficiency in maize through increasing production of sustainable maize. However, these efforts have faced several obstacles, one of them is the presence of plant diseases such as sheat blight, caused by *Rhizoctonia solani* Kuhn, and bacterial wilt caused by (*Pantoea* sp.). *R. solani* can infect up to the midrib of the cob (Djaenuddin et al. 2017), resulting in a decrease in the yield of up to 100%. (Muis 2007). *Pantoea* sp. can attack all stages of the plant causing wilting and leaf blight, and is known as Stewart's wilt (Pataky 2004; Ammar et al. 2014). The pathogens can cause 40-100% yield loss.

Over the past 3 decades, the concept of sustainable and environmentally friendly agriculture have been carried out by minimizing the use of chemicals, both synthetic fertilizers and synthetic pesticides. In the management of pests and plant diseases, biological control is developed by applying biological control agents including the endophytic bacteria (Shanti and Vittal 2013). Many endophytic bacteria can pass the endodermic barrier across from the root cortex to the vascular system, and subsequently develop as endophytes in stems, leaves, tubers, and other organs (Compant et al. 2005). The use of endophytic bacteria as biological agents has an advantage compared to rhizosphere bacteria because endophytic bacteria live and survive in the plant tissue during plant development, thus protecting the plants.

*Bacillus* sp. and fluorescents *Pseudomonads* are reported to be able to live as endophytes and are widely used as biological control agents for soil-borne and air-borne diseases. The endophytic bacteria could control plant diseases through several mechanisms including competition, hyperparasite, producing microbial inhibiting compounds (antibiotics, lysis enzymes, other physical or chemical disorders), enhancing plant resistance, and promoting plant growth (Compant et al 2005, Pal and McSpadden 2006; Rosenblueth and Martinez Romero 2006;).

Based on the mechanisms, the use of endophytic bacteria isolated from maize, both upland and lowland, suggested potentially alternative control for sheath blight (*R. solani*) and bacterial wilt (*Pantoea* sp). The research aimed to isolate and characterize morphologically and biochemically the endophytic bacterial as well as their potential to control pathogens that cause disease in maize especially *R. solani* and *Pantoea* sp.

## MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Protection, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia, from April to August 2019

### Isolation *R. solani*

*R. solani* was isolated from maize with sheath blight symptoms and there was sclerotium as a resistant structure from the pathogenic fungi in Banyumas. Samples were isolated on PDA medium to obtain pure *R. solani* isolates.

**Isolation *Pantoea* sp.**

*Pantoea* sp. isolated from diseased maize, which was taken from the maize growing area in Banyumas Regency. *Pantoea* sp. was isolated according to Coplin et al. 2012; Aini et al. 2013 and Desi et al. 2014. Diseased leaves or stems were washed with running water, then dried with a tissue. Diseased samples were cut 1.5 x 5 cm, then sterilized with ethanol 70% and rinsed with sterile water 3 times. Furthermore, the sample was crushed with 5 ml of sterile distilled water using a sterile mortar. The bacterial suspension was streaked on nutrient agar and incubated 3-5 days. Bacterial colonies that exhibit the character of *Pantoea* sp. are yellow, shiny, slimy, flat or convex, then separated as pure cultures of stewartii candidates. The culture was then tested by Gram Reaction (KOH test), Hugh-Leifson test, pigment production in YDC medium, oxidase test, hypersensitivity test, and pathogenicity test on maize.

**Isolation and characterization of endophytic bacteria**

Sampling for the isolation of endophytic bacteria was carried out in Banyumas and Purbalingga, Central Java, Indonesia, with purposive stratified random sampling. Samples were taken from two areas of altitude, i.e., low-moderate lands (0-500 m above sea level), and highlands (> 500 m above sea level) (Nuryanto et al., 2014). In each district, 2 locations were selected for the low-medium lands, and 1 location for the highlands. Age of maize plants was 20-30 days after planting, when the number of endophytic microbial populations that can be cultured is in the highest population (Cavaglieri et al. 2009).

The endophytic bacteria are isolated from the roots and stems of healthy maize plants. Roots and stems are washed, sterilized with 70% ethanol (1 minute), 20% natrium hypochlorite (5 minutes) and Ringer's thiosulfate solution (5 minutes). Separately, the roots and stems of 10 g each were crushed with 90 ml PBS on a sterile mortar. Subsequently, samples were plated on NA and Kings B media (Cavaglieri et al. 2009). To isolate *Bacillus* sp., the suspension is heated for 10 minutes at 80 ° C, before plating on NA. Bacterial isolates were further purified and characterized, such as morphological characteristics, gram properties, catalase tests, and hypersensitivity tests

**The antagonism test of endophytic bacterial to *R solani***

The antagonism test of endophytic bacteria on *R. solani* was carried out using the dual culture method. The level of inhibition of antagonist is calculated using the formula (Abidin et al., 2015).

$$I = \frac{C - T}{C} \times 100\%$$

I = The level of inhibition of antagonist (%)
C = The radius of pathogen colonies opposite antagonist
T = The radius of the colony of pathogens towards antagonist

**The antagonism test of endophytic bacterial to bacterial pathogens**

Antagonism testing was carried out using the double-layer test method (Santiago et al. 2015). Endophytic bacteria to be tested were grown on the NA medium, incubated at 28 C for 48 hours. In the upside-down position, 1 ml of chloroform was added to the cup lid and left for 2 hours. Next, add 5 mL so that 0.6% water containing 0.5 mL of P. stewartii bacterial suspension. The culture was re-incubated for 24 hours, and there were clear zones around the antagonistic bacterial colony. The antibiotic activity was assessed based on the diameter of the clear zone compared to the diameter of the colony. Characterization of the type of antibiosis can be divided into bactericidal and bacteriostatic types according to the method of Djatmiko et al. (2017).

The mechanism test of endophytic bacteria as controlling agents biological and plant growth-promoting microbial

The testing mechanism of endophytic bacteria is carried out for bacteria that have the potential in testing the antagonism of the fungus *R. solani* and *Pantoea* sp.

*Protease Test*

The activity of the ability of antagonistic bacteria to produce extracellular protease enzymes was tested using Skim Milk Agar (SMA) medium. Each bacterium to be tested was grown in a medium SMA and incubated at 28 C for 24-48 hours. The presence of clear zones around the colony shows that positive bacteria produce protease enzymes (Abed et al. 2016). The protease activity index is assessed based on the diameter of the clear zone compared to the diameter of the colony..

$$\text{Protease index} = \frac{\text{(clear zone diameter} - \text{colony diameter)}}{\text{colony diameter}}$$

*Lipase test*

Detection of the ability of bacteria to produce the enzyme lipase was done by growing the antagonistic bacteria on a medium containing 1% Tween 80. The presence of lipase enzyme activity was demonstrated by milky white sediment around the bacterial colony, after incubating at 28 C for 4-7 days. The lipolytic index was measured using a formula Djuric et al. (201).

$$\text{Lipolytic index} = \frac{\text{(milky white diameter - colony diameter)}}{\text{colony diameter}}$$

*Uji fosfatase*

Detection of the ability of bacteria to produce the enzyme phosphatase was done by growing bacterial isolates on Pikovkaya medium. After incubating for 7 days at 28 C, the presence of a clear zone around the bacterial colony shows that the bacteria has the ability to produce the phosphatase enzyme to dissolve phosphates. The solubility index is measured using a formula (Farooq and Bano 2013)

$$\text{Phosphatase Index} = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diamater}}$$

## RESULTS AND DISCUSSION

**Isolation and characterization of endophytic bacteria**

The results of the exploration, isolation, and characterization of endophytic bacteria obtained 23 isolates of endophytic bacteria, consisted of 9 isolates of the *fluorescent Pseudomonads* and 14 isolates of *Bacillus* sp. (Table 1). *Fluorescent Pseudomonads* colony on King's B is round, with a flat edge, and yellowish-white, to greenish-yellow, gram-negative, rod-shaped, non-spore and fluorescent under ultraviolet light. According to Arwiyanto et al. (2007), *P. fluorescens* have round, flat-edged, fluidal and release greenish-yellow colony in the King's B. Individual rod-shaped bacteria with a size (0.5-1.0) - (1.5-4.0) µm. The *P. fluorescens* isolates is gram-negative, which can form catalase, a positive oxidase, needed to grow aerobes.

*Bacillus* sp. has a spherical colony, cell rod-shaped, gram-positive, and endospores within cells (Table 1.). Menurut …..*Bacillus* sp. has the characteristics of a circular colony and punctiform (small round), variations in the entire margin and lobate, white dull, non-slimy, gram-positive, has endospores, flagellum and some are motile.

Based on its distribution, *fluorescent Pseudomonads* and *Bacillus* sp. found in all sampling locations, high or low-medium lands. This shows that *fluorescent Pseudomonads* and *Bacillus* sp spread and can live in various altitudes, both high and low-medium land. According to Bacon and Hilton 2002 and Ganeshan and Kumar 2005, *P. fluoresscens* and *Bacillus* sp, are species of bacteria with a wide range of life and are very adaptive in various environments. Both types of bacteria are also found in the roots or corn stalks. According to Ganeshan and Kumar 2005; Orole and Adejumo 2011; Costa et al. 2013, *Bacillus* sp, and *Pseudomonas* sp. including a group of endophytic bacteria have a wide range of life and more isolated in maize.

**Antagonism test between the endophytic bacteria against *R. solani***

Based on the results of in vitro tests (Table 2), 24 isolates of the endophytic bacteria were able to inhibit the growth of *R. solani*, with varying degrees of inhibition. The endophytic bacteria that have inhibition rates above 50%, i.e *Pseudomonas* Pf BK.A1 (51%), *Bacillus* sp. B.K.A1 (55.39%), *Bacillus* sp BK.A3 (51.52%), PP.A5 (50.66%), and PPD.B2 ( 50.8%). The effect of the endophytic bacteria in inhibiting the growth *of R. solani* is inversely proportional to the dry weight mycelium. The greater the percentage of inhibition of endophytic bacteria to the growth of *R. solani*, the smaller the dry weight mycelium (Table 2.)

The endophytic bacteria can inhibit the growth of *R solani* shown by the inhibition zone around the bacterial colony (Fig. 1). The endophytic bacteria have anti-pathogenic properties and can produce antibiotic compounds. The ability of the endophytic bacteria to control plant pathogens occurs through the mechanism of antibiosis, competition, lysis, inducing resistance and producing growth substances. Bacteria capable of producing secondary metabolites that can inhibit growth or damage pathogens (Hastuti et al. 2014). These compounds, including alkaloids, phenols, flavonoids, glycosides, and phytoalexin (Soesanto et al. 2010).

**Table 1.** Isolation and characterization of endophytic bacteria

| Land | Sampling location | sample | Gram test | Catalase test | oxidase test | Colony morphology | colony pigment | Fluorescence on KB Medium | Cell morphology | Endo spores | Isolat |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Highland | 1. Purbalingga, Pratin 7.13'33" LS, 109.17'21" BT, TT 1.190 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PP.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PP.A3 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. PP.A5 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PP. B4 |
| | 2.Banyumas, Baturaden 7.19"1" LS, 109.14'29" BT, TT 520 m dpl | Root | - | + | + | round | Greenish yellow | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BB.A2 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BB.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB.B4 |
| Medium-Lowland | 1.Banyumas, Sumbang7.21'54" LS, 109.17'33"BT, TT 200 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BS.A2 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BS.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS.A3 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS. B1 |
| | 2.Purbalingga, Bojongsari, 7.20'12" LS, 109.20'22" BT, TT 190 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | *Fluorescent Pseudomonads (Pf)* PB. A 4 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PB. B1 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB. B3 |
| | 3.Purbalingga, Padamara, 7.22'28" LS, 109.13'24" BT, TT 180 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | *Fluorescent Pseudomonads (Pf)* PPD A1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PPD. B1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PPD. B5 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B2 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B4 |
| | 4.Banyumas, Kembaran 7.23'47" LS, 109.17'9" BT, TT 110 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BK. A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.B3 |

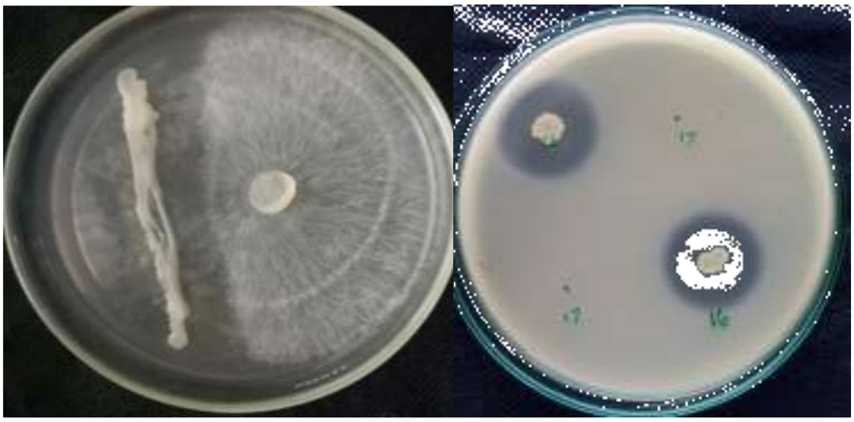

**Figure 1.** Antagonism test between the endophytic bacteria against *R. solani* (a) and *Pantoea* sp..

**Table 2.** Inhibition of endophytic bacteria against *R. solani*

| No | Isolate | Inhibition rate (%) | Dry weights Mycelium |
|---|---|---|---|
| 1 | Control | 0 | 0,093 |
| Endophytic bacteria from the root | | | |
| 2 | *Pseudomonas* Pf BB.A2 | 49,00 | 0,038 |
| 3 | *Pseudomonas* Pf BS.A 2 | 45,00 | 0,027 |
| 4 | *Pseudomonas* Pf BK.A1 | 51,00 | 0,017 |
| 5 | *Pseudomonas* Pf PPD.A1 | 10,33 | 0,059 |
| 6 | *Pseudomonas* Pf PP.A1 | 38,33 | 0,017 |
| 7 | *Pseudomonas* Pf PB.A4 | 18,00 | 0,037 |
| 8 | *Bacillus* sp.BB.A3 | 40,42 | 0,030 |
| 9 | *Bacillus* sp.BS.A1 | 48,73 | 0,016 |
| 10 | *Bacillus* sp. BSA3 | 37,42 | 0,039 |
| 11 | *Bacillus* sp. B.K.A1 | 55,39 | 0,002 |
| 12 | *Bacillus* sp. B.K.A3 | 51,52 | 0,003 |
| 13 | *Bacillus* sp.PP.A3 | 46,65 | 0,019 |
| 14 | *Bacillus* sp.PP.A5 | 50,66 | 0,009 |
| Endophytic bacteria from the stem | | | |
| 15 | *Pseudomonas* Pf PPD.B1 | 27,00 | 0,020 |
| 16 | *Pseudomonas* Pf PPD.B5 | 49,33 | 0,013 |
| 17 | *Pseudomonas* Pf PP.B4 | 65,67 | 0,004 |
| 18 | *Bacillus* sp. BB.B4 | 44,44 | 0,026 |
| 19 | *Bacillus* sp. BS.B1 | 49,74 | 0,012 |
| 20 | *Bacillus* sp. BK. B3 | 40,36 | 0,031 |
| 21 | *Bacillus* sp.PPD.B2 | 50,8 | 0,007 |
| 22 | *Bacillus* sp. PPD.B4 | 39,44 | 0,036 |
| 23 | *Bacillus* sp. PB.B1 | 37,29 | 0,047 |
| 24 | *Bacillus* sp. PB.B3 | 44,9 | 0,022 |

**The endophytic bacterial antagonism test against *Pantoea* sp.**

The results of antagonism between the endophytic bacteria and *Pantoea* sp. show varied results. The endophytic bacteria that can inhibit bacterial growth are indicated by the presence of clear zones around the endophytic bacterial colonies (Fig.1). From the nine isolate *Pseudomonas* sp. were tested, only three isolates were able to inhibit the growth of the *Pantoea* sp., i.e Pf BS.A2, Pf BK.A1 Pf PPD.B5. While the isolates Pf BB.A2, Pf PPD.A1, Pf PP.A1, Pf PPD.B1, and Pf PP.B4 are not able to inhibit the growth of pathogenic bacteria. Meanwhile, all isolate *Bacillus* sp tested (thirteen isolates) were able to inhibit the growth of *Pantoea* sp. (Table 3).

The presence of clear zones around endophytic bacterial colonies shows the ability of endophytic bacteria to produce antibiotics to inhibit the growth of *Pantoea* sp. *P. fluorescens* P60 can produce antibiotics that inhibit the growth of pathogens (Soesanto 2011). *Pseudomonas fluorescens* is reported to produce phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4 diacetyl phloroglucinol (DAPG), pyrrolnitrin (PRN) and or pyoluteorin (Plt) (Heydari and Pessarakli 2010). Nasrun and Burhanudin (2016) mention that *P. fluorescens* produce secondary metabolites, i.e. antimicrobial, cyanide acid and 2,4-diacetyl phloroglucinol phenazine, pyrrolnitrin, pyoluteorin antibiotics.

The level of bacteria's ability to inhibit growth can be shown by the large diameter of the clear zone. The results showed that the antagonism index ranged from 1.67 - 8.17. Based on this index, most endophytic bacteria have a strong antagonism (index of antagonism> 4) (Davis and Stout 1971). Furthermore, bacterial isolates that showed antagonistic activity were tested for types of antagonism based on Djatmiko (2007). Based on the type of antagonistic activity, ten isolates the endophytic bacteria were bacteriostatic and nine isolates the endophytic bacteria were bactericidal. Bacteriostatic activity, growth inhibition is temporary, it is shown that regrowth of bacteria after being transferred to a new medium, which is free from the influence of antagonistic bacteria. Bactericidal activity, inhibition is permanent. Bacteria were unable to grow even though they are transferred to new medium.

**Table 3.** Inhibition of endophytic bacteria against *Pantoea* sp.

| No | Isolate | Antagonism | Antagonism index | Antagonism catagory* | Antagonism activity |
|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | |
| 1 | *Pseudomonas* Pf BB.A2 | - | 0 | - | - |
| 2 | *Pseudomonas* Pf BS.A 2 | + | 4,91 | strong | bacteriostatic |
| 3 | *Pseudomonas* Pf BK.A1 | + | 4,42 | strong | bacteriostatic |
| 4 | *Pseudomonas* Pf PPD.A1 | - | 0 | - | - |
| 5 | *Pseudomonas* Pf PP.A1 | - | 0 | - | - |
| 6 | *Pseudomonas* Pf PB.A4 | + | 5,29 | strong | bactericidal |
| 7 | *Bacillus* sp.BB.A3 | + | 8,17 | strong | bacteriostatic |
| 8 | *Bacillus* sp.BS.A1 | + | 4,00 | strong | bacteriostatic |
| 9 | *Bacillus* sp. BSA3 | + | 5,07 | strong | bactericidal |
| 10 | *Bacillus* sp. B.K.A1 | + | 4,01 | strong | bakteriostatik |
| 11 | *Bacillus* sp. B.K.A3 | + | 4,91 | strong | bacteriostatic |
| 12 | *Bacillus* sp.PP.A3 | + | 6,63 | strong | bactericidal |
| 13 | *Bacillus* sp.PP.A5 | + | 6,56 | strong | bactericidal |
| Endophytic bacteria from the stem | | | | | |
| 14 | *Pseudomonas* PPD.B1 | - | 0 | - | - |
| 15 | *Pseudomonas* PPD.B5 | + | 5,86 | strong | bactericidal |
| 16 | *Pseudomonas* PP.B4 | - | 0 | - | - |
| 17 | *Bacillus* sp. BB.B4 | + | 7,80 | strong | bactericidal |
| 18 | *Bacillus* sp. BS.B1 | + | 6,22 | strong | bacteriostatic |
| 19 | *Bacillus* sp. BK. B3 | + | 5,33 | strong | bacteriostatic |
| 20 | *Bacillus* sp.PPD.B2 | + | 5,00 | strong | bacteriostatic |
| 21 | *Bacillus* sp. PPD.B4 | + | 8,75 | strong | bacteriostatic |
| 22 | *Bacillus* sp. PB.B1 | + | 1,67 | weak | bacteriostatic |
| 23 | *Bacillus* sp. PB.B3 | + | 5,67 | strong | bactericidal |

•Based on Davis and Stout, 1971

**Test the mechanism of endophytic bacteria as biological control agents and plant growth-promoting microbes**

The mechanism test is carried out on endophytic bacteria that have the potential to control the fungus *R. solani* and *Pantoea* sp., i.e. *Bacillus* sp. B.K.A1, *Bacillus* sp. B.K.A3, *Bacillus* sp. PP.A5, *Bacillus* sp. PPD.B2. The results of enzyme activity tests are as shown in Table 4. The production of compounds related to biocontrol of pathogens and/or promotion of plant growth in bacterial isolates was evaluated by measuring the production of antimicrobial compounds and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and phosphate solubilization. the isolates that have high protein and fat hydrolysis enzymes have the potential as biological control agents because proteins and fats are constituents of pathogen cells (Mota et al 2016).

The four isolates *Bacillus* sp. tested were able to produce protease, lipase and phosphatase enzymes, with varied activity indexes. All isolates of *Bacillus* sp. those tested had a high index of protease and lipase enzymes (> 3) (Table 4., Fig 2.). Protease and lipase enzymes, related to the ability of the endophytic bacteria as biological control agents. According to Anderson et al. (2014), the extracellular protease enzyme produced by P. fluorescens can inactivate antibiotic compounds produced by *Pantoea agglomerans.* The phosphate solubilization is related to the ability of endophytic bacteria as a plant growth promoter, providing phosphates for plants.

**Table 4.** Test results of proteases, lipases and phosphate solubilization.

| No | Isolate | Protease Test activity | Protease Test index | Lipase Test activity | Lipase Test index | Phosphate solubilization activity | Phosphate solubilization index |
|---|---|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | | | |
| 1 | *Bacillus* sp. B.K.A1 | + | 3.75 | + | 3.23 | + | 1.17 |
| 2 | *Bacillus* sp. B.K.A3 | + | 3.20 | + | 3.73 | + | 1.27 |
| 3 | *Bacillus* sp.PP.A5 | + | 5.00 | + | 4.40 | + | 1.46 |
| Endophytic bacteria from the stem | | | | | | | |
| 4 | *Bacillus* sp.PPD.B2 | + | 3.00 | + | 3.90 | + | 2.60 |


**Figure 2.** Hydrolysis enzyme activity, (a) protease , (b) lipase and (c) phosphate solubilization.

The author should expand the discussion by looking at previous published studies and compare with current findings.

## CONCLUSION

Based on research carried out, it has been successfully isolated, morphologically and biochemically characterized four the endophytic bacteria that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

## ACKNOWLEDGEMENTS

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**Naskah Koreksi 2**

# Short Communication: Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control

**Abstract.** The present was aimed to isolate and characterize the endophytic bacteria morphologically and biochemically and to studytheir potential to control maize diseases, especially sheat blight and bacterial wilt causing pathogens. The study was conducted at the Plant Protection Laboratory, Faculty of Agriculture, Jenderal Soedirman University, from April to August 2019. The study consisted of four stages: isolation and characterization of endophytic bacteria, the antagonism test of the endophytic bacterial to *R solani*, the antagonism test of the endophytic bacteria to *Pantoea* sp., and the mechanism test of the endophytic bacteria as biological control agents and plant growth-promoting bacteria. Based on the research, four endophytic bacteria isolates has been successfully isolated, and characterized successfully and found have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. *Bacillus* sp, endophytic from the root (BK.A1; BK.A3; PP.A5) and *Bacillus* sp. endophytic from the stem (PPD.B2) can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

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**Key words:** *Bacillus* sp., Fluorescents Pseudomonads, *Pantoea* sp, *Rhizoctoni solani*

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**Running title:** Isolation and characterization of the endophytic

## INTRODUCTION

Maize is a strategic food commodity in the world. In Indonesia, the government seeks to achieve self-sufficiency in maize through increasing production of sustainable maize. However, these efforts have faced several obstacles; one of them is the presence of plant diseases such as sheath blightcaused by *Rhizoctonia solani* Kuhn, and bacterial wilt caused by (*Pantoea* sp.). *R. solani* can infect up to the midrib of the cob (Djaenuddin et al. 2017), resulting in up to 100% decrease in the yield (Muis 2007). *Pantoea* sp. can attack all stages of the plant causing wilting and leaf blight, and is known as Stewart's wilt (Pataky 2004; Ammar et al. 2014). The pathogens can cause 40-100% yield loss.

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Over the past 3 decades, the concept of sustainable and environmentally friendly agriculture has been carried out by minimizing the use of chemicals, both synthetic fertilizers and pesticides. In the management of pests and plant diseases, biological control is developed by applying biological agents including the endophytic bacteria (Shanti and Vittal 2013). Many endophytic bacteria can pass the endodermic barrier across from the root cortex to the vascular system, and subsequently develop as endophytes in stems, leaves, tubers, and other organs (Compant et al. 2005). The use of endophytic bacteria as biological agents has an advantage compared to rhizosphere bacteria because endophytic bacteria live and survive in the plant tissue during plant development, thus protecting the plants.

*Bacillus* sp. and fluorescents *Pseudomonads* are reported to be able to live as endophytes and are widely used as biological control agents for soil-borne and air-borne diseases. The endophytic bacteria could control plant diseases through several mechanisms including competition, hyperparasitism, producing microbial inhibiting compounds (antibiotics, lysis enzymes, other physical or chemical disorders), enhancing plant resistance, and promoting plant growth (Compant et al 2005, Pal and McSpadden 2006; Rosenblueth and Martinez Romero 2006).

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Based on the mechanisms, the use of endophytic bacteria isolated from maize, both upland and lowland, suggested potentially alternative control for sheath blight (*R. solani*) and bacterial wilt (*Pantoea* sp). The research aimed to isolate and characterize morphologically and biochemically the endophytic bacteria as well as their potential to control pathogens that cause disease in maize especially *R. solani* and *Pantoea* sp.

## MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Protection, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia, from April to August 2019

**Isolation *R. solani***

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*R. solani* was isolated from maize with sheath blight symptoms and there was sclerotium as a resistant structure from the pathogenic fungi in Banyumas. Samples were isolated on PDA medium to obtain pure *R. solani* isolates.

**Isolation *Pantoea* sp.**

*Pantoea* sp. was isolated from diseased maizesamples taken from the maize growing area in Banyumas Regency according to Coplin et al. (2012); Aini et al.(2013) and Desi et al. (2014). Diseased leaves or stems were washed with running water, then dried with a tissue. Diseased samples were cut 1.5 x 5 cm, then sterilized with ethanol 70% and rinsed with sterile water 3 times. Furthermore, the sample was crushed with 5 ml of sterile distilled water using a sterile mortar. The bacterial suspension was streaked on nutrient agar and incubated 3-5 days. Bacterial colonies that exhibit the character of *Pantoea* sp. were yellow, shiny, slimy, flat or convex, then separated as pure cultures of stewartii candidates. The culture was then tested by Gram Reaction (KOH test), Hugh-Leifson test, pigment production in YDC medium, oxidase test, hypersensitivity test, and pathogenicity test on maize.

**Isolation and characterization of endophytic bacteria**

Sampling for the isolation of endophytic bacteria was carried out in Banyumas and Purbalingga, Central Java, Indonesia, with purposive stratified random sampling. Samples were taken from two areas of altitude, i.e., low-moderate lands (0-500 m above sea level), and highlands (> 500 m above sea level) (Nuryanto et al., 2014). In each district, 2 locations were selected for the low-medium lands, and 1 location for the highlands. Age of maize plants was 20-30 days after planting, when the number of endophytic microbial populations that can be cultured is in the highest population (Cavaglieri et al. 2009).

The endophytic bacteria were isolated from the roots and stems of healthy maize plants. Roots and stems were washed, sterilized with 70% ethanol (1 minute), 20% natrium hypochlorite (5 minutes) and Ringer's thiosulfate solution (5 minutes). Separately, the roots and stems of 10 g each were crushed with 90 ml PBS on a sterile mortar. Subsequently, samples were plated on NA and Kings B media (Cavaglieri et al. 2009). To isolate *Bacillus* sp., the suspension was heated for 10 minutes at 80 ° C, before plating on NA. Bacterial isolates were further purified and characterized, such as morphological characteristics, gram properties, catalase tests, and hypersensitivity tests

**The antagonism test of endophytic bacterial to *R solani***

The antagonism test of endophytic bacteria on *R. solani* was carried out using the dual culture method. The level of inhibition of antagonist is calculated using the formula (Abidin et al., 2015).

$$I = \frac{C - T}{C} \times 100\%$$

I = The level of inhibition of antagonist (%)
C = The radius of pathogen colonies opposite antagonist
T = The radius of the colony of pathogens towards antagonist

**The antagonism test of endophytic bacterial to bacterial pathogens**

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Antagonism testing was carried out using the double-layer test method (Santiago et al. 2015). Endophytic bacteria to be tested were grown on the NA medium, incubated at 28 C for 48 hours. In the upside-down position, 1 ml of chloroform was added to the cup lid and left for 2 hours. Next, add 5 mL so that 0.6% water containing 0.5 mL of *P. stewartii* bacterial suspension. The culture was re-incubated for 24 hours, and there were clear zones around the antagonistic bacterial colony. The antibiotic activity was assessed based on the diameter of the clear zone compared to the diameter of the colony. Characterization of the type of antibiosis can be divided into bactericidal and bacteriostatic types according to the method of Djatmiko et al. (2007).

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The mechanism test of endophytic bacteria as controlling agents biological and plant growth-promoting microbial

The testing mechanism of endophytic bacteria was carried out for bacteria that have the potential in testing the antagonism of the fungus *R. solani* and *Pantoea* sp.

*Protease Test*

The activity of the ability of antagonistic bacteria to produce extracellular protease enzymes was tested using Skim Milk Agar (SMA) medium. Each bacterium to be tested was grown in a medium SMA and incubated at 28 C for 24-48 hours. The presence of clear zones around the colony shows that positive bacteria produce protease enzymes (Abed et al.

2016). The protease activity index is assessed based on the diameter of the clear zone compared to the diameter of the colony..

$$\text{Protease index} = \frac{\text{(clear zone diameter} - \text{colony diameter)}}{\text{colony diameter}}$$

*Lipase test*

Detection of the ability of bacteria to produce the enzyme lipase was done by growing the antagonistic bacteria on a medium containing 1% Tween 80. The presence of lipase enzyme activity was demonstrated by milky white sediment around the bacterial colony, after incubating at 28 C for 4-7 days. The lipolytic index was measured using a formula Djuric et al. (201).

$$\text{Lipolytic index} = \frac{\text{(milky white diameter - colony diameter)}}{\text{colony diameter}}$$

*Uji fosfatase*

Detection of the ability of bacteria to produce the enzyme phosphatase was done by growing bacterial isolates on Pikovkaya medium. After incubating for 7 days at 28 C, the presence of a clear zone around the bacterial colony shows that the bacteria has the ability to produce the phosphatase enzyme to dissolve phosphates. The solubility index is measured using a formula (Farooq and Bano 2013)

$$\text{Phosphatase Index} = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diamater}}$$

## RESULTS AND DISCUSSION

**Isolation and characterization of endophytic bacteria**

The results of the exploration, isolation and characterization of endophytic bacteria obtained 23 isolates of endophytic bacteria, consisted of 9 isolates of the fluorescent *Pseudomonads* and 14 isolates of *Bacillus* sp. (Table 1). *Fluorescent Pseudomonads* colony on King's B was round, with a flat edge, and yellowish-white, to greenish-yellow, gram-negative, rod-shaped, non-spore and fluorescent under ultraviolet light. According to Arwiyanto et al. (2007), *P. fluorescens* have round, flat-edged, fluidal and release greenish-yellow colony in the King's B. Individual rod-shaped bacteria with a size (0.5-1.0) - (1.5-4.0) µm. The *P. fluorescens* isolates is gram-negative, which can form catalase, a positive oxidase, needed to grow aerobes.

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*Bacillus* sp. was observed with spherical colony having cell rod-shaped, gram-positive, and endospores within cells (Table 1.). According to Slepecky and Hempill 2006; Amin et al. 2015, *Bacillus* sp. has the characteristics of a circular colony and punctiform (small round), variations in the entire margin and lobate, white dull, non-slimy, gram-positive, has endospores, flagellum and some are motile.

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Based on its distribution, *fluorescent Pseudomonads* and *Bacillus* sp. found in all sampling locations, high or low-medium lands. This shows that *fluorescent Pseudomonads* and *Bacillus* sp. spread and can live in various altitudes, both high and low-medium land. According to Bacon and Hilton 2002 and Ganeshan and Kumar 2005, *P. fluoresscens* and *Bacillus* sp., are species of bacteria with a wide range of life and are very adaptive in various environments. Both types of bacteria are also found in the roots or corn stalks. According to Ganeshan and Kumar 2005; Orole and Adejumo 2011; Costa et al. 2013, *Bacillus* sp. and *Pseudomonas* sp. including a group of endophytic bacteria have a wide range of life and more isolated in maize.

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**Commented [A12]:** if authors are doing characterization, then why characterize *Bacillus* only up to genus level?

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**Antagonism test between the endophytic bacteria against *R. solani***

Based on the results of *in vitro* tests (Table 2), 24 isolates of the endophytic bacteria were able to inhibit the growth of *R. solani*, with varying degrees of inhibition. The endophytic bacteria that have inhibition rates above 50%, i.e *Pseudomonas* Pf BK.A1 (51%), *Bacillus* sp. B.K.A1 (55.39%), *Bacillus* sp BK.A3 (51.52%), PP.A5 (50.66%), and PPD.B2 ( 50.8%). The effect of the endophytic bacteria in inhibiting the growth *of R. solani* is inversely proportional to the dry weight mycelium. The greater the percentage of inhibition of endophytic bacteria to the growth of *R. solani*, the smaller the dry weight mycelium (Table 2.)

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The endophytic bacteria can inhibit the growth of *R. solani* shown by the inhibition zone around the bacterial colony (Fig. 1). The endophytic bacteria have anti-pathogenic properties and can produce antibiotic compounds. The ability of the endophytic bacteria to control plant pathogens occurs through the mechanism of antibiosis, competition, lysis, inducing resistance and producing growth substances. Bacteria capable of producing secondary metabolites that can inhibit growth or damage pathogens (Hastuti et al. 2014). These compounds, including alkaloids, phenols, flavonoids, glycosides, and phytoalexin (Soesanto et al. 2010).

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**Table 1.** Isolation and characterization of endophytic bacteria

| Land | Sampling location | sample | Gram test | Catalase test | oxidase test | Colony morphology | colony pigment | Fluorescence on KB Medium | Cell morphology | Endo spores | Isolat |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Highland | 1. Purbalingga, Pratin 7.13'33" LS, 109.17'21" BT, TT 1.190 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PP.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PP.A3 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. PP.A5 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PP. B4 |
| | 2.Banyumas, Baturaden 7.19"1" LS, 109.14'29" BT, TT 520 m dpl | Root | - | + | + | round | Greenish yellow | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BB.A2 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BB.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB.B4 |
| Medium-Lowland | 1.Banyumas, Sumbang7.21'54" LS, 109.17'33"BT, TT 200 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BS.A2 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BS.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS.A3 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS. B1 |
| | 2.Purbalingga, Bojongsari, 7.20'12" LS, 109.20'22" BT, TT 190 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | *Fluorescent Pseudomonads (Pf)* PB. A 4 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PB. B1 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB. B3 |
| | 3.Purbalingga, Padamara, 7.22'28" LS, 109.13'24" BT, TT 180 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | *Fluorescent Pseudomonads (Pf)* PPD A1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PPD. B1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* PPD. B5 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B2 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B4 |
| | 4.Banyumas, Kembaran 7.23'47" LS, 109.17'9" BT, TT 110 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | *Fluorescent Pseudomonads (Pf)* BK. A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.B3 |

**Commented [A18]:** Check rules of binomial system of nomenclature???

**Figure 1.** Antagonism test between the endophytic bacteria against *R. solani* (a) and *Pantoea* sp..

**Table 2.** Inhibition of endophytic bacteria against *R. solani*

| No | Isolate | Inhibition rate (%) | Dry weights Mycelium |
|---|---|---|---|
| 1 | Control | 0 | 0,093 |
| Endophytic bacteria from the root | | | |
| 2 | *Pseudomonas* Pf BB.A2 | 49,00 | 0,038 |
| 3 | *Pseudomonas* Pf BS.A 2 | 45,00 | 0,027 |
| 4 | *Pseudomonas* Pf BK.A1 | 51,00 | 0,017 |
| 5 | *Pseudomonas* Pf PPD.A1 | 10,33 | 0,059 |
| 6 | *Pseudomonas* Pf PP.A1 | 38,33 | 0,017 |
| 7 | *Pseudomonas* Pf PB.A4 | 18,00 | 0,037 |
| 8 | *Bacillus* sp.BB.A3 | 40,42 | 0,030 |
| 9 | *Bacillus* sp.BS.A1 | 48,73 | 0,016 |
| 10 | *Bacillus* sp. BSA3 | 37,42 | 0,039 |
| 11 | *Bacillus* sp. B.K.A1 | 55,39 | 0,002 |
| 12 | *Bacillus* sp. B.K.A3 | 51,52 | 0,003 |
| 13 | *Bacillus* sp.PP.A3 | 46,65 | 0,019 |
| 14 | *Bacillus* sp.PP.A5 | 50,66 | 0,009 |
| Endophytic bacteria from the stem | | | |
| 15 | *Pseudomonas* Pf PPD.B1 | 27,00 | 0,020 |
| 16 | *Pseudomonas* Pf PPD.B5 | 49,33 | 0,013 |
| 17 | *Pseudomonas* Pf PP.B4 | 65,67 | 0,004 |
| 18 | *Bacillus* sp. BB.B4 | 44,44 | 0,026 |
| 19 | *Bacillus* sp. BS.B1 | 49,74 | 0,012 |
| 20 | *Bacillus* sp. BK. B3 | 40,36 | 0,031 |
| 21 | *Bacillus* sp.PPD.B2 | 50,8 | 0,007 |
| 22 | *Bacillus* sp. PPD.B4 | 39,44 | 0,036 |
| 23 | *Bacillus* sp. PB.B1 | 37,29 | 0,047 |
| 24 | *Bacillus* sp. PB.B3 | 44,9 | 0,022 |

**The endophytic bacterial antagonism test against *Pantoea* sp.**

The results of antagonism between the endophytic bacteria and *Pantoea* sp. show varied results. The endophytic bacteria that can inhibit bacterial growth were indicated by the presence of clear zones around the endophytic bacterial colonies (Fig.1). From the nine isolate *Pseudomonas* sp. were tested, only three isolates were able to inhibit the growth of the *Pantoea* sp., i.e Pf BS.A2, Pf BK.A1 Pf PPD.B5. While the isolates Pf BB.A2, Pf PPD.A1, Pf PP.A1, Pf PPD.B1, and Pf PP.B4 are not able to inhibit the growth of pathogenic bacteria. Meanwhile, all isolate *Bacillus* sp. tested (thirteen isolates) were able to inhibit the growth of *Pantoea* sp. (Table 3).

**Commented [A19]:**

The presence of clear zones around endophytic bacterial colonies showed the ability of endophytic bacteria to produce antibiotics to inhibit the growth of *Pantoea* sp. *P. fluorescens* P60 can produce antibiotics that inhibit the growth of pathogens (Soesanto 2011). *Pseudomonas fluorescens* is reported to produce phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4 diacetyl phloroglucinol (DAPG), pyrrolnitrin (PRN) and or pyoluteorin (Plt) (Heydari and Pessarakli 2010). Nasrun and Burhanudin (2016) mention that *P. fluorescens* produce secondary metabolites, i.e. antimicrobial, cyanide acid and 2,4-diacetyl phloroglucinol phenazine, pyrrolnitrin, pyoluteorin antibiotics.

The level of bacteria's ability to inhibit growth can be shown by the large diameter of the clear zone. The results showed that the antagonism index ranged from 1.67 - 8.17. Based on this index, most endophytic bacteria have a strong antagonism (index of antagonism> 4) (Davis and Stout 1971). Furthermore, bacterial isolates that showed antagonistic activity were tested for types of antagonism based on Djatmiko (2007). Based on the type of antagonistic activity, ten isolates the endophytic bacteria were bacteriostatic and nine isolates the endophytic bacteria were bactericidal. Bacteriostatic activity, growth inhibition is temporary, it is shown that regrowth of bacteria after being transferred to a new medium, which is free from the influence of antagonistic bacteria. Bactericidal activity, inhibition is permanent. Bacteria were unable to grow even though they are transferred to new medium.

**Table 3.** Inhibition of endophytic bacteria against *Pantoea* sp.

| No | Isolate | Antagonism | Antagonism index | Antagonism catagory* | Antagonism activity |
|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | |
| 1 | *Pseudomonas* Pf BB.A2 | - | 0 | - | - |
| 2 | *Pseudomonas* Pf BS.A 2 | + | 4,91 | strong | bacteriostatic |
| 3 | *Pseudomonas* Pf BK.A1 | + | 4,42 | strong | bacteriostatic |
| 4 | *Pseudomonas* Pf PPD.A1 | - | 0 | - | - |
| 5 | *Pseudomonas* Pf PP.A1 | - | 0 | - | - |
| 6 | *Pseudomonas* Pf PB.A4 | + | 5,29 | strong | bactericidal |
| 7 | *Bacillus* sp.BB.A3 | + | 8,17 | strong | bacteriostatic |
| 8 | *Bacillus* sp.BS.A1 | + | 4,00 | strong | bacteriostatic |
| 9 | *Bacillus* sp. BSA3 | + | 5,07 | strong | bactericidal |
| 10 | *Bacillus* sp. B.K.A1 | + | 4,01 | strong | bakteriostatik |
| 11 | *Bacillus* sp. B.K.A3 | + | 4,91 | strong | bacteriostatic |
| 12 | *Bacillus* sp.PP.A3 | + | 6,63 | strong | bactericidal |
| 13 | *Bacillus* sp.PP.A5 | + | 6,56 | strong | bactericidal |
| Endophytic bacteria from the stem | | | | | |
| 14 | *Pseudomonas* PPD.B1 | - | 0 | - | - |
| 15 | *Pseudomonas* PPD.B5 | + | 5,86 | strong | bactericidal |
| 16 | *Pseudomonas* PP.B4 | - | 0 | - | - |
| 17 | *Bacillus* sp. BB.B4 | + | 7,80 | strong | bactericidal |
| 18 | *Bacillus* sp. BS.B1 | + | 6,22 | strong | bacteriostatic |
| 19 | *Bacillus* sp. BK. B3 | + | 5,33 | strong | bacteriostatic |
| 20 | *Bacillus* sp.PPD.B2 | + | 5,00 | strong | bacteriostatic |
| 21 | *Bacillus* sp. PPD.B4 | + | 8,75 | strong | bacteriostatic |
| 22 | *Bacillus* sp. PB.B1 | + | 1,67 | weak | bacteriostatic |
| 23 | *Bacillus* sp. PB.B3 | + | 5,67 | strong | bactericidal |

•Based on Davis and Stout, 1971

**Test the mechanism of endophytic bacteria as biological control agents and plant growth-promoting microbes**

The mechanism test was carried out on endophytic bacteria that have the potential to control the fungus *R. solani* and *Pantoea* sp., i.e. *Bacillus* sp. B.K.A1, *Bacillus* sp. B.K.A3, *Bacillus* sp. PP.A5, *Bacillus* sp. PPD.B2. The production of compounds related to biocontrol of pathogens and promotion of plant growth in bacterial isolates was evaluated by measuring the production of antimicrobial compounds and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and phosphate solubilization. The results of enzyme activity tests are as shown in Table 4.

**Table 4.** Test results of proteases, lipases and phosphate solubilization.

| No | Isolate | Protease Test activity | index | Lipase Test activity | index | Phosphate solubilization Activity | index |
|---|---|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | | | |
| 1 | *Bacillus* sp. B.K.A1 | + | 3.75 | + | 3.23 | + | 1.17 |
| 2 | *Bacillus* sp. B.K.A3 | + | 3.20 | + | 3.73 | + | 1.27 |
| 3 | *Bacillus* sp. PP.A5 | + | 5.00 | + | 4.40 | + | 1.46 |
| Endophytic bacteria from the stem | | | | | | | |
| 4 | *Bacillus* sp.PPD.B2 | + | 3.00 | + | 3.90 | + | 2.60 |

The four isolates *Bacillus* sp. tested were able to produce protease, lipase and phosphatase enzymes, with varied activity indexes. All isolates of *Bacillus* sp. those tested had a high index of protease and lipase enzymes (> 3) (Table 4., Fig 2.). Protease and lipase enzymes, related to the ability of the endophytic bacteria as biological control agents. Based on the protease and lipase indexes, *Bacillus* sp. PP.A5 can produce the highest proteases and lipase enzymes compared to other isolates. The isolates that have high protein and fat hydrolysis enzymes have the potential as biological control agents because proteins and fats are constituents of pathogen cells (Mota et al 2016). Besides, the protease enzyme is thought to degrade antibiotics produced by fungal or bacterial pathogens. According to Anderson et al. (2014), the extracellular protease enzyme produced by *P. fluorescens* can inactivate antibiotic compounds produced by *Pantoea agglomerans.*

*Bacillus* sp. PPD.B2 has the highest phosphate solubility index. The phosphate solubilization is related to the ability of endophytic bacteria as a plant growth promoter, providing phosphates for plants. Microbes with high phosphate solubility activity are capable of producing and releasing metabolites such as organic acids that chelate cations that are bound to phosphate (especially calcium) and converting them into soluble forms. Solubilization of different forms of phosphate by microbes associated with plants, and increasing its availability for plants, will increase growth and production of the plant (Djuric et al., 2011)


**Figure 2.** Hydrolysis enzyme activity, (a) protease , (b) lipase and (c) phosphate solubilization.

## CONCLUSION

Based on research carried out, it has been successfully isolated, morphologically and biochemically characterized four the endophytic bacteria that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

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# Naskah Perbaikan 2

## Short Communication: Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control

**Abstract.** Sheath blight and bacterial wilt are diseases that can reduce maize production. Biological control with the endophytic bacteria offers environmentally friendly control for these pathogens. The study was aimed to isolate and characterize the endophytic bacteria morphologically and biochemically and to study their potential to control maize diseases, especially sheat blight and bacterial wilt causing pathogens. The study was conducted at the Plant Protection Laboratory, Faculty of Agriculture, Jenderal Soedirman University, from April to August 2019. The study consisted of four stages: isolation and characterization of endophytic bacteria, the antagonism test of the endophytic bacterial to *R solani*, the antagonism test of the endophytic bacteria to *Pantoea* sp., and the mechanism test of the endophytic bacteria as biological control agents and plant growth-promoting bacteria. Based on the research, four endophytic bacteria isolates have -been successfully isolated, and characterized successfully and found have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. *Bacillus* sp, endophytic from the root (BK.A1; BK.A3; PP.A5) and *Bacillus* sp. endophytic from the stem (PPD.B2) can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

**Key words:** *Bacillus* sp., fluorescent *Pseudomonas*, *Pantoea* sp, *Rhizoctoni solani*

**Running title:** Isolation and characterization of the endophytic

## INTRODUCTION

Maize is a strategic food commodity in the world. In Indonesia, the government seeks to achieve self-sufficiency in maize through increasing production of sustainable maize. However, these efforts have faced several obstacles; one of them is the presence of plant diseases such as sheath blight caused by *Rhizoctonia solani* Kuhn, and bacterial wilt caused by (*Pantoea stewartii)*. *R. solani* can infect up to the midrib of the cob (Djaenuddin et al. 2017), resulting in up to 100% decrease in the yield (Muis 2007). *Pantoea* sp. can attack all stages of the plant causing wilting and leaf blight, and is known as Stewart's wilt (Pataky 2004; Ammar et al. 2014). The pathogens can cause 40-100% yield loss.

Over the past 3 decades, the concept of sustainable and environmentally friendly agriculture has been carried out by minimizing the use of chemicals, both synthetic fertilizers and pesticides. In the management of pests and plant diseases, biological control is developed by applying biological agents including the endophytic bacteria (Shanti and Vittal 2013). Many endophytic bacteria can pass the endodermic barrier across from the root cortex to the vascular system, and subsequently develop as endophytes in stems, leaves, tubers, and other organs (Compant et al. 2005). The use of endophytic bacteria as biological agents has an advantage compared to rhizosphere bacteria because endophytic bacteria live and survive in the plant tissue during plant development, thus protecting the plants.

*Bacillus* sp. and fluorescent *Pseudomonas* are reported to be able to live as endophytes and are widely used as biological control agents for soil-borne and air-borne diseases. The endophytic bacteria could control plant diseases through several mechanisms including competition, hyperparasitism, producing microbial inhibiting compounds (antibiotics, lysis enzymes, other physical or chemical disorders), enhancing plant resistance, and promoting plant growth (Compant et al 2005, Pal and McSpadden 2006; Rosenblueth and Martinez Romero 2006).

Based on the mechanisms, the use of endophytic bacteria isolated from maize, both upland and lowland, suggested potentially alternative control for sheath blight (*R. solani*) and bacterial wilt (*Pantoea* sp). The research aimed to isolate and characterize morphologically and biochemically the endophytic bacteria as well as their potential to control pathogens that cause disease in maize especially *R. solani* and *Pantoea* sp.

## MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Protection, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia, from April to August 2019

### Isolation *R. solani*

*R. solani* was isolated from maize with sheath blight symptoms and there was sclerotium as a resistant structure from the pathogenic fungi in Banyumas. *R. solani* isolation was carried out based on Al-Fadhal et al. 2019. Disease samples were cut 0.5 x 0,5 cm, then sterilized with NaOCl (1%) for 2 min, and rinsed with sterile water 3 times. Disease samples pieces were then dried using sterile filter papers, and transferred to Petri dishes containing PDA medium to obtain pure *R. solani* isolates.

### Isolation *Pantoea* sp.

*Pantoea* sp. was isolated from diseased maize samples taken from the maize growing area in Banyumas Regency according to Coplin et al. (2012); Aini et al.(-2013) and Desi et al. (2014). Diseased leaves or stems were washed with running water, then dried with a tissue. Diseased samples were cut 1.5 x 5 cm, then sterilized with ethanol 70% and rinsed with sterile water 3 times. Furthermore, the sample was crushed with 5 ml of sterile distilled water using a sterile mortar. The bacterial suspension was streaked on nutrient agar and incubated 3-5 days. Bacterial colonies that exhibit the character of *Pantoea* sp. were yellow, shiny, slimy, flat or convex, then separated as pure cultures of stewartii candidates. The culture was then tested by Gram Reaction (KOH test), Hugh-Leifson test, pigment production in YDC medium, oxidase test, hypersensitivity test, and pathogenicity test on maize.

### Isolation and characterization of endophytic bacteria

Sampling for the isolation of endophytic bacteria was carried out in Banyumas and Purbalingga, Central Java, Indonesia, with purposive stratified random sampling. Samples were taken from two areas of altitude, i.e., low-moderate lands (0-500 m above sea level), and highlands (> 500 m above sea level) (Nuryanto et al., 2014). In each district, 2 locations were selected for the low-medium lands, and 1 location for the highlands. Age of maize plants was 20-30 days after planting, when the number of endophytic microbial populations that can be cultured is in the highest population (Cavaglieri et al. 2009).

The endophytic bacteria were isolated from the roots and stems of healthy maize plants. Roots and stems were washed, sterilized with 70% ethanol (1 minute), 20% natrium hypochlorite (5 minutes) and Ringer's thiosulfate solution (5 minutes). Separately, the roots and stems of 10 g each were crushed with 90 ml PBS on a sterile mortar. Subsequently, samples were plated on NA and Kings B media (Cavaglieri et al. 2009). To isolate *Bacillus* sp., the suspension was heated for 10 minutes at 80 ° C, before plating on NA. Bacterial isolates were further purified and characterized, such as morphological characteristics, gram properties, catalase tests, and hypersensitivity tests

### The antagonism test of endophytic bacterial to *R solani*

The antagonism test of endophytic bacteria on *R. solani* was carried out using the dual culture method. The level of inhibition of antagonist is calculated using the formula (Abidin et al., 2015).

$$I = \frac{C - T}{C} \times 100\%$$

I = The level of inhibition of antagonist (%)
C = The radius of pathogen colonies opposite antagonist
T = The radius of the colony of pathogens towards antagonist

### The antagonism test of endophytic bacterial to *Pantoea* sp.

Antagonism testing was carried out using the double-layer test method (Santiago et al. 2015). Endophytic bacteria to be tested were grown on the nutrien agar medium, incubated at 28 C for 48 hours. In the upside-down position, 1 ml of chloroform was added to the cup lid and left for 2 hours. Next, add 5 mL so that 0.6% water containing 0.5 mL of *Pantoea* sp. bacterial suspension. The culture was re-incubated for 24 hours, and there were clear zones around the antagonistic bacterial colony. The antibiotic activity was assessed based on the diameter of the clear zone compared to the diameter of the colony. Characterization of the type of antibiosis can be divided into bactericidal and bacteriostatic types according to the method of Djatmiko et al. (2007).

### The mechanism test of endophytic bacteria as controlling agents biological and plant growth-promoting microbial

The testing mechanism of endophytic bacteria was carried out for bacteria that have the potential in testing the antagonism of the fungus *R. solani* and *Pantoea* sp.

*Protease Test*

The activity of the ability of antagonistic bacteria to produce extracellular protease enzymes was tested using Skim Milk Agar (SMA) medium. Each bacterium to be tested was grown in a medium SMA and incubated at 28 C for 24-48 hours. The presence of clear zones around the colony shows that positive bacteria produce protease enzymes (Abed et al. 2016). The protease activity index is assessed based on the diameter of the clear zone compared to the diameter of the colony..

$$\text{Protease index} = \frac{(\text{clear zone diameter} - \text{colony diameter})}{\text{colony diameter}}$$

*Lipase test*

Detection of the ability of bacteria to produce the enzyme lipase was done by growing the antagonistic bacteria on a medium containing 1% Tween 80. The presence of lipase enzyme activity was demonstrated by milky white sediment around the bacterial colony, after incubating at 28 C for 4-7 days. The lipolytic index was measured using a formula Djuric et al. (201).

$$\text{Lipolytic index} = \frac{(\text{milky white diameter} - \text{colony diameter})}{\text{colony diameter}}$$

*Uji fosfatase*

Detection of the ability of bacteria to produce the enzyme phosphatase was done by growing bacterial isolates on Pikovkaya medium. After incubating for 7 days at 28 C, the presence of a clear zone around the bacterial colony shows that the bacteria has the ability to produce the phosphatase enzyme to dissolve phosphates. The solubility index is measured using a formula (Farooq and Bano 2013)

$$\text{Phosphatase Index} = \frac{\text{clear zone diameter} - \text{colony diameter}}{\text{colony diamater}}$$

## RESULTS AND DISCUSSION

**Isolation and characterization of endophytic bacteria**

The results of the exploration, isolation, and characterization of endophytic bacteria obtained 23 isolates of endophytic bacteria, consisted of 9 isolates of the fluorescent *Pseudomonas* and 14 isolates of *Bacillus* sp. (Table 1). *Fluorescent Pseudomonas* colony on King's B was round, with a flat edge, and yellowish-white, to greenish-yellow, gram-negative, rod-shaped, non-spore and fluorescent under ultraviolet light. Singh et al. 2017 reported fluorescent *Pseudomonas* showed light green, yellowish, creamy, circular, slimy, regular-irregular characteristics. Bacteria have short-long rod forms. The Fluorescent *Pseudomonas* isolates is gram-negative, which can form catalase, a positive oxidase, needed to grow aerobes.

*Bacillus* sp. was observed with spherical colony having cell rod-shaped, gram-positive, and endospores within cells (Table 1.). Slepecky and Hempill 2006; Amin et al. 2015 reported *Bacillus* sp. has the characteristics of a circular colony and punctiform (small round), variations in the entire margin and lobate, white dull, non-slimy, gram-positive, has endospores, flagellum and some are motile.

Based on its distribution, fluorescent *Pseudomonas* and *Bacillus* sp. found in all sampling locations, high or low-medium lands. This shows that fluorescent *Pseudomonas* and *Bacillus* sp. spread and can live in various altitudes, both high and low-medium land. Bacon and Hilton 2002; Ganeshan and Kumar 2005 reported *P. fluoresscens* and *Bacillus* sp., are species of bacteria with a wide range of life and are very adaptive in various environments. Both types of bacteria are also found in the roots or corn stalks. *Bacillus* sp. and *Pseudomonas* sp. including a group of endophytic bacteria have a wide range of life and more isolated in maize (Ganeshan and Kumar 2005; Orole and Adejumo 2011; Costa et al. 2013)

**Antagonism test between the endophytic bacteria against *R. solani***

Based on the results of *in vitro* tests (Table 2), 24 isolates of the endophytic bacteria were able to inhibit the growth of *R. solani*, with varying degrees of inhibition. The endophytic bacteria that have inhibition rates above 50%, i.e fluorescent *Pseudomonas* BK.A1 (51%), *Bacillus* sp. B.K.A1 (55.39%), *Bacillus* sp BK.A3 (51.52%), PP.A5 (50.66%), and PPD.B2 ( 50.8%). The effect of the endophytic bacteria in inhibiting the growth *of R. solani* is inversely proportional to the dry weight mycelium. The greater the percentage of inhibition of endophytic bacteria to the growth of *R. solani*, the smaller the dry weight mycelium (Table 2.)

Endophytic bacteria can inhibit the growth of *R. solani*, which were shown by the inhibitory zone in the area bordering the bacterial streak (Fig. 1a). The endophytic bacteria have anti-pathogenic properties and can produce antibiotic compounds. The ability of the endophytic bacteria to control plant pathogens occurs through the mechanism of antibiosis, competition, lysis, inducing resistance and producing growth substances. Bacteria capable of producing secondary metabolites that can inhibit growth or damage pathogens (Hastuti et al. 2014). These compounds, including alkaloids, phenols, flavonoids, glycosides, and phytoalexin (Soesanto et al. 2010). Fluorescent *Pseudomonas* can produce various types of antibiotics including phenazine-1-carboxylic acid, pyocyanin, pyrrolnitrin, and pyoluteorin and

**Table 1.** Isolation and characterization of endophytic bacteria

| Land | Sampling location | sample | Gram test | Catalase test | oxidase test | Colony morphology | colony pigment | Fluorescence on KB Medium | Cell morphology | Endo spores | Isolat |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Highland | 1. Purbalingga, Pratin 7.13'33" LS, 109.17'21" BT, TT 1.190 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* PP.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PP.A3 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. PP.A5 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* PP. B4 |
| | 2.Banyumas, Baturaden 7.19"1" LS, 109.14'29" BT, TT 520 m dpl | Root | - | + | + | round | Greenish yellow | + | Medium rod | - | fluorescent *Pseudomonas* BB.A2 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BB.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB.B4 |
| Medium-Lowland | 1.Banyumas, Sumbang7.21'54" LS, 109.17'33"BT, TT 200 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas (Pf)* BS.A2 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BS.A1 |
| | | Root | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS.A3 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. BS. B1 |
| | 2.Purbalingga, Bojongsari, 7.20'12" LS, 109.20'22" BT, TT 190 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | fluorescent *Pseudomonas (Pf)* PB. A 4 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PB. B1 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BB. B3 |
| | 3.Purbalingga, Padamara, 7.22'28" LS, 109.13'24" BT, TT 180 m dpl | Root | - | + | + | round | Greenish yellow | + | Small rod | - | fluorescent *Pseudomonas (Pf)* PPD A1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas (Pf)* PPD. B1 |
| | | Stem | - | + | + | round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas (Pf)* PPD. B5 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B2 |
| | | Stem | + | + | + | round | white | - | Small rod | + | *Bacillus* sp. PPD. B4 |
| | 4.Banyumas, Kembaran 7.23'47" LS, 109.17'9" BT, TT 110 m dpl | Root | - | + | + | round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas (Pf)* BK. A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A1 |
| | | Root | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.A3 |
| | | Stem | + | + | + | round | white | - | Medium rod | + | *Bacillus* sp. BK.B3 |

2,4-diacetylphloroglucinol (Phl). Phl is a phenolic metabolite with antibacterial and antifungal (Jain and Das 2016). Bacillus species can produce various kinds of volatile compounds and diffusible with strong inhibitory activity against plant pathogens (Lim et al. 2017).

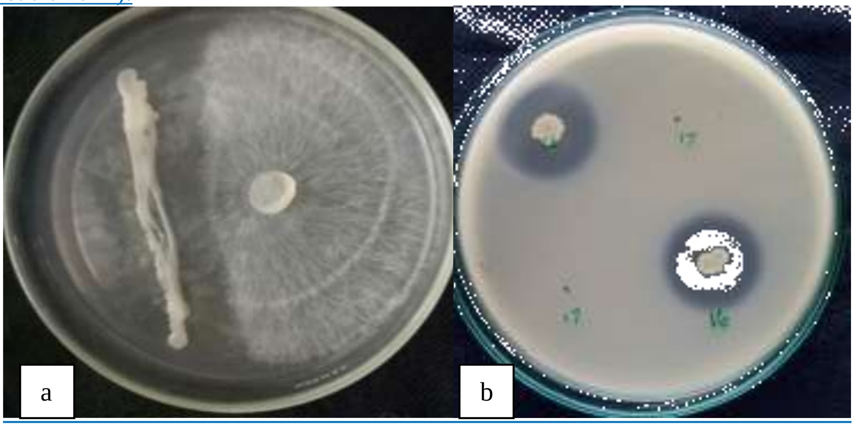


**Figure 1.** Antagonism test between the endophytic bacteria against *R. solani* (a) and *Pantoea* sp (b)

**Table 2.** Inhibition of endophytic bacteria against *R. solani*

| No | Isolate | Inhibition rate (%) | Dry weights mycelium |
|---|---|---|---|
| 1 | Control | 0 | 0,093 |
| Endophytic bacteria from the root | | | |
| 2 | fluorescent *Pseudomonas* BB.A2 | 49,00 | 0,038 |
| 3 | fluorescent *Pseudomonas* BS.A 2 | 45,00 | 0,027 |
| 4 | fluorescent *Pseudomonas* BK.A1 | 51,00 | 0,017 |
| 5 | fluorescent *Pseudomonas* PPD.A1 | 10,33 | 0,059 |
| 6 | fluorescent *Pseudomonas* PP.A1 | 38,33 | 0,017 |
| 7 | fluorescent *Pseudomonas* PB.A4 | 18,00 | 0,037 |
| 8 | *Bacillus* sp.BB.A3 | 40,42 | 0,030 |
| 9 | *Bacillus* sp.BS.A1 | 48,73 | 0,016 |
| 10 | *Bacillus* sp. BSA3 | 37,42 | 0,039 |
| 11 | *Bacillus* sp. B.K.A1 | 55,39 | 0,002 |
| 12 | *Bacillus* sp. B.K.A3 | 51,52 | 0,003 |
| 13 | *Bacillus* sp.PP.A3 | 46,65 | 0,019 |
| 14 | *Bacillus* sp.PP.A5 | 50,66 | 0,009 |
| Endophytic bacteria from the stem | | | |
| 15 | fluorescent *Pseudomonas* PPD.B1 | 27,00 | 0,020 |
| 16 | fluorescent *Pseudomonas* PPD.B5 | 49,33 | 0,013 |
| 17 | fluorescent *Pseudomonas* PP.B4 | 65,67 | 0,004 |
| 18 | *Bacillus* sp. BB.B4 | 44,44 | 0,026 |
| 19 | *Bacillus* sp. BS.B1 | 49,74 | 0,012 |
| 20 | *Bacillus* sp. BK. B3 | 40,36 | 0,031 |
| 21 | *Bacillus* sp.PPD.B2 | 50,8 | 0,007 |
| 22 | *Bacillus* sp. PPD.B4 | 39,44 | 0,036 |
| 23 | *Bacillus* sp. PB.B1 | 37,29 | 0,047 |
| 24 | *Bacillus* sp. PB.B3 | 44,9 | 0,022 |

**The endophytic bacterial antagonism test against *Pantoea* sp.**

The results of antagonism between the endophytic bacteria and *Pantoea* sp. show varied results. The endophytic bacteria that can inhibit bacterial growth were indicated by the presence of clear zones around the endophytic bacterial colonies (Fig.1). From the nine isolate fluorescent *Pseudomonas* were tested, only three isolates were able to inhibit the growth of the *Pantoea* sp., i.e Pf BS.A2, Pf BK.A1 Pf PPD.B5. While the isolates Pf BB.A2, Pf PPD.A1, Pf PP.A1, Pf

PPD.B1, and Pf PP.B4 are not able to inhibit the growth of pathogenic bacteria. Meanwhile, all isolate *Bacillus* sp. tested (thirteen isolates) were able to inhibit the growth of *Pantoea* sp. (Table 3).

The presence of clear zones around endophytic bacterial colonies showed the ability of endophytic bacteria to produce antibiotics to inhibit the growth of *Pantoea* sp. *P. fluorescens* P60 can produce antibiotics that inhibit the growth of pathogens (Soesanto 2011). *Pseudomonas fluorescens* is reported to produce phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4 diacetyl phloroglucinol (DAPG), pyrrolnitrin (PRN) and or pyoluteorin (Plt) (Heydari and Pessarakli 2010). Nasrun and Burhanudin (2016) mention that *P. fluorescens* produce secondary metabolites, i.e. antimicrobial, cyanide acid and 2,4-diacetyl phloroglucinol phenazine, pyrrolnitrin, pyoluteorin antibiotics.

The level of bacteria's ability to inhibit growth can be shown by the large diameter of the clear zone. The results showed that the antagonism index ranged from 1.67 - 8.17. Based on this index, most endophytic bacteria have a strong antagonism (index of antagonism> 4) (Davis and Stout 1971). Furthermore, bacterial isolates that showed antagonistic activity were tested for types of antagonism based on Djatmiko (2007). Based on the type of antagonistic activity, ten isolates the endophytic bacteria were bacteriostatic and nine isolates the endophytic bacteria were bactericidal. Bacteriostatic activity, growth inhibition is temporary, it is shown that regrowth of bacteria after being transferred to a new medium, which is free from the influence of antagonistic bacteria. Bactericidal activity, inhibition is permanent. Bacteria were unable to grow even though they are transferred to new medium.

**Test the mechanism of endophytic bacteria as biological control agents and plant growth-promoting microbes**

The mechanism test was carried out on endophytic bacteria that have the potential to control the fungus *R. solani* and *Pantoea* sp., i.e. *Bacillus* sp. B.K.A1, *Bacillus* sp. B.K.A3, *Bacillus* sp. PP.A5, *Bacillus* sp. PPD.B2. The production of compounds related to biocontrol of pathogens and promotion of plant growth in bacterial isolates was evaluated by measuring the production of antimicrobial compounds and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and phosphate solubilization. The results of enzyme activity tests are as shown in Table 4. The four isolates *Bacillus* sp. tested were able to produce protease, lipase and phosphatase enzymes, with varied activity indexes. All isolates of *Bacillus* sp. those tested had a high index of protease and lipase enzymes (> 3) (Table 4., Fig 2.). Protease and lipase enzymes, related to the ability of the endophytic bacteria as biological control agents. Based on the protease and lipase indexes, *Bacillus* sp. PP.A5 can produce the highest proteases and lipase enzymes compared to other isolates. The isolates that have high protein and fat hydrolysis enzymes have the potential as biological control agents because proteins and fats are constituents of pathogen cells (Mota et al 2016). Besides, the protease enzyme is thought to degrade antibiotics produced by fungal or bacterial pathogens. According to Anderson et al. (2014), the extracellular protease enzyme produced by *P. fluorescens* can inactivate antibiotic compounds produced by *Pantoea agglomerans.*

*Bacillus* sp. PPD.B2 has the highest phosphate solubility index. The phosphate solubilization is related to the ability of endophytic bacteria as a plant growth promoter, providing phosphates for plants. Microbes with high phosphate solubility activity are capable of producing and releasing metabolites such as organic acids that chelate cations that are bound to phosphate (especially calcium) and converting them into soluble forms. Solubilization of different forms of phosphate by microbes associated with plants, and increasing its availability for plants, will increase growth and production of the plant (Djuric et al., 2011).

**Figure 2.** Hydrolysis enzyme activity, (a) protease , (b) lipase and (c) phosphate solubilization.

**Table 3.** Inhibition of endophytic bacteria against *Pantoea* sp.

| No | Isolate | Antagonism | Antagonism index | Antagonism catagory* | Antagonism activity |
|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | |
| 1 | fluorescent *Pseudomonas* BB.A2 | - | 0 | - | - |
| 2 | fluorescent *Pseudomonas* BS.A 2 | + | 4,91 | strong | bacteriostatic |
| 3 | fluorescent *Pseudomonas* BK.A1 | + | 4,42 | strong | bacteriostatic |
| 4 | fluorescent *Pseudomonas* PPD.A1 | - | 0 | - | - |
| 5 | fluorescent *Pseudomonas* PP.A1 | - | 0 | - | - |
| 6 | fluorescent *Pseudomonas* PB.A4 | + | 5,29 | strong | bactericidal |
| 7 | *Bacillus* sp.BB.A3 | + | 8,17 | strong | bacteriostatic |
| 8 | *Bacillus* sp.BS.A1 | + | 4,00 | strong | bacteriostatic |
| 9 | *Bacillus* sp. BSA3 | + | 5,07 | strong | bactericidal |
| 10 | *Bacillus* sp. B.K.A1 | + | 4,01 | strong | bakteriostatik |
| 11 | *Bacillus* sp. B.K.A3 | + | 4,91 | strong | bacteriostatic |
| 12 | *Bacillus* sp.PP.A3 | + | 6,63 | strong | bactericidal |
| 13 | *Bacillus* sp.PP.A5 | + | 6,56 | strong | bactericidal |
| Endophytic bacteria from the stem | | | | | |
| 14 | fluorescent *Pseudomonas* PPD.B1 | - | 0 | - | - |
| 15 | fluorescent *Pseudomonas* PPD.B5 | + | 5,86 | strong | bactericidal |
| 16 | fluorescent *Pseudomonas* PP.B4 | - | 0 | - | - |
| 17 | *Bacillus* sp. BB.B4 | + | 7,80 | strong | bactericidal |
| 18 | *Bacillus* sp. BS.B1 | + | 6,22 | strong | bacteriostatic |
| 19 | *Bacillus* sp. BK. B3 | + | 5,33 | strong | bacteriostatic |
| 20 | *Bacillus* sp.PPD.B2 | + | 5,00 | strong | bacteriostatic |
| 21 | *Bacillus* sp. PPD.B4 | + | 8,75 | strong | bacteriostatic |
| 22 | *Bacillus* sp. PB.B1 | + | 1,67 | weak | bacteriostatic |
| 23 | *Bacillus* sp. PB.B3 | + | 5,67 | strong | bactericidal |

•Based on Davis and Stout, 1971

**Table 4.** Test results of proteases, lipases and phosphate solubilization.

| No | Isolate | Protease Test activity | index | Lipase Test activity | index | Phosphate solubilization Activity | index |
|---|---|---|---|---|---|---|---|
| Endophytic bacteria from the root | | | | | | | |
| 1 | *Bacillus* sp. B.K.A1 | + | 3.75 | + | 3.23 | + | 1.17 |
| 2 | *Bacillus* sp. B.K.A3 | + | 3.20 | + | 3.73 | + | 1.27 |
| 3 | *Bacillus* sp. PP.A5 | + | 5.00 | + | 4.40 | + | 1.46 |
| Endophytic bacteria from the stem | | | | | | | |
| 4 | *Bacillus* sp.PPD.B2 | + | 3.00 | + | 3.90 | + | 2.60 |

## CONCLUSION

Based on research carried out, it has been successfully isolated, morphologically and biochemically characterized four the endophytic bacteria that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

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# Short Communication: Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control

**ENDANG MUGIASTUTI♥, SUPRAYOGI, NUR PRIHATININGSIH, LOEKAS SOESANTO**
Faculty of Agriculture, Universitas Jenderal Soedirman. Jl. Dr. Suparno, Karangwangkal, Purwokerto Utara, Banyumas 53123, Central Java, Indonesia. Tel./fax.: +62-281-638791, ♥email: endangmugiastuti@gmail.com.

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**Abstract.** *Mugiastuti E, Suprayogi, Prihatiningsih N, Soesanto L. 2020. Short Communication: Isolation And Characterization Of The Endophytic Bacteria, And Their Potential As Maize Diseases Control. Biodiversitas 21: xxxx.* Sheath blight and bacterial wilt are diseases that can reduce maize production. Biological control with the endophytic bacteria offers environmentally friendly control for these pathogens. The study ~~was~~ aimed to isolate and characterize the endophytic bacteria morphologically and biochemically and to study their potential to control maize diseases, especially sheat blight and bacterial wilt causing pathogens. The study was conducted at the Plant Protection Laboratory, Faculty of Agriculture, Jenderal Soedirman University, from April to August 2019. The study consisted of four stages: isolation and characterization of endophytic bacteria, the antagonism test of the endophytic bacterial to *R solani,* the antagonism test of the endophytic bacteria to *Pantoea* sp., and the mechanism test of the endophytic bacteria as biological control agents and plant growth-promoting bacteria. Based on the research, four endophytic bacteria isolates have been successfully isolated, and characterized successfully and found have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. *Bacillus* sp, endophytic from the root (BK.A1; BK.A3; PP.A5) and *Bacillus* sp. endophytic from the stem (PPD.B2) can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

**Keywords:** *Bacillus*, fluorescent *Pseudomonas, Pantoea, Rhizoctoni solani*

## INTRODUCTION

Maize is a strategic food commodity in the world. In Indonesia, the government seeks to achieve self-sufficiency in maize through increasing production of sustainable maize. However, these efforts have faced several obstacles; one of them is the presence of plant diseases such as sheath blight caused by *Rhizoctonia solani* Kuhn, and bacterial wilt caused by ~~(~~*Pantoea stewartii*~~)~~. *R. solani* can infect up to the midrib of the cob (Djaenuddin et al. 2017), resulting in up to 100% decrease in the yield (Muis 2007). *Pantoea* sp. can attack all stages of the plant causing wilting and leaf blight, and is known as Stewart's wilt (Pataky 2004; Ammar et al. 2014). The pathogens can cause 40-100% yield loss.

Over the past 3 decades, the concept of sustainable and environmentally friendly agriculture has been carried out by minimizing the use of chemicals, both synthetic fertilizers and pesticides. In the management of pests and plant diseases, biological control is developed by applying biological agents including the endophytic bacteria (Shanti and Vittal 2013). Many endophytic bacteria can pass the endodermic barrier across from the root cortex to the vascular system, and subsequently develop as endophytes in stems, leaves, tubers, and other organs (Compant et al. 2005). The use of endophytic bacteria as biological agents has an advantage compared to rhizosphere bacteria because endophytic bacteria live and survive in the plant tissue during plant development, thus protecting the plants.

*Bacillus* sp. and fluorescent *Pseudomonas* are reported to be able to live as endophytes and are widely used as biological control agents for soil-borne and air-borne diseases. The endophytic bacteria could control plant diseases through several mechanisms including competition, hyperparasitism, producing microbial inhibiting compounds (antibiotics, lysis enzymes, other physical or chemical disorders), enhancing plant resistance, and promoting plant growth (Compant et al 2005, Pal and McSpadden 2006; Rosenblueth and Martinez-~~—~~Romero 2006).

Based on the mechanisms, the use of endophytic bacteria isolated from maize, both upland and lowland, suggested potentially alternative control for sheath blight (*R. solani*) and bacterial wilt (*Pantoea* sp). The research aimed to isolate and characterize morphologically and biochemically the endophytic bacteria as well as their potential to control pathogens that cause disease in maize especially *R. solani* and *Pantoea* sp.

## MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Protection, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto, Central Java, Indonesia, from April to August 2019

**Isolation *R. solani***

*R. solani* was isolated from maize with sheath blight symptoms and there was sclerotium as a resistant structure from the pathogenic fungi in Banyumas. *R. solani* isolation was carried out based on Al-Fadhal et al. 2019. Disease samples were cut 0.5 x 0,5 cm, then sterilized with NaOCl (1%) for 2 min, and rinsed with sterile water 3 times. Disease samples pieces were then dried using sterile filter papers, and transferred to Petri dishes containing PDA medium to obtain pure *R. solani* isolates.

**Isolation *Pantoea* sp.**

*Pantoea* sp. was isolated from diseased maize samples taken from the maize growing area in Banyumas Regency according to Coplin et al. (2012); Aini et al. (2013) and Desi et al. (2014). Diseased leaves or stems were washed with running water, then dried with a tissue. Diseased samples were cut 1.5 x 5 cm, then sterilized with ethanol 70% and rinsed with sterile water 3 times. Furthermore, the sample was crushed with 5 ml of sterile distilled water using a sterile mortar. The bacterial suspension was streaked on nutrient agar and incubated 3-5 days. Bacterial colonies that exhibit the character of *Pantoea* sp. were yellow, shiny, slimy, flat or convex, then separated as pure cultures of stewartii candidates. The culture was then tested by Gram Reaction (KOH test), Hugh-Leifson test, pigment production in YDC medium, oxidase test, hypersensitivity test, and pathogenicity test on maize.

**Isolation and characterization of endophytic bacteria**

Sampling for the isolation of endophytic bacteria was carried out in Banyumas and Purbalingga, Central Java, Indonesia, with purposive stratified random sampling. Samples were taken from two areas of altitude, i.e., low-moderate lands (0-500 m above sea level), and highlands (> 500 m above sea level) (Nuryanto et al., 2014). In each district, 2 locations were selected for the low-medium lands, and 1 location for the highlands. Age of maize plants was 20-30 days after planting, when the number of endophytic microbial populations that can be cultured is in the highest population (Cavaglieri et al. 2009).

The endophytic bacteria were isolated from the roots and stems of healthy maize plants. Roots and stems were washed, sterilized with 70% ethanol (1 minute), 20% natrium hypochlorite (5 minutes) and Ringer's thiosulfate solution (5 minutes). Separately, the roots and stems of 10 g each were crushed with 90 ml PBS on a sterile mortar. Subsequently, samples were plated on NA and Kings B media (Cavaglieri et al. 2009). To isolate *Bacillus* sp., the suspension was heated for 10 minutes at 80 ° C, before plating on NA. Bacterial isolates were further purified and characterized, such as morphological characteristics, gram properties, catalase tests, and hypersensitivity tests

**The antagonism test of endophytic bacterial to *R solani***

The antagonism test of endophytic bacteria on *R. solani* was carried out using the dual culture method. The level of inhibition of antagonist is calculated using the formula (Abidin et al., 2015).

$$I = \frac{C-T}{C} \times 100\%$$

Where:
I : The level of inhibition of antagonist (%)
C: The radius of pathogen colonies opposite antagonist
T: The radius of the colony of pathogens towards antagonist

**The antagonism test of endophytic bacterial to *Pantoea* sp.**

Antagonism testing was carried out using the double-layer test method (Santiago et al. 2015). Endophytic bacteria to be tested were grown on the nutrien agar medium, incubated at 28 C for 48 hours. In the upside-down position, 1 ml of chloroform was added to the cup lid and left for 2 hours. Next, add 5 mL so that 0.6% water [U1]containing 0.5 mL of *Pantoea* sp. bacterial suspension. The culture was re-incubated for 24 hours, and there were clear zones around the antagonistic bacterial colony. The antibiotic activity was assessed based on the diameter of the clear zone compared to the diameter of the colony. Characterization of the type of antibiosis can be divided into bactericidal and bacteriostatic types ~~according to the method of~~ based on Djatmiko et al. (2007).

**The mechanism test of endophytic bacteria as controlling agents biological and plant growth-promoting microbial.**

The testing mechanism of endophytic bacteria was carried out for bacteria that have the potential in testing the antagonism of the fungus *R. solani* and *Pantoea* sp.

*Protease Test*

The activity of the ability of antagonistic bacteria to produce extracellular protease enzymes was tested using Skim Milk Agar (SMA) medium. Each bacterium to be tested was grown in a medium SMA and incubated at 28 C for 24-48 hours. The presence of clear zones around the colony shows that positive bacteria produce protease enzymes (Abed et al. 2016). The protease activity index is assessed based on the diameter of the clear zone compared to the diameter of the colony.

$$\text{Protease index} = \frac{\text{(clear zone diameter-colony diameter)}}{\text{colony diameter}}$$

*Lipase test*

Detection of the ability of bacteria to produce the enzyme lipase was done by growing the antagonistic bacteria on a medium containing 1% Tween 80. The presence of lipase enzyme activity was demonstrated by milky white sediment around the bacterial colony, after incubating at 28 C for 4-7 days. The lipolytic index was measured using a formula Djuric et al. (201).

Lipolytic index = (milky white diameter-colony diameter) / colony diameter

*Phosfatase test*

Detection of the ability of bacteria to produce the enzyme phosphatase was done by growing bacterial isolates on Pikovkaya medium. After incubating for 7 days at 28 C, the presence of a clear zone around the bacterial colony shows that the bacteria has the ability to produce the phosphatase enzyme to dissolve phosphates. The solubility index is measured using a formula (Farooq and Bano 2013)

Phosphatase Index = (clear zone diameter-colony diameter) / colony diameter

## RESULTS AND DISCUSSION

**Isolation and characterization of endophytic bacteria**

The results of the exploration, isolation, and characterization of endophytic bacteria obtained 23 isolates of endophytic bacteria, consisted of 9 isolates of the fluorescent *Pseudomonas* and 14 isolates of *Bacillus* sp. (Table 1). *Fluorescent Pseudomonas* colony on King's B was round, with a flat edge, and yellowish-white, to greenish-yellow, gram-negative, rod-shaped, non-spore and fluorescent under ultraviolet light. Singh et al. 2017 reported fluorescent *Pseudomonas* showed light green, yellowish, creamy, circular, slimy, regular-irregular characteristics. Bacteria have short-long rod forms. The Fluorescent *Pseudomonas* isolates is gram-negative, which can form catalase, a positive oxidase, needed to grow aerobes. *Bacillus* sp. was observed with a spherical colony having cell rod-shaped, gram-positive, and endospores within cells (Table 1.). Slepecky and Hempill 2006; Amin et al. 2015 reported *Bacillus* sp. has the characteristics of a circular colony and punctiform (small round), variations in the entire margin and lobate, white dull, non-slimy, gram-positive, has endospores, flagellum and some are motile.

Based on its distribution, fluorescent *Pseudomonas* and *Bacillus* sp. found in all sampling locations, high or low-medium lands. This shows that fluorescent *Pseudomonas* and *Bacillus* sp. spread and can live in various altitudes, both high and low-medium land. Bacon and Hilton 2002; Ganeshan and Kumar 2005 reported *P. fluoresscens* and *Bacillus* sp., are species of bacteria with a wide range of life and are very adaptive in various environments. Both types of bacteria are also found in the roots or corn stalks. *Bacillus* sp. and *Pseudomonas* sp. including a group of endophytic bacteria have a wide range of life and more isolated in maize (Ganeshan and Kumar 2005; Orole and Adejumo 2011; Costa et al. 2013)

**Antagonism test between the endophytic bacteria against *R. solani***

Based on the results of *in vitro* tests (Table 2), 24 isolates of the endophytic bacteria were able to inhibit the growth of *R. solani*, with varying degrees of inhibition. The endophytic bacteria that have inhibition rates above 50%, i.e fluorescent *Pseudomonas* BK.A1 (51%), *Bacillus* sp. B.K.A1 (55.39%), *Bacillus* sp BK.A3 (51.52%), PP.A5 (50.66%), and PPD.B2 ( 50.8%). The effect of the endophytic bacteria in inhibiting the growth *of R. solani* is inversely proportional to the dry weight mycelium. The greater the percentage of inhibition of endophytic bacteria to the growth of *R. solani*, the smaller the dry weight mycelium (Table 2.) Endophytic bacteria can inhibit the growth of *R. solani*, which were shown by the inhibitory zone in the area bordering the bacterial streak (Figure 1a). The endophytic bacteria have anti-pathogenic properties and can produce antibiotic compounds. The ability of the endophytic bacteria to control plant pathogens occurs through the mechanism of antibiosis, competition, lysis, inducing resistance and producing growth substances. Bacteria capable of producing secondary metabolites that can inhibit growth or damage pathogens (Hastuti et al. 2014). These compounds, including alkaloids, phenols, flavonoids, glycosides, and phytoalexin (Soesanto et al. 2010). Fluorescent *Pseudomonas* can produce various types of antibiotics including phenazine-1-carboxylic acid, pyocyanin, pyrrolnitrin, and pyoluteorin, 2,4-diacetylphloroglucinol (Phl). Phl is a phenolic metabolite with antibacterial and antifungal (Jain and Das 2016). Bacillus species can produce various kinds of volatile compounds and diffusible with strong inhibitory activity against plant pathogens (Lim et al. 2017).[U2]

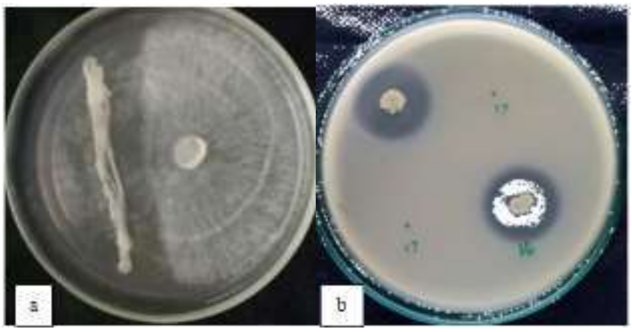


**Figure 1.** Antagonism test between the endophytic bacteria against *R. solani* (a) and *Pantoea* sp (b)

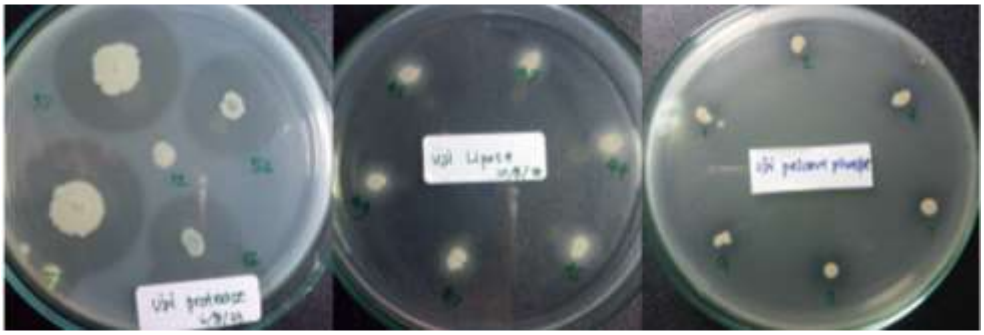

**Figure 2.** Hydrolysis enzyme activity, (a) protease , (b) lipase and (c) phosphate solubilization.

**Table 1.** Isolation and characterization of endophytic bacteria.

| Land | Sampling location | Sample | Gram test | Catalase test | Oxidase test | Colony morpho-logy | Colony pigment | Fluorescence on KB medium | Cell morphology | Endo-spores | Isolate |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Highland | Purbalingga, Pratin | Root | - | + | + | Round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* PP.A1 |
| | 7.13'33" S, 109.17'21" E, | Root | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. PP.A3 |
| | 1.190 m asl | Root | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. PP.A5 |
| | | Stem | - | + | + | Round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* PP. B4 |
| | Banyumas, Baturaden | Root | - | + | + | Round | Greenish yellow | + | Medium rod | - | fluorescent *Pseudomonas* BB.A2 |
| | 7.19"1" S, 109.14'29" E, | Root | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. BB.A3 |
| | 520 m asl | Stem | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BB.B4 |
| Medium-Lowland | Banyumas, Sumbang | Root | - | + | + | Round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* ~~(*Pf*)~~ BS.A2 |
| | 7.21'54" S, 109.17'33"E, | Root | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BS.A1 |
| | 200 m asl | Root | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. BS.A3 |
| | | Stem | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. BS. B1 |
| | Purbalingga, Bojongsari | Root | - | + | + | Round | Greenish yellow | + | Small rod | - | fluorescent *Pseudomonas* ~~(*Pf*)~~ PB. A 4 |
| | 7.20'12" S, 109.20'22" E, | Stem | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. PB. B1 |
| | 190 m asl | Stem | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BB. B3 |
| | Purbalingga, Padamara | Root | - | + | + | Round | Greenish yellow | + | Small rod | - | fluorescent *Pseudomonas* ~~(*Pf*)~~ PPD A1 |
| | 7.22'28" S, 109.13'24" E, | Stem | - | + | + | Round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* ~~(*Pf*)~~ PPD. B1 |
| | 180 m asl | Stem | - | + | + | Round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* ~~(*Pf*)~~ PPD. B5 |
| | | Stem | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. PPD. B2 |
| | | Stem | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. PPD. B4 |
| | Banyumas, Kembaran | Root | - | + | + | Round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* ~~(*Pf*)~~ BK. A1 |
| | 7.23'47" S, 109.17'9" E, | Root | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BK.A1 |
| | 110 m asl | Root | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BK.A3 |
| | | Stem | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BK.B3 |

**Table 2.** Inhibition of endophytic bacteria against *R. solani.*

| Isolate | Inhibition rate (%) | Dry weights mycelium |
|---|---|---|
| Control | 0 | 0.093 |
| Endophytic bacteria from the root | | |
| fFluorescent *Pseudomonas* BB.A2 | 49.00 | 0.038 |
| fFluorescent *Pseudomonas* BS.A 2 | 45.00 | 0.027 |
| fluorescent *Pseudomonas* BK.A1 | 51.00 | 0.017 |
| fluorescent *Pseudomonas* PPD.A1 | 10.33 | 0.059 |
| fluorescent *Pseudomonas* PP.A1 | 38.33 | 0.017 |
| fluorescent *Pseudomonas* PB.A4 | 18.00 | 0.037 |
| *Bacillus* sp.BB.A3 | 40.42 | 0.030 |
| *Bacillus* sp.BS.A1 | 48.73 | 0.016 |
| *Bacillus* sp. BSA3 | 37.42 | 0.039 |
| *Bacillus* sp. B.K.A1 | 55.39 | 0.002 |
| *Bacillus* sp. B.K.A3 | 51.52 | 0.003 |
| *Bacillus* sp.PP.A3 | 46.65 | 0.019 |
| *Bacillus* sp.PP.A5 | 50.66 | 0.009 |
| Endophytic bacteria from the stem | | |
| fluorescent *Pseudomonas* PPD.B1 | 27.00 | 0.020 |
| fluorescent *Pseudomonas* PPD.B5 | 49.33 | 0.013 |
| fluorescent *Pseudomonas* PP.B4 | 65.67 | 0.004 |
| *Bacillus* sp. BB.B4 | 44.44 | 0.026 |
| *Bacillus* sp. BS.B1 | 49.74 | 0.012 |
| *Bacillus* sp. BK. B3 | 40.36 | 0.031 |
| *Bacillus* sp.PPD.B2 | 50.8 | 0.007 |
| *Bacillus* sp. PPD.B4 | 39.44 | 0.036 |
| *Bacillus* sp. PB.B1 | 37.29 | 0.047 |
| *Bacillus* sp. PB.B3 | 44.9 | 0.022 |

2[U3],4 diacetylphloroglucinol (Phl). Phl is a phenolic metabolite with antibacterial and antifungal (Jain and Das 2016). Bacillus species can produce various kinds of volatile compounds and diffusible with strong inhibitory activity against plant pathogens (Lim et al. 2017).

**The endophytic bacterial antagonism test against *Pantoea* sp.**

The results of antagonism between the endophytic bacteria and *Pantoea* sp. show varied results. The endophytic bacteria that can inhibit bacterial growth were indicated by the presence of clear zones around the endophytic bacterial colonies (Fig.1). From the nine isolate fluorescent *Pseudomonas* were tested, only three isolates were able to inhibit the growth of the *Pantoea* sp., i.e fluorescent *Pseudomonas* (Pf) BS.A2, Pf BK.A1 Pf PPD.B5. While the isolates Pf BB.A2, Pf PPD.A1, Pf PP.A1, Pf PPD.B1, and Pf PP.B4 are not able to inhibit the growth of pathogenic bacteria. Meanwhile, all isolate *Bacillus* sp. tested (thirteen isolates) were able to inhibit the growth of *Pantoea* sp. (Table 3).

The presence of clear zones around endophytic bacterial colonies showed the ability of endophytic bacteria to produce antibiotics to inhibit the growth of *Pantoea* sp. *P. fluorescens* P60 can produce antibiotics that inhibit the growth of pathogens (Soesanto 2011). *Pseudomonas fluorescens* is reported to produce phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4 diacetyl phloroglucinol (Phl)DAPG), pyrrolnitrin (PRN) and or pyoluteorin (Plt) (Heydari and Pessarakli 2010). Nasrun and Burhanudin (2016) mention that *P. fluorescens* produce secondary metabolites, i.e. antimicrobial, cyanide acid and 2,4-diacetyl phloroglucinol phenazine, pyrrolnitrin, pyoluteorin antibiotics.

**Table 3.** Inhibition of endophytic bacteria against *Pantoea* sp.

| Isolate | Antagonism | Antagonism index | Antagonism catagory* | Antagonism activity |
|---|---|---|---|---|
| Endophytic bacteria from the root | | | | |
| fluorescent *Pseudomonas* BB.A2 | - | 0 | - | - |
| fluorescent *Pseudomonas* BS.A 2 | + | 4,91 | Strong | Bacteriostatic |
| fluorescent *Pseudomonas* BK.A1 | + | 4,42 | Strong | Bacteriostatic |
| fluorescent *Pseudomonas* PPD.A1 | - | 0 | - | - |
| fluorescent *Pseudomonas* PP.A1 | - | 0 | - | - |
| fluorescent *Pseudomonas* PB.A4 | + | 5,29 | Strong | Bactericidal |
| *Bacillus* sp.BB.A3 | + | 8,17 | Strong | Bacteriostatic |
| *Bacillus* sp.BS.A1 | + | 4,00 | Strong | Bacteriostatic |
| *Bacillus* sp. BSA3 | + | 5,07 | Strong | Bactericidal |
| *Bacillus* sp. B.K.A1 | + | 4,01 | Strong | Bakteriostatik |
| *Bacillus* sp. B.K.A3 | + | 4,91 | Strong | Bacteriostatic |
| *Bacillus* sp.PP.A3 | + | 6,63 | Strong | Bactericidal |
| *Bacillus* sp.PP.A5 | + | 6,56 | Strong | Bactericidal |
| Endophytic bacteria from the stem | | | | |
| fluorescent *Pseudomonas* PPD.B1 | - | 0 | - | - |
| fluorescent *Pseudomonas* PPD.B5 | + | 5,86 | Strong | Bactericidal |
| fluorescent *Pseudomonas* PP.B4 | - | 0 | - | - |
| *Bacillus* sp. BB.B4 | + | 7,80 | Strong | Bactericidal |
| *Bacillus* sp. BS.B1 | + | 6,22 | Strong | Bacteriostatic |
| *Bacillus* sp. BK. B3 | + | 5,33 | Strong | Bacteriostatic |
| *Bacillus* sp.PPD.B2 | + | 5,00 | Strong | Bacteriostatic |
| *Bacillus* sp. PPD.B4 | + | 8,75 | Strong | Bacteriostatic |
| *Bacillus* sp. PB.B1 | + | 1,67 | Weak | Bacteriostatic |

| | | | | |
|---|---|---|---|---|
| *Bacillus* sp. PB.B3 | + | 5,67 | Strong | Bactericidal |

Note: •Based on Davis and Stout, 1971

**Table 4.** Test results of proteases, lipases and phosphate solubilization.

| **Isolate** | **Protease test** | | **Lipase test** | | **Phosphate solubilization** | |
|---|---|---|---|---|---|---|
| | **Activity** | **Index** | **Activity** | **Index** | **Activity** | **Index** |
| Endophytic bacteria from the root | | | | | | |
| *Bacillus* sp. B.K.A1 | + | 3.75 | + | 3.23 | + | 1.17 |
| *Bacillus* sp. B.K.A3 | + | 3.20 | + | 3.73 | + | 1.27 |
| *Bacillus* sp. PP.A5 | + | 5.00 | + | 4.40 | + | 1.46 |
| Endophytic bacteria from the stem | | | | | | |
| *Bacillus* sp.PPD.B2 | + | 3.00 | + | 3.90 | + | 2.60 |

The level of bacteria's ability to inhibit growth can be shown by the large diameter of the clear zone. The results showed that the antagonism index ranged from 1.67-8.17. Based on this index, most endophytic bacteria have a strong antagonism (index of antagonism> 4) (Davis and Stout 1971). Furthermore, bacterial isolates that showed antagonistic activity were tested for types of antagonism based on Djatmiko (2007). Based on the type of antagonistic activity, ten isolates the endophytic bacteria were bacteriostatic and nine isolates the endophytic bacteria were bactericidal. Bacteriostatic activity, growth inhibition is temporary, it is shown that regrowth of bacteria after being transferred to a new medium, which is free from the influence of antagonistic bacteria. Bactericidal activity, inhibition is permanent. Bacteria were unable to grow even though they are transferred to new medium.

**Test the mechanism of endophytic bacteria as biological control agents and plant growth-promoting microbes**

The mechanism test was carried out on endophytic bacteria that have the potential to control the fungus *R. solani* and *Pantoea* sp., i.e. *Bacillus* sp. B.K.A1, *Bacillus* sp. B.K.A3, *Bacillus* sp. PP.A5, *Bacillus* sp. PPD.B2. The production of compounds related to biocontrol of pathogens and promotion of plant growth in bacterial isolates was evaluated by measuring the production of antimicrobial compounds and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and phosphate solubilization. The results of enzyme activity tests are as shown in Table 4. The four isolates *Bacillus* sp. tested were able to produce protease, lipase and phosphatase enzymes, with varied activity indexes. All isolates of *Bacillus* sp. those tested had a high index of protease and lipase enzymes (> 3) (Table 4., Figure 2.). Protease and lipase enzymes, related to the ability of the endophytic bacteria as biological control agents. Based on the protease and lipase indexes, *Bacillus* sp. PP.A5 can produce the highest proteases and lipase enzymes compared to other isolates. The isolates that have high protein and fat hydrolysis enzymes have the potential as biological control agents because proteins and fats are constituents of pathogen cells (Mota et al 2016). Besides, the protease enzyme is thought to degrade antibiotics produced by fungal or bacterial pathogens. According to Anderson et al. (2014), the extracellular protease enzyme produced by *P. fluorescens* can inactivate antibiotic compounds produced by *Pantoea agglomerans.*

*Bacillus* sp. PPD.B2 has the highest phosphate solubility index. The phosphate solubilization is related to the ability of endophytic bacteria as a plant growth promoter, providing phosphates for plants. Microbes with high phosphate solubility activity are capable of producing and releasing metabolites such as organic acids that chelate cations that are bound to phosphate (especially calcium) and converting them into soluble forms. Solubilization of different forms of phosphate by microbes associated with plants, and increasing its availability for plants, will increase growth and production of the plant (Djuric et al., 2011).

In conclusion, based on research carried out, it has been successfully isolated, morphologically and biochemically characterized four the endophytic bacteria that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

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# Short Communication: Isolation and characterization of the endophytic bacteria, and their potential as maize diseases control

**ENDANG MUGIASTUTI♥, SUPRAYOGI, NUR PRIHATININGSIH, LOEKAS SOESANTO**
Faculty of Agriculture, Universitas Jenderal Soedirman. Jl. Dr. Suparno, Karangwangkal, Purwokerto Utara, Banyumas 53123, Central Java, Indonesia. Tel./fax.: +62-281-638791, ♥email: endangmugiastuti@gmail.com.

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**Abstract.** *Mugiastuti E, Suprayogi, Prihatiningsih N, Soesanto L. 2020. Short Communication: Isolation And Characterization Of The Endophytic Bacteria, And Their Potential As Maize Diseases Control. Biodiversitas 21: 1809-1815.* Sheath blight and bacterial wilt are diseases that can reduce maize production. Biological control with the endophytic bacteria offers environmentally friendly control for these pathogens. The study aimed to isolate and characterize the endophytic bacteria morphologically and biochemically and to study their potential to control maize diseases, especially sheat blight and bacterial wilt causing pathogens. The study was conducted at the Plant Protection Laboratory, Faculty of Agriculture, Jenderal Soedirman University, from April to August 2019. The study consisted of four stages: isolation and characterization of endophytic bacteria, the antagonism test of the endophytic bacterial to *R solani,* the antagonism test of the endophytic bacteria to *Pantoea* sp., and the mechanical test of the endophytic bacteria as biological control agents and plant growth-promoting bacteria. Based on the research, four endophytic bacteria isolates have been successfully isolated, and characterized successfully and found have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea s*p. *Bacillus* sp, endophytic from the root (BK.A1; BK.A3; PP.A5) and *Bacillus* sp. endophytic from the stem (PPD.B2) can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

**Keywords:** *Bacillus,* fluorescent *Pseudomonas, Pantoea, Rhizoctonia solani*

## INTRODUCTION

Maize is a strategic food commodity in the world. In Indonesia, the government seeks to achieve self-sufficiency in maize through increasing production of sustainable maize. However, these efforts have faced several obstacles; one of them is the presence of plant diseases such as sheath blight caused by *Rhizoctonia solani* Kuhn, and bacterial wilt caused by *Pantoea stewartii*. *R. solani* can infect up to the midrib of the cob (Djaenuddin et al. 2017), resulting in up to 100% decrease in the yield (Muis 2007). *Pantoea* sp. can attack all stages of the plant causing wilting and leaf blight, and is known as Stewart's wilt (Pataky 2004; Ammar et al. 2014). The pathogens can cause 40-100% yield loss.

Over the past 3 decades, the concept of sustainable and environmentally friendly agriculture has been carried out by minimizing the use of chemicals, both synthetic fertilizers, and pesticides. In the management of pests and plant diseases, biological control is developed by applying biological agents including endophytic bacteria (Shanti and Vittal 2013). Many endophytic bacteria can pass the endodermic barrier across from the root cortex to the vascular system, and subsequently develop as endophytes in stems, leaves, tubers, and other organs (Compant et al. 2005). The use of endophytic bacteria as biological agents has an advantage compared to rhizosphere bacteria because endophytic bacteria live and survive in the plant tissue during plant development, thus protecting the plants.

*Bacillus* sp. and fluorescent *Pseudomonas* are reported to be able to live as endophytes and are widely used as biological control agents for soil-borne and air-borne diseases. The endophytic bacteria could control plant diseases through several mechanisms including competition, hyperparasitism, producing microbial inhibiting compounds (antibiotics, lysis enzymes, other physical or chemical disorders), enhancing plant resistance, and promoting plant growth (Compant et al 2005, Pal and McSpadden-Gardener 2006; Rosenblueth and Martinez-Romero 2006).

Based on the mechanisms, the use of endophytic bacteria isolated from maize, both upland and lowland, suggested potentially alternative control for sheath blight (*R. solani*) and bacterial wilt (*Pantoea* sp). The research aimed to isolate and characterize morphologically and biochemically the endophytic bacteria as well as their potential to control pathogens that cause disease in maize especially *R. solani* and *Pantoea* sp.

## MATERIALS AND METHODS

This research was conducted at the Laboratory of Plant Protection, Faculty of Agriculture, Jenderal Soedirman

University, Purwokerto, Central Java, Indonesia, from April to August 2019

**Isolation *Rhizoctonia solani***

*Rhizoctonia solani* was isolated from maize with sheath blight symptoms and there was sclerotium as a resistant structure from the pathogenic fungi in Banyumas. *R. solani* isolation was carried out based on Al-Fadhal et al. 2019. Disease samples were cut 0.5 x 0,5 cm, then sterilized with NaOCl (1%) for 2 min, and rinsed with sterile water 3 times. Disease samples pieces were then dried using sterile filter papers, and transferred to Petri dishes containing PDA medium to obtain pure *R. solani* isolates.

**Isolation *Pantoea* sp.**

*Pantoea* sp. was isolated from diseased maize samples taken from the maize growing area in Banyumas Regency according to Coplin et al. (2012); Aini et al. (2013) and Desi et al. (2014). Diseased leaves or stems were washed with running water, then dried with a tissue. Diseased samples were cut 1.5 x 5 cm, then sterilized with ethanol 70% and rinsed with sterile water 3 times. Furthermore, the sample was crushed with 5 ml of sterile distilled water using a sterile mortar. The bacterial suspension was streaked on nutrient agar and incubated 3-5 days. Bacterial colonies that exhibit the character of *Pantoea* sp. were yellow, shiny, slimy, flat or convex, then separated as pure cultures of P. stewartii candidates. The culture was then tested by Gram Reaction (KOH test), Hugh-Leifson test, pigment production in YDC medium, oxidase test, hypersensitivity test, and pathogenicity test on maize.

**Isolation and characterization of endophytic bacteria**

Sampling for the isolation of endophytic bacteria was carried out in Banyumas and Purbalingga, Central Java, Indonesia, with purposive stratified random sampling. Samples were taken from two areas of altitude, i.e., low-moderate lands (0-500 m above sea level), and highlands (> 500 m above sea level) (Nuryanto et al., 2014). In each district, 2 locations were selected for the low-medium lands, and 1 location for the highlands. Age of maize plants was 20-30 days after planting, when the number of endophytic microbial populations that can be cultured is in the highest population (Cavaglieri et al. 2009).

The endophytic bacteria were isolated from the roots and stems of healthy maize plants. Roots and stems were washed, sterilized with 70% ethanol (1 minute), 20% sodium hypochlorite (5 minutes) and Ringer's thiosulfate solution (5 minutes). Separately, the roots and stems of 10 g each were crushed with 90 ml PBS on a sterile mortar. Subsequently, samples were plated on NA and Kings B media (Cavaglieri et al. 2009). To isolate *Bacillus* sp., the suspension was heated for 10 minutes at 80 ° C, before plating on NA. Bacterial isolates were further purified and characterized, such as morphological characteristics, gram properties, catalase tests, and hypersensitivity tests

**The antagonism test of endophytic bacterial to *Rhizoctonia solani***

The antagonism test of endophytic bacteria on *R. solani* was carried out using the dual culture method. The level of inhibition of antagonist is calculated using the formula (Abidin et al. 2015).

$$I = \frac{C-T}{C} \times 100\%$$

Where:
I : The level of inhibition of antagonist (%)
C: The radius of pathogen colonies opposite antagonist
T: The radius of the colony of pathogens towards antagonist

**The antagonism test of endophytic bacterial to *Pantoea* sp.**

Antagonism testing was carried out using the double-layer test method (Santiago et al. 2015). Endophytic bacteria to be tested were grown on the nutrient agar medium, incubated at 28 C for 48 hours. In the upside-down position, 1 ml of chloroform was added to the cup lid and left for 2 hours. Next, add 5 mL so that 0.6% water containing 0.5 mL of *Pantoea* sp. bacterial suspension. The culture was re-incubated for 24 hours, and there were clear zones around the antagonistic bacterial colony. The antibiotic activity was assessed based on the diameter of the clear zone compared to the diameter of the colony. Characterization of the type of antibiosis can be divided into bactericidal and bacteriostatic types based on Djatmiko et al. (2007).

**The mechanism test of endophytic bacteria as controlling agents biological and plant growth-promoting microbial**

The testing mechanism of endophytic bacteria was carried out for bacteria that have the potential in testing the antagonism of the fungus *R. solani* and *Pantoea* sp.

*Protease test*

The activity of the ability of antagonistic bacteria to produce extracellular protease enzymes was tested using Skim Milk Agar (SMA) medium. Each bacterium to be tested was grown in a medium SMA and incubated at 28 C for 24-48 hours. The presence of clear zones around the colony shows that positive bacteria produce protease enzymes (Abed et al. 2016). The protease activity index is assessed based on the diameter of the clear zone compared to the diameter of the colony.

$$\text{Protease index} = \frac{\text{(clear zone diameter-colony diameter)}}{\text{Colony diameter}}$$

*Lipase test*

Detection of the ability of bacteria to produce the enzyme lipase was done by growing the antagonistic bacteria on a medium containing 1% Tween 80. The presence of lipase enzyme activity was demonstrated by milky white sediment around the bacterial colony, after incubating at 28 C for 4-7 days. The lipolytic index was measured using a formula Djuric et al. (2011).

Lipolytic index = (Milky white diameter-colony diameter) / Colony diameter

*Phosphatase test*

Detection of the ability of bacteria to produce the enzyme phosphatase was done by growing bacterial isolates on Pikovskaya medium. After incubating for 7 days at 28 C, the presence of a clear zone around the bacterial colony shows that the bacteria has the ability to produce the phosphatase enzyme to dissolve phosphates. The solubility index is measured using a formula (Farooq and Bano 2013)

Phosphatase Index = (Clear zone diameter-Colony diameter) / Colony diameter

## RESULTS AND DISCUSSION

**Isolation and characterization of endophytic bacteria**

The results of the exploration, isolation, and characterization of endophytic bacteria obtained 23 isolates of endophytic bacteria, consisted of 9 isolates of the fluorescent *Pseudomonas* and 14 isolates of *Bacillus* sp. (Table 1). *Fluorescent Pseudomonas* colony on King's B was round, with a flat edge, and yellowish-white, to greenish-yellow, gram-negative, rod-shaped, non-spore and fluorescent under ultraviolet light. Singh et al. 2017 reported fluorescent *Pseudomonas* showed light green, yellowish, creamy, circular, slimy, regular-irregular characteristics. Bacteria have short-long rod forms. The Fluorescent *Pseudomonas* isolates is gram-negative, which can form catalase, a positive oxidase, needed to grow aerobes. *Bacillus* sp. was observed with a spherical colony having cell rod-shaped, gram-positive, and endospores within cells (Table 1.). Slepecky and Hempill (2006); Amin et al. (2015) reported *Bacillus* sp. has the characteristics of a circular colony and punctiform (small round), variations in the entire margin and lobate, white dull, non-slimy, gram-positive, has endospores, flagellum and some are motile.

Based on its distribution, fluorescent *Pseudomonas* and *Bacillus* sp. found in all sampling locations, high or low-medium lands. This shows that fluorescent *Pseudomonas* and *Bacillus* sp. spread and can live in various altitudes, both high and low-medium land. Bacon and Hilton 2002; Ganeshan and Kumar 2005 reported *P. fluorescens* and *Bacillus* sp., are species of bacteria with a wide range of life and are very adaptive in various environments. Both types of bacteria are also found in the roots or corn stalks. *Bacillus* sp. and *Pseudomonas* sp. including a group of endophytic bacteria have a wide range of life and more isolated in maize (Ganeshan and Kumar 2005; Orole and Adejumo 2011; Costa et al. 2013)

**Antagonism test between the endophytic bacteria against *R. solani***

Based on the results of *in vitro* tests (Table 2), 24 isolates of the endophytic bacteria were able to inhibit the growth of *R. solani*, with varying degrees of inhibition. The endophytic bacteria that have inhibition rates above 50%, i.e fluorescent *Pseudomonas* BK.A1 (51%), *Bacillus* sp. B.K.A1 (55.39%), *Bacillus* sp BK.A3 (51.52%), PP.A5 (50.66%), and PPD.B2 (50.8%). The effect of the endophytic bacteria in inhibiting the growth *of R. solani* is inversely proportional to the dry weight mycelium. The greater the percentage of inhibition of endophytic bacteria to the growth of *R. solani*, the smaller the dry weight mycelium (Table 2) Endophytic bacteria can inhibit the growth of *R. solani*, which were shown by the inhibitory zone in the area bordering the bacterial streak (Figure 1.A).

The endophytic bacteria have anti-pathogenic properties and can produce antibiotic compounds. The ability of the endophytic bacteria to control plant pathogens occurs through the mechanism of antibiosis, competition, lysis, inducing resistance and producing growth substances. Bacteria capable of producing secondary metabolites that can inhibit growth or damage pathogens (Hastuti et al. 2014). These compounds, including alkaloids, phenols, flavonoids, glycosides, and phytoalexin (Soesanto et al. 2010). Fluorescent *Pseudomonas* can produce various types of antibiotics including phenazine-1-carboxylic acid, pyocyanin, pyrrolnitrin, and pyoluteorin, 2,4-diacetyl phloroglucinol (Phl). Phl is a phenolic metabolite with antibacterial and antifungal (Jain and Das 2016). *Bacillus* species can produce various kinds of volatile compounds and diffusible with strong inhibitory activity against plant pathogens (Lim et al. 2017).

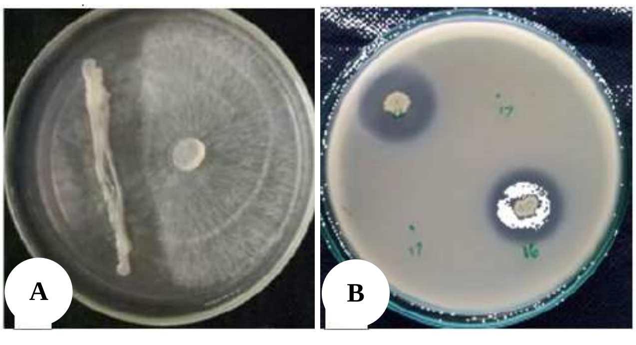


**Figure 1.** Antagonism test between the endophytic bacteria against *R. solani* (A) and *Pantoea* sp. (B)

**Table 1.** Isolation and characterization of endophytic bacteria.

| Land | Sampling location | Sample | Gram test | Catalase test | Oxidase test | Colony morpho-logy | Colony pigment | Fluorescence on KB medium | Cell morphology | Endo-spores | Isolate |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Highland | Purbalingga, Pratin 7.13'33" S, 109.17'21" E, 1.190 m asl | Root | - | + | + | Round | yellowish-white | + | Medium rod | - | fluorescent *Pseudomonas* PP.A1 |
| | | Root | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. PP.A3 |
| | | Root | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. PP.A5 |
| | | Stem | - | + | + | Round | yellowish-white | + | Medium rod | - | fluorescent *Pseudomonas* PP. B4 |
| | Banyumas, Baturaden 7.19"1" S, 109.14'29" E, 520 m asl | Root | - | + | + | Round | Greenish-yellow | + | Medium rod | - | fluorescent *Pseudomonas* BB.A2 |
| | | Root | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. BB.A3 |
| | | Stem | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BB.B4 |
| Medium-Lowland | Banyumas, Sumbang 7.21'54" S, 109.17'33"E, 200 m asl | Root | - | + | + | Round | yellowish-white | + | Medium rod | - | fluorescent *Pseudomonas* BS.A2 |
| | | Root | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BS.A1 |
| | | Root | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. BS.A3 |
| | | Stem | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. BS. B1 |
| | Purbalingga, Bojongsari 7.20'12" S, 109.20'22" E, 190 m asl | Root | - | + | + | Round | Greenish-yellow | + | Small rod | - | fluorescent *Pseudomonas* PB. A 4 |
| | | Stem | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. PB. B1 |
| | | Stem | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BB. B3 |
| | Purbalingga, Padamara 7.22'28" S, 109.13'24" E, 180 m asl | Root | - | + | + | Round | Greenish-yellow | + | Small rod | - | fluorescent *Pseudomonas* PPD A1 |
| | | Stem | - | + | + | Round | yellowish white | + | Medium rod | - | fluorescent *Pseudomonas* PPD. B1 |
| | | Stem | - | + | + | Round | yellowish-white | + | Medium rod | - | fluorescent *Pseudomonas* PPD. B5 |
| | | Stem | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. PPD. B2 |
| | | Stem | + | + | + | Round | White | - | Small rod | + | *Bacillus* sp. PPD. B4 |
| | Banyumas, Kembaran 7.23'47" S, 109.17'9" E, 110 m asl | Root | - | + | + | Round | yellowish-white | + | Medium rod | - | fluorescent *Pseudomonas* BK. A1 |
| | | Root | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BK.A1 |
| | | Root | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BK.A3 |
| | | Stem | + | + | + | Round | White | - | Medium rod | + | *Bacillus* sp. BK.B3 |

**Table 2.** Inhibition of endophytic bacteria against *R. solani.*

| Isolate | Inhibition rate (%) | Dry weight mycelium |
|---|---|---|
| Control | 0 | 0.093 |
| Endophytic bacteria from the root | | |
| fluorescent *Pseudomonas* BB.A2 | 49.00 | 0.038 |
| fluorescent *Pseudomonas* BS.A 2 | 45.00 | 0.027 |
| fluorescent *Pseudomonas* BK.A1 | 51.00 | 0.017 |
| fluorescent *Pseudomonas* PPD.A1 | 10.33 | 0.059 |
| fluorescent *Pseudomonas* PP.A1 | 38.33 | 0.017 |
| fluorescent *Pseudomonas* PB.A4 | 18.00 | 0.037 |
| *Bacillus* sp.BB.A3 | 40.42 | 0.030 |
| *Bacillus* sp.BS.A1 | 48.73 | 0.016 |
| *Bacillus* sp. BSA3 | 37.42 | 0.039 |
| *Bacillus* sp. B.K.A1 | 55.39 | 0.002 |
| *Bacillus* sp. B.K.A3 | 51.52 | 0.003 |
| *Bacillus* sp.PP.A3 | 46.65 | 0.019 |
| *Bacillus* sp.PP.A5 | 50.66 | 0.009 |
| Endophytic bacteria from the stem | | |
| fluorescent *Pseudomonas* PPD.B1 | 27.00 | 0.020 |
| fluorescent *Pseudomonas* PPD.B5 | 49.33 | 0.013 |
| fluorescent *Pseudomonas* PP.B4 | 65.67 | 0.004 |
| *Bacillus* sp. BB.B4 | 44.44 | 0.026 |
| *Bacillus* sp. BS.B1 | 49.74 | 0.012 |
| *Bacillus* sp. BK. B3 | 40.36 | 0.031 |
| *Bacillus* sp.PPD.B2 | 50.8 | 0.007 |
| *Bacillus* sp. PPD.B4 | 39.44 | 0.036 |
| *Bacillus* sp. PB.B1 | 37.29 | 0.047 |
| *Bacillus* sp. PB.B3 | 44.9 | 0.022 |

**The endophytic bacterial antagonism test against *Pantoea* sp.**

The results of antagonism between the endophytic bacteria and *Pantoea* sp. show varied results. The endophytic bacteria that can inhibit bacterial growth were indicated by the presence of clear zones around the endophytic bacterial colonies (Figure1). From the nine isolate fluorescent, *Pseudomonas* were tested, only three isolates were able to inhibit the growth of the *Pantoea* sp., i.e fluorescent *Pseudomonas* (Pf) BS.A2, Pf BK.A1 Pf PPD.B5. While the isolates Pf BB.A2, Pf PPD.A1, Pf PP.A1, Pf PPD.B1, and Pf PP.B4 are not able to inhibit the growth of pathogenic bacteria. Meanwhile, all isolate *Bacillus* sp. tested (thirteen isolates) were able to inhibit the growth of *Pantoea* sp. (Table 3).

The presence of clear zones around endophytic bacterial colonies showed the ability of endophytic bacteria to produce antibiotics to inhibit the growth of *Pantoea* sp. *P. fluorescens* P60 can produce antibiotics that inhibit the growth of pathogens (Soesanto 2011). *Pseudomonas fluorescens* is reported to produce phenazine-1-carboxylic acid (PCA) and other derivatives, 2,4 diacetyl phloroglucinol (Phl)), pyrrolnitrin (PRN) and or pyoluteorin (Plt) (Heydari and Pessarakli 2010). Nasrun and Burhanudin (2016) mention that *P. fluorescens* produce secondary metabolites, i.e. antimicrobial, cyanide acid and 2,4-diacetyl phloroglucinol phenazine, pyrrolnitrin, pyoluteorin antibiotics.

**Table 3.** Inhibition of endophytic bacteria against *Pantoea* sp.

| Isolate | Antagonism | Antagonism index | Antagonism category* | Antagonism activity |
|---|---|---|---|---|
| Endophytic bacteria from the root | | | | |
| fluorescent *Pseudomonas* BB.A2 | - | 0 | - | - |
| fluorescent *Pseudomonas* BS.A 2 | + | 4.91 | Strong | Bacteriostatic |
| fluorescent *Pseudomonas* BK.A1 | + | 4.42 | Strong | Bacteriostatic |
| fluorescent *Pseudomonas* PPD.A1 | - | 0 | - | - |
| fluorescent *Pseudomonas* PP.A1 | - | 0 | - | - |
| fluorescent *Pseudomonas* PB.A4 | + | 5.29 | Strong | Bactericidal |
| *Bacillus* sp.BB.A3 | + | 8.17 | Strong | Bacteriostatic |
| *Bacillus* sp.BS.A1 | + | 4.00 | Strong | Bacteriostatic |
| *Bacillus* sp. BSA3 | + | 5.07 | Strong | Bactericidal |
| *Bacillus* sp. B.K.A1 | + | 4.01 | Strong | Bacteriostatic |
| *Bacillus* sp. B.K.A3 | + | 4.91 | Strong | Bacteriostatic |
| *Bacillus* sp.PP.A3 | + | 6.63 | Strong | Bactericidal |
| *Bacillus* sp.PP.A5 | + | 6.56 | Strong | Bactericidal |
| Endophytic bacteria from the stem | | | | |
| fluorescent *Pseudomonas* PPD.B1 | - | 0 | - | - |
| fluorescent *Pseudomonas* PPD.B5 | + | 5.86 | Strong | Bactericidal |
| fluorescent *Pseudomonas* PP.B4 | - | 0 | - | - |
| *Bacillus* sp. BB.B4 | + | 7.80 | Strong | Bactericidal |
| *Bacillus* sp. BS.B1 | + | 6.22 | Strong | Bacteriostatic |
| *Bacillus* sp. BK. B3 | + | 5.33 | Strong | Bacteriostatic |
| *Bacillus* sp.PPD.B2 | + | 5.00 | Strong | Bacteriostatic |
| *Bacillus* sp. PPD.B4 | + | 8.75 | Strong | Bacteriostatic |
| *Bacillus* sp. PB.B1 | + | 1.67 | Weak | Bacteriostatic |
| *Bacillus* sp. PB.B3 | + | 5.67 | Strong | Bactericidal |

Note: •Based on Davis and Stout (1971)

**Table 4.** Test results of proteases, lipases and phosphate solubilization

| Isolate | Protease test | | Lipase test | | Phosphate solubilization | |
|---|---|---|---|---|---|---|
| | Activity | Index | Activity | Index | Activity | Index |
| Endophytic bacteria from the root | | | | | | |
| *Bacillus* sp. B.K.A1 | + | 3.75 | + | 3.23 | + | 1.17 |
| *Bacillus* sp. B.K.A3 | + | 3.20 | + | 3.73 | + | 1.27 |
| *Bacillus* sp. PP.A5 | + | 5.00 | + | 4.40 | + | 1.46 |
| Endophytic bacteria from the stem | | | | | | |
| *Bacillus* sp.PPD.B2 | + | 3.00 | + | 3.90 | + | 2.60 |

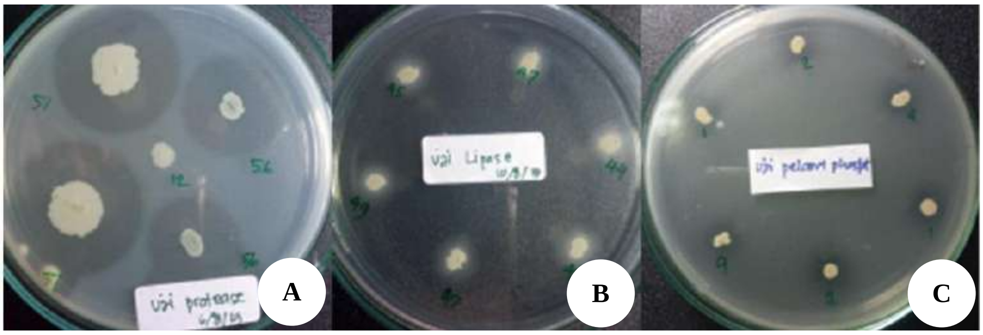


**Figure 2.** Hydrolysis enzyme activity, (A) protease, (B) lipase and (C) phosphate solubilization

The level of bacteria's ability to inhibit growth can be shown by the large diameter of the clear zone. The results showed that the antagonism index ranged from 1.67-8.17. Based on this index, most endophytic bacteria have a strong antagonism (index of antagonism> 4) (Davis and Stout 1971). Furthermore, bacterial isolates that showed antagonistic activity were tested for types of antagonism based on Djatmiko (2007). Based on the type of antagonistic activity, ten isolates the endophytic bacteria were bacteriostatic and nine isolates the endophytic bacteria were bactericidal. Bacteriostatic activity, growth inhibition is temporary, it is shown that regrowth of bacteria after being transferred to a new medium, which is free from the influence of antagonistic bacteria. Bactericidal activity, inhibition is permanent. Bacteria were unable to grow even though they are transferred to new medium.

**Test the mechanism of endophytic bacteria as biological control agents and plant growth-promoting microbes**

The mechanism test was carried out on endophytic bacteria that have the potential to control the fungus *R. solani* and *Pantoea* sp., i.e. *Bacillus* sp. B.K.A1, *Bacillus* sp. B.K.A3, *Bacillus* sp. PP.A5, *Bacillus* sp. PPD.B2. The production of compounds related to biocontrol of pathogens and promotion of plant growth in bacterial isolates was evaluated by measuring the production of antimicrobial compounds and hydrolytic enzymes (amylases, lipases, proteases, and chitinases) and phosphate solubilization. The results of enzyme activity tests are as shown in Table 4. The four isolates *Bacillus* sp. tested were able to produce protease, lipase and phosphatase enzymes, with varied activity indexes. All isolates of *Bacillus* sp. those tested had a high index of protease and lipase enzymes (> 3) (Table 4, Figure 2). Protease and lipase enzymes, related to the ability of the endophytic bacteria as biological control agents. Based on the protease and lipase indexes, *Bacillus* sp. PP.A5 can produce the highest proteases and lipase enzymes compared to other isolates. The isolates that have high protein and fat hydrolysis enzymes have the potential as biological control agents because proteins and fats are constituents of pathogen cells (Mota et al 2016). Besides, the protease enzyme is thought to degrade antibiotics produced by fungal or bacterial pathogens. According to Anderson et al. (2014), the extracellular protease enzyme produced by *P. fluorescens* can inactivate antibiotic compounds produced by *Pantoea agglomerans.*

*Bacillus* sp. PPD.B2 has the highest phosphate solubility index. The phosphate solubilization is related to the ability of endophytic bacteria as a plant growth promoter, providing phosphates for plants. Microbes with high phosphate solubility activity are capable of producing and releasing metabolites such as organic acids that chelate cations that are bound to phosphate (especially calcium) and converting them into soluble forms. Solubilization of different forms of phosphate by microbes associated with plants, and increasing its availability for plants, will increase growth and production of the plant (Djuric et al., 2011).

In conclusion, based on research carried out, it has been successfully isolated, morphologically and biochemically characterized four the endophytic bacteria that have the potential to be developed as biopesticides to control maize disease, especially *R. solani* and *Pantoea* sp. They can suppress the growth of *R.solani* by more than 50%, have a strong antagonistic index against *Pantoea* sp (> 4), and can produce protease and lipase enzyme, and phosphate solubilization.

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