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# Strategy For Controlling Bacterial Leaf Blight (*Xanthomonas sp*.) on *Eucalyptus Pellita* Plants by Administering *P. aeruginosa* RE081

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**ABSTRACT**

The demand for Eucalyptus Pellita seeds as a high-throughput inoculum (HTI) crop commodity in the field is extremely high. However, Xanthomonas sp., a bacteria that causes bacterial leaf blight, poses a significant challenge in ensuring a steady supply of healthy seedlings in nurseries. Researchers have explored using the rhizobacteria P. aeruginosa RE081 as an environmentally friendly biocontrol agent to address this issue. This study aims to determine the optimal concentration of P. aeruginosa RE081 that can effectively reduce the incidence and severity of bacterial leaf blight caused by Xanthomonas sp. The research was conducted using a completely randomized design, with different concentrations of P. aeruginosa RE081 ($10^5$, $10^6$, $10^7$, $10^8$ CFU/mL) being tested. The findings indicate that the biological agent's concentration significantly impacts plant disease occurrence and severity. In vivo testing revealed that P. aeruginosa RE081 reduced disease severity by only 2.13% at a 108 CFU/mL concentration. Interestingly, concentrations lower than $10^8$ CFU/mL increased disease severity compared to the control group.

Keywords: Concentration, biological agents, disease incidence, disease severity, *Pseudomonas aeruginosa*

## 1. INTRODUCTION

Eucalyptus Pellita, a prominent plant species, possesses valuable chemical properties that contribute to advancing industrial forest plantations (HTI) in the pulp and paper industry. (Fatimah et al., 2013). (Fatimah et al., 2013). The demand for seeds as a commodity for HTI cultivation is exceedingly high. However, the presence of Xanthomonas sp. bacteria, which leads to bacterial leaf blight, poses a significant challenge in ensuring the availability of healthy seedlings in nurseries. The reduction in leaf cross-sectional area caused by lesions and continuous defoliation hampered photosynthesis, resulting in stunted growth and decreased survival rates of eucalyptus seedlings. To address this issue, using rhizobacteria, an environmentally friendly biocontrol, presents a potential solution.

Pseudomonas, a bacterium commonly found in the rhizosphere, has garnered significant attention as a rhizobacterium with remarkable biological capabilities. Among its various mechanisms, Pseudomonas aeruginosa, in particular, has been extensively researched for its ability to serve as a biological agent. This species employs multiple strategies to combat plant diseases, including synthesizing secondary metabolites like hydrogen cyanide, siderophores, and antibiotics. Additionally, it induces plant defense responses and competes for nutrients, further enhancing its effectiveness in disease control (Lukkani & Reddy, 2014).

Pseudomonas aeruginosa is a type of bacteria that generates antibacterials known as bacterial pigments, including the pigment pyocyanin. Pyocyanin, a soluble derivative of the blue-green phenazine compound, is one of these pigments. Its mechanism of action involves disrupting the metabolism of normal cells, as it is released as an exotoxin compound by P. aeruginosa, effectively inhibiting bacteria (Moustafa et al., 1980)

In a previous in vitro study conducted by Marfungah et al. (2023), it was found that Pseudomonas aeruginosa RE081, isolated from the rhizosphere of Eucalyptus Pellita plants, exhibited antagonistic solid activity against Xanthomonas sp., resulting in the formation of a highly effective inhibition zone. The research also highlighted the biocontrol characteristics of P. aeruginosa RE081, including the production of HCN, siderophores, proteases, IAA, and solvent P. Similarly, Sandilya et al. (2017) conducted a study on Pseudomonas aeruginosa strain MAJPIA03, a rhizobacteria derived from the rhizosphere of castor plants in India. This strain produced NH3, HCN, siderophores, ACC deaminase, IAA, and Gibberellins. It exhibited the ability to enhance the growth of Ricinus communis plants while inhibiting the growth of five plant pathogen isolates, namely Fusarium oxysporum Ciceri, Fusarium monoliformes, Fusarium oxysporum, Fusarium glycopersicum, and Rhizoctonia solani. Furthermore, Ghadamgahi et al. (2022) reported that Pseudomonas aeruginosa strain FG106, isolated from the rhizosphere of tomato plants, demonstrated the ability to inhibit the growth of various pathogens including P. infestans, A. alternate, P. colocasiae, B. cinerea, R. solani, A. alternate, C. michiganensis subsp. Michiganensis, and X. euvesicatoria pv. Perforance..

Based on the description above, it is necessary to research the right concentration of Pseudomonas aeruginosa RE081 bacteria to control bacterial leaf blight (Xanthomonas sp.) through in vivo.

## 2. MATERIAL AND METHODS

### Research Time and Place

The research was conducted in PT Arara Abadi Research and Development, Perawang's growth chamber and greenhouse, from August to October 2022.

### Research Implementation

The isolate used was Pseudomonas aeruginosa RE081, sourced from the rhizosphere of the Eucalyptus Pellita plant, isolated by Marfungah et al. (2023) and Xanthomonas sp. (Plant pathogen E. Pellita, R&D collection of PT. Arara Abadi). The research was carried out using a completely randomized design by administering P. aeruginosa RE081 at different concentrations to E. Pellita plants infected with Xanthomonas sp. Data analysis was done using variance analysis (ANOVA) and the Duncan Multiple Range Test (DMRT) at the 5% level.

The treatments used as test parameters were differences in the concentration of RE081 rhizobacteria, namely: K0 (No treatment), P1 (concentration 105 CFU/mL), P2 (106 CFU/mL), P3 (107 CFU/mL), and P4 (108 CFU /mL). The stages of the research carried out are described in the following flow diagram:

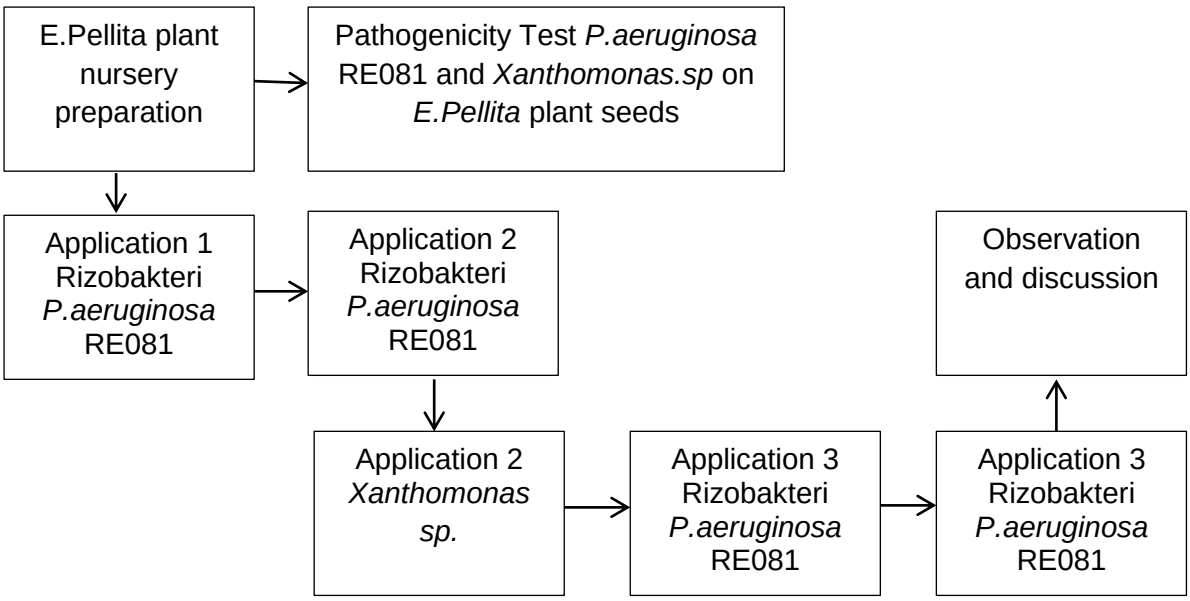


**Figure 1**: Research Stages

### Preparing E. Pellita plant seeds

The Eucalyptus Pellita clone EP0361WK seeds were utilized for the in vivo testing of plants. The plants, which were 28 days old, were randomly arranged using a Completely Randomized Design. There were four replications and six treatments in total. Each replication included four units of plant seeds, while each treatment consisted of 16 units of plant seeds. In total, 96 plant seeds were used for this research. The experiment occurred in a greenhouse, where the humidity and temperature were maintained at 80-90% and 25-30oC, respectively. Additionally, there was a 12-hour periodic photosynthesis cycle.

### Pathogenicity test of P. aeruginosa RE081 and Xanthomonas sp. on E. Pellita plant seeds

Each bacteria was tested for pathogenicity on E. Pellita plant seeds in a growth chamber (temperature 27-30oC,

humidity 80-90%). Each 48-hour-old isolate was inoculated on NB medium and incubated on a shaker at 100 rpm for 24 hours. The bacterial suspension is aged for 24 hours, and the density is adjusted to $10^8$ CFU/mL (OD600= 0.6), then sprayed on the surface of the plant leaves until wet (Bophela et al., 2019). Plants were observed up to 21 days after bacterial inoculation.

**Applying Rhizobacterium Pseudomonas aeruginosa**

The concentration of the bacterial solution was determined using a spectrophotometer at a wavelength of 600 nm, with each concentration measured at:
Absorbance [$10^5$ CFU/mL]= 0.001 (number of colonies 3.1 x $10^5$ CFU/mL)
Absorbance [$10^6$ CFU/mL]= 0.01 (number of colonies 4 x $10^6$ CFU/mL)
Absorbance [$10^7$ CFU/mL]= 0.1 (Number of colonies is 3.25 x $10^7$ CFU/mL)
Absorbance [$10^8$ CFU/mL]= 1 (Number of colonies is 3.35 x $10^8$ CFU/mL)

The roots of 28-day-old eucalyptus plants were soaked in 500 mL of P. aeruginosa RE081 suspension along with the pot tray according to the treatment for 30 minutes (Heo et al., 2022). Apart from soaking the roots, the P. aeruginosa RE081 suspension is sprayed on the plant leaves until wet. Next, once a week, 10 mL of the rhizobacteria suspension is poured into the planting medium according to the specified concentration and sprayed on the leaves until wet. The treatment was repeated twice before and after pathogen application, with a week's interval between each application.

**Applying the Pathogen Xanthomonas sp.**

Inoculation of the pathogen Xanthomonas sp. This is done by spraying plant leaves with a suspension of Xanthomonas sp bacteria. At a density of $10^8$ CFU/mL ($A_{600}$=0.6) until wet. Inoculation of plants with bacterial isolates of Xanthomonas sp. was carried out once, 24 hours after the second application of rhizobacteria. The pathogen is applied in the afternoon between 16.00 and 18.00 so that the bacteria are not exposed to too hot temperatures. After pathogen application, observations were made on days 7, 14, and 21.

**Maintenance**

Caring for eucalyptus seedlings in a greenhouse includes watering, fertilizing, and pest control. The fertilizer used is Simplot (macro and micronutrients) which is given by mixing it into the cocopeat planting medium at the beginning of planting. Watering is done using misting (automatic mist watering) 4x a day for 10 minutes per watering. Pest control is carried out by mechanical means.

**Observation Variable**

**(1) Incidence of Disease**

Disease incidence is determined by observing external symptoms in plants. Observations were made on days 7, 14, and 21 after pathogen inoculation. Disease incidence was calculated using the Abbolt method with the following formula:

$$KP = \frac{n}{N} \; x100\%$$

Note;
KP : Disease incidence rate / Disease incidence;
N : Number of symptomatic plants observed;
N : Total number of plants observed.

**(2) Disease Severity (I)**

Observations were made on the upper, middle, and lower leaves (4 per plant) on days 7, 14, and 21 after pathogen inoculation. Calculations are carried out using the Townsend and Heuberger formula (Santosa and Triyono, 1999 (Rahayu & Nurcahyanti, 2020) as follows:

$$I = \frac{\sum_{i-0}^{n}(\text{n1 x v1})\text{x } 100\%}{Z\ x\ N}$$

Note: I: Severity of disease;

n1 : 1st infected plant leaf;
v1 : Score with the 1st transmission category;
N : Number of plants observed;
Z : The highest transmission scale value (Score 5).

The scoring method is determined based on (Rivera-Zabala et al., 2017) modified:
Score (0): No symptoms
Score (1): Blight area >0-5.6%
Score (2): Blight area 5.6-19%
Score (3): Blight area >19– 48.1%
Score (4): Blight area 48.1 – 78.7%
Score (5): Blight area >78.7%

## 3. RESULT AND DISCUSSION

### 3.1 Pathogenicity test of P. aeruginosa RE081 and Xanthomonas sp. on E. Pellita plant seeds

Pathogenicity tests on the two bacteria gave different results. After incubation for 21 days, the rhizobacteria P. aeruginosa RE081 did not show disease symptoms on E. Pellita seedlings, while Xanthomonas sp. showed symptoms of bacterial leaf blight since the 7th day and continued to develop until the 21st day. This indicates that P. aeruginosa RE081 is not a pathogenic bacteria, while Xanthomonas is pathogenic on E. Pellita plants.

### 3.2 Test the effect of P. aeruginosa RE081 concentration as a biocontrol for Xanthomonas sp. on E. Pellita seedlings using in Vivo.

P. aeruginosa RE81 is applied in two areas: the rhizosphere and leaves. The rhizosphere is the area around the roots. Application to the rhizosphere aims to induce a plant immune response, while in leaves, it is hoped that there will be direct competition with pathogens through antagonism.

The results showed that symptoms of bacterial leaf blight in the form of wet spots parallel to the leaf veins were first observed on the 7th day after pathogen inoculation in all treatments except the control (K0). The analysis of variance showed that administration of P. aeruginosa RE081 at various concentrations significantly affected the percentage of bacterial leaf blight incidence. The results of the Duncan Multiple Range Test (DMRT) at the 5% level on the percentage of bacterial leaf blight caused by Xanthomonas sp. can be seen in Table 1.

Table 1. Occurrence of bacterial leaf blight disease by Xanthomonas sp. on Eucalyptus Pellita plant seeds Clone EP0361WK

| Treatment | Observation Time | | |
|---|---|---|---|
| | 7 HSI (%) | 14 HSI (%) | 21 HSI (%) |
| K0 (Without Treatment) | 00 a | 00 a | 38 a |
| K1 (+ *Xanthomonas* sp.) | 81 c | 94 b | 100 b |
| P1 ($10^5$ CFU/mL) | 94 c | 100 b | 100 b |
| P2 ($10^6$ CFU/mL) | 94 c | 100 b | 100 b |
| P3 ($10^7$ CFU/mL) | 94 c | 100 b | 100 b |
| P4 ($10^8$ CFU/mL) | 50 b | 94 b | 100 b |

Note: Numbers followed by different letters in each column indicate significantly different effects using the DMRT test at the 5% significance level.

Observation results On the 7th day after pathogen inoculation, the disease incidence in the treatment with the highest concentration of rhizobacteria (P4=108 CFU/mL) showed the smallest percentage, namely 50% significantly different from all treatments. Treatments P1, P2, and P3 were not substantially different from the treatment without rhizobacteria (K1) but showed higher numbers (94%) compared to K1 (81%). The incidence of disease on control plants (K0) was 0%, indicating that of the 16 plant units, not a single leaf was infected with Xanthomonas sp.

On observation days 14 to 21 after pathogen inoculation, the disease incidence in all treatments increased and was not significantly different. The incidence of disease in treating rhizobacteria with concentrations of P1, P2, and P3 reached 100% at 14 days, meaning that the pathogen had attacked all plant units. This incidence was higher than without rhizobacteria (K1) administration, namely 94%. The incidence of disease without rhizobacteria (K1) administration on day 14 was the same as the highest concentration treatment (P4), namely 94%.

The incidence of bacterial leaf blight on the 21st day in all treatments except K0 had reached 100%. The incidence of bacterial leaf blight was also observed in the control (K0) at 38%.

Increased incidence of leaf blight caused by Xanthomonas sp. in all treatments, including control. This occurred due to disease transmission through contact between leaves or splashing water when watering, an environment with high humidity, and the pathogen's virulence.

In the disease severity analysis results, the percentage also increased from the 7th day to the 21st day after pathogen inoculation, as shown in Table 2 below.

Table 2. The severity of bacterial leaf blight disease by Xanthomonas sp. on Eucalyptus Pellita plants Clone EP0361WK

| Treatment | Observation Time | | |
|---|---|---|---|
| | 7 HSI (%) | 14 HSI (%) | 21 HSI (%) |
| K0 (Without Treatment) | 0.00 a | 0.00 a | 13.75 a |
| K1 (+ *Xanthomonas* sp.) | 26.25 bc | 47.50 b | 58.75 b |
| P1 ($10^5$ CFU/mL) | 42.50 d | 60.00 c | 80,00 c |
| P2 ($10^6$ CFU/mL) | 30.00 cd | 53.75 c | 60.42 b |
| P3 ($10^7$ CFU/mL) | 33.75 cd | 60.00 c | 78.75 c |
| P4 ($10^8$ CFU/mL) | 18.75 b | 43.75 b | 57.50 b |

Note: Numbers followed by different letters in each column indicate significantly different effects according to the DMRT follow-up test at the 5% level

The lowest percentage of bacterial leaf blight disease severity at 21 days, namely 57.5%, was observed in the treatment with the highest concentration of rhizobacteria (P4=108 CFU/mL). This value is 2.13% lower than the severity of the disease in seedlings without rhizobacteria (K1), namely 58.75%.

The higher severity of disease in the treatment given P. aeruginosa RE081 compared to the control is thought to be due to increased nutrients, especially P and N in the growing media for E. Pellita seedlings. Research conducted in vitro by Marfungah et al. (2023) reported that the characteristics of P. aeruginosa RE081 as a biocontrol agent could produce siderophores, HCN, Protease, IAA, and solvent P. This was confirmed by other research reported by Sandilya et al. al. (2017), who stated that the Pseudomonas aeruginosa strain MAJPIA03 bacteria is capable of producing NH3, HCN, siderophores, ACC deaminase, IAA, and gibberellins.

The increased phosphorus and nitrogen resulting from the administration of P. aeruginosa RE081 in treatments P1, P2, and P3 may negatively affect the spread of the disease. Nitrogen is the primary macronutrient needed for plant metabolism. Nitrogen can affect the strength of the plant's physical barrier by reducing the thickness of the wax layer and lignin content, making it easier for pathogens to penetrate. Nitrogen also influences the formation of virulent compounds present in pathogens (Sun et al., 2020).

However, at the highest rhizobacterial concentration (P4), P. aeruginosa RE081 reduced disease severity by 2.13%. It is possible that at this concentration, P. aeruginosa RE081 has reached quorum to form compounds that can prevent the formation of virulence factors from Xanthomonas sp. so that the severity of plant disease decreases, as reported by Kanugala et al. (2019), Pseudomonas aeruginosa strain CGK-KS-1 produces Chumacin-1 and Chumacin-2 as inhibitors of quorum sensing signaling for biocontrol of rice bacterial blight. Mechanistic studies revealed that both electrolytes inhibited the production of quorum sensing signaling factor (cis-11-methyl-2-dodecenoic acid), suppressed xanthan gum secretion, and inhibited biofilms formed by various Xanthomonas pathovars. However, in this study, the number of colonizers was still too small, so the reduction in disease severity was also small. Another possibility is that the presence of rhizobacteria RE081 in P4 can trigger systemic plant resistance through the mechanism of systemic acquired resistance (SAR) in Eucalyptus Pellita seedlings.

This result aligns with the research results observed visually that in the K1 treatment (administration of Xanthomonas sp.), the plant leaves were tougher, and the color of the leaves turned yellow, with smaller leaf

diameters. Treatment with P. aeruginosa RE081 rhizobacteria at all concentrations resulted in plants with greener leaf color, softer and brittle leaf surface structure, and wider leaf diameter. At a P. aeruginosa RE081 concentration of 105,106,107 CFU/mL, around 3-4 pairs of lower leaves experienced loss, while at a concentration of 108 CFU/mL, the old leaves could still be maintained without falling off until the last day of observation (Figure 2).

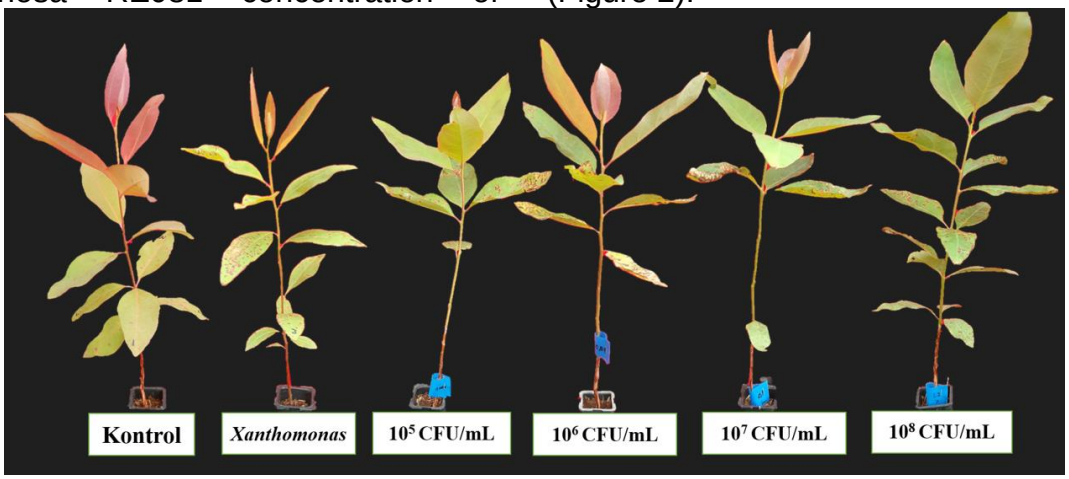


**Figure 2**. The in-vivo test results on day 21 after inoculation of the pathogen Xanthomonas sp.

The rhizobacteria P. aeruginosa RE081 are able to produce HCN. HCN is a very toxic volatile compound. However, Eucalyptus plants are tolerant to HCN exposure. This is consistent with the pathogenicity test of the rhizobacteria P. aeruginosa RE081 on Eucalyptus Pellita plant seeds in the growth chamber. The results of the pathogenicity test showed that after RE081 inoculation and incubation for 21 days, the plant seeds still looked healthy, without any symptoms of disease or wilting. Visually, the average growth was better than the control without rhizobacteria. Rijavec and Lapanje (2016) reported that HCN in the soil does not act as a direct biocontrol agent but is involved in geochemical processes in the substrate (e.g., metal chelation), which indirectly increases the availability of nutrients for rhizobacteria and their host plants, such as phosphate.

Thus, in this study, P. aeruginosa RE081 could not maximally reduce the incidence and severity of bacterial leaf blight caused by Xanthomonas sp. According to the author, the presence of HCN produced by P. aeruginosa RE081 does not directly influence the incidence and severity of bacterial leaf blight on E. Pellita clone EP0361WK seedlings. However, it indirectly contributes to increasing disease severity through increasing rhizosphere nutrition.

Another possibility is that the rhizobacteria P. aeruginosa RE081 cannot colonize the rhizosphere or leaves properly because they are washed away during watering, unsuitable environmental conditions, or competition with other microbes. Thus, these bacteria require a carrier medium, adhesive material, or even the application of technology that has recently been developed, namely microencapsulation, so that the rhizobacteria can survive unfavorable environments so that they can colonize the rhizosphere and leaves optimally (Fathi et al., 2021; Riseh et al., 2022).

The ability of rhizobacteria to colonize roots is essential for effective Plant growth promoting Rhizobacteria (PGPR) activity. Heo et al. (2022) reported that rhizobacteria colonization of Burkholderia contaminants strain AY001 was tested by quantitative measurements of bacterial populations in tomato roots infected with

the pathogen. The AY001 population increased to 3×107 CFU/g at 14 days post-inoculation, indicating that this bacterium could colonize the tomato root system well. RT-PCR and real-time qRT-PCR tests show that AY001 bacteria can protect tomato plants from pathogen infection by triggering ISR by expressing genes coding for jasmonic acid or ethylene. Kirtanayasa (2022) stated that one of the antibacterial activities is influenced by the concentration of the antibacterial extract produced. The ability of rhizobacteria to colonize the rhizosphere is influenced by environmental factors such as soil type, soil moisture, pH, temperature, and plant age and condition (Reisberg et al., 2013).

## 4. CONCLUSION

The concentration of biological agents influences the incidence and severity of plant diseases; however, the reduction in disease severity is closely related to the relationship between pathogens, biological agents, the environment, and the plants themselves. In vivo test results, P. aeruginosa RE081 was able to reduce disease severity by 2.13% at a concentration of 108 CFU/mL. At lower concentrations, P. aeruginosa RE081 increased bacterial leaf blight severity.

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