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Tracking GFP-Marked Osmoprotectant Rhizobacteria in the Rhizosphere of Mung Bean Under Sterile Conditions of 60% FC

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Abstract

This study aims to investigate osmoprotectant rhizobacteria producing glycine betaine, marked with the Green Fluorescent Protein (GFP) gene, in the rhizosphere of mung bean plants under drought stress conditions. The transformation of the GFP gene was performed on isolates Bl-19 and Am-7. Seeds were soaked for 30 minutes in bacterial suspensions containing either Bl-19-gfp or Am-7-gfp at a density of 106 cells mL-1, in a volume of 500 mL. The seeds were then planted under drought stress at 60% field ca-pacity and sterile media. The results showed that inoculation of Bl-19-gfp isolates with an initial density of 10^6 CFU led to a decrease in bacterial density to 1.4×10^5 CFU 1 week after inoculation. Similarly, inoculation of Am-7-gfp isolates with an initial density of 10^6 CFU caused a decrease to 1.7×10^5 CFU after 1 week of inoculation. Subsequently, the densities of both isolates remained stable from week 2 to week 4. The dry weight of mung bean plants at soil moisture conditions of 60% field capacity using isolates Bl-19 and Am-7 increased by 40.13% and 52.44%, respectively. Based on the results, both isolate increased dry weight below 60% of field capacity in sterile media.

Keywords: drought stress; GFP-tagging; glycine betaine-producing rhizobacteria; rhizosphere colonization; *Vigna radiata*

1. Introduction

The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance. The current state of the research field should be carefully reviewed and key publications cited. Please highlight controversial and diverging hypotheses when necessary. Finally, briefly mention the main aim of the work and highlight the principal conclusions. As far as possible, please keep the introduction comprehensible to scientists outside your particular field of research. References should be numbered in order of appearance and indicated by a numeral or numerals in square brackets—e.g., [1] or [2,3], or [4–6]. See the end of the document for further details on references.

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2. Materials and Methods

2.1. Isolates

Osmotolerant rhizobacterial isolates Bl-19 and Am-7 have the ability to produce osmolyte glycine betaine, which increases osmotic pressure, particularly under drought stress conditions. Glycine betaine measurements were conducted in previous research. Isolates Bl-19 and Am-7 synthesized glycine betaine at 10.3212 mg g^{-1} and 13.8463 mg g^{-1}, respectively. Both isolates were obtained from the isolation and screening of dryland plant rhizospheres in Bantul, Yogyakarta, Indonesia. In addition, the samples exhibited green fluorescence under UV light. Morphological observations of isolates were carried out in previous research. Isolat Bl-19 has Colony Shape Circular, Colony Color Yellowish White, Elevation Low convec, Internal Structure Finely granuler, Colony Edge Entire. Isolate Am-7 has Colony Shape Circular, Colony Color Yellowish White, Elevation Low convec, Internal Structure Finely granuler, Colony Edge Lobate.

2.2. GFP Gene Transformation

Recipient bacteria must be resistant to both kanamycin and rifamycin [12]. Antibiotic testing was carried out on 10 isolates capable of forming glycine and the osmoprotectant rhizobacteria isolates Bl-19 and Am-7 were resistant to kanamycin 50 µg mL-1 and rifamycin 25 µg mL-1. However, E. coli did not possess resistance to rifamycin, as a result, rifamycin-containing medium was used as a selective medium [12].

The modified gfp gene was mutagenated and expressed under the control of the bacteriophage lambda P L promoter. A cassette consisting of the transposon arms of the bacteriophage Mu flanking the gfp gene was irreversibly integrated as an in vitro assembled transposition complex into the genomes of Escherichia coli and Salmonella spp. Fluorescent E. coli and Salmonella strains carrying the gfp gene cassette were easily distinguished from their non-fluorescent parent strains on various growth media by visualization under UV light [23] . The method used was conjugative transformation with antibiotic selection.

Isolate Bl-19 or Am-7 were grown overnight in liquid LB medium containing 50 µg mL-1 rifamycin. Donor bacteria E. coli carrying the green fluorescent gene (E. coli -gfp) were grown overnight in liquid LB medium. GFP gene is resistant to kanamycin. E. coli-gfp is not resistant to the antibiotic kanamycin, so E. coli-gfp dies and gets a piece of DNA that has the GFP gene. Isolates Bl-19 and Am-7 were resistant to kanamycin and rifamycin based on antibiotic testing. Subsequently, 0.5 mL of Am-7 suspension, 0.5 mL of E. coli-gfp suspension and 50 µg mL-1 kanamycin were mixed in an Eppendorf tube, and centrifuged at 13,000 rpm for 5 minutes. The resulting pellet was collected and washed with 1 mL of 10 mM MgSO4, followed by centrifugation at 13,000 rpm for 5 minutes, and the pellet was taken. The pellet was then resuspended in 10 µL of 10 mM MgSO4, vortexed, and spotted onto LB agar medium, followed by inoculation for 24 hours. In addition, the growing colonies were collected and grown in liquid LB with shaking overnight. The bacterial suspension was then spread onto selective agar medium containing rifamycin and petri dishes and incubated for 48 hours. Colonies were observed under UV light. The appearance of green fluorescence in Bl-19 or Am-7 colonies showed successful transfer of the GFP gene.

2.3. Inoculation of Bl-19-gfp and Am-7-gfp Isolates

Medium sterization. The planting medium was sterilized by autoclaving. The soil medium was sterilized by autoclaving 3 times. Autoclaving is set at a temperature of 121°C, a pressure of 15 psi for 60 minutes.

Field capacity. Field capacity is determined by watering 500 g of soil until saturated and allowing the water to drip off for 1 day. The soil is weighed as the wet soil weight (FC) and oven-dried at 105°C to a constant weight as the oven-dry soil. Calculate using the formula:

$$\mathbf{FC\ (100\%) = \frac{Wet\ Soil\ Weight\ (FC) - Oven{-}Dry\ Soil\ Weight}{Oven{-}Dry\ Soil\ Weight}\ x\ 100\%}$$

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Once the 100% FC value is known (e.g., as a percentage of the soil mass), use this formula to achieve 60% FC:

$$\textbf{Water Required} = \frac{\textbf{W1} - \textbf{W2 x Dry Soil Weight (g)}}{\textbf{100}}$$

W1: Soil water content at the desired 60% KL (60% of the actual KL value).

W2: Initial soil water content (air-dried soil).

Field capacity value 29.6%, dry weight of soil media in polybag = 500 g, water added for 60% field capacity = 0.296 x 0.60 x 500 g = 88.8 g. weight of soil and water condition 60% field capacity 588.8 g. To maintain the condition of 60% field capacity, then every 2 days the weight of soil and water is returned to the weight of 588.8 g.

2.3.1. Seed Sterilization

The bacteriocidal agent contains Cu Oxide (Nordox 56 WP), administered at a dose of 1g per 1 kg of mung bean seeds, mixed with a little water, stirred, and air-dried for 30 minutes. The seeds are then soaked in sterile water for 30 minutes and rinsed with sterile water three times to prevent the bactericide from affecting the inoculation of rhizobacteria isolates.

The sterilized seeds were then treated according to the respective treatments, while control seeds were soaked in sterile distilled water.

2.3.2. Inoculation

The experiment was carried out in a completely randomized design with 3 treatments and 3 replications, so there are 9 treatment units and each treatment unit has 30 plants. A 15 x 15 polybag contains 500 g of soil for 1 plant. The inoculation treatment of osmoprotectant rhizobacteria isolates included no bacterial inoculation (I0), inoculation with Bl-19-gfp isolate (I1), and inoculation with Am-7-gfp isolate (I2). For the inoculation treatments, Seeds were soaked for 30 minutes in bacterial suspensions containing either Bl-19-gfp or Am-7-gfp at a density of 106 cells mL-1. After treatment, the seeds were planted in sterile soil and watered every two days to 60% field capacity with sterile water. Observations of rhizobacterial densities in the dinizosphere were conducted by taking 1 g of soil samples when the plants were 1 week, 2 weeks, 3 weeks, and 4 weeks old. CFU observation was repeated 3 times. After 4 weeks, samples were taken from each treatment for rhizobacterial density observations on the root surface and endophytic properties testing.

The experiment was carried out in a completely randomized design with 3 treatments and 5 replications, so there are 15 treatment units and each treatment unit has 20 plants. A 15 x 15 polybag contains 500 g of soil for 1 plant. The inoculation treatment of osmoprotectant rhizobacteria isolates included no bacterial inoculation (I0), inoculation with Bl-19-gfp isolate (I1), and inoculation with Am-7-gfp isolate (I2). For the inoculation treatments, Seeds were soaked for 30 minutes in bacterial suspensions containing either Bl-19-gfp or Am-7-gfp at a density of 106 cells mL-1. After treatment, the seeds were planted in sterile soil and watered every two days to 60% field capacity with sterile water. After four weeks, samples were taken from each treatment to measure dry weight to monitor plant growth.

2.4. Searching for the Presence of Osmoprotectant Rhizobacteria in the Rhizosphere

The detection of osmoprotectant rhizobacteria was carried out by collecting 1 g of soil from the rhizosphere of 2-week-old mung bean plants and suspending it in sterile water to a final volume of 100 mL. The solution was then filtered through filter paper. A loop of the resulting filtrate was streaked onto selective agar medium supplemented with kanamycin and rifamycin and incubated for 48 hours. The colonies were subsequently observed under UV light. The appearance of green fluorescence in Bl-19 or Am-7 colonies showed successful transfer of the GFP gene.

The density of osmotolerant rhizobacteria was determined using the serial dilution and colony counting methods, expressed as colony-forming units (CFU). A 1 g sample of mung bean rhizosphere soil was collected according to the treatment and suspended in sterile water to a final volume of 100

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mL. The suspension was then filtered through filter paper. Serial dilutions were prepared by transferring 1 mL of the suspension into 9 mL of sterile water. The dilution process was repeated sequentially up to 10-10. After incubation, the growing bacterial colonies are counted using the Total Plate Count (TPL) standard. Select a petri dish with a colony count between 30 and 300. Osmotolerant rhizobacterial colonies are counted using a Colony Counter to determine bacterial density. Density of rhizobacteria isolates was determined in weeks 1, 2, 3, and 4 with 3 replications.

2.5. Detection of Osmoprotective Rhizobacteria in Mung Bean Plant Tissue (Endophytic)

The presence of osmoprotective rhizobacteria in plant tissues was tested by collecting plant samples according to the treatment. For each treatment, osmotolerant rhizobacteria were re-isolated from leaf, stem, and root tissue. Sterilization was performed on the roots for indophytic observation. The roots were washed under running water to remove soil. The roots were cut into 3 cm lengths with a sterile knife. The roots were soaked for 30 seconds in 70% ethanol, then the roots were soaked in 1% NaOCl for 3 minutes. Then the roots were soaked again in 70% ethanol for 30 seconds. Finally, the roots were rinsed 3 times with sterile distilled water. Sterile roots, stems, and leaves were sectioned and placed on solid LB medium supplemented with 25 µg of rifamycin. The cultures were observed under UV light every 2 days for 10 days to detect fluorescent colonies.

2.6. Root Surface Observations

Mung bean roots at 28 days of age (vegetative stage) were surface sterilized with 70% ethanol and shaken for 5 minutes. The roots were then sterilized in 6.25% sodium hypochlorite for 10 minutes, followed by 5 rinses with sterile water. Observations of osmotolerant rhizobacteria on the root surface were performed using a fluorescence microscope at 100x magnification.

The density of osmotolerant rhizobacteria was determined using the serial dilution and colony counting method, expressed as CFU. A 1 gr root sample was collected and washed in sterile water to obtain the root wash suspension, which was adjusted to a final volume of 100 mL. Serial dilutions were prepared by transferring 1 mL of suspension into 9 mL of sterile water. The dilution process was repeated sequentially up to 10-10. From each dilution, 1 mL was plated onto agar medium and incubated to allow colony formation. The colonies that developed were counted to determine the density of osmotolerant rhizobacteria. Density of rhizobacteria isolates was determined in weeks 1, 2, 3, and 4 with 3 replications.

2.7. Plant Growth

Plant sampling was conducted weekly for 4 weeks. A total of 5 plant samples from each treatment were cleaned to remove adhering soil and oven-dried at 105°C until a constant weight was obtained.

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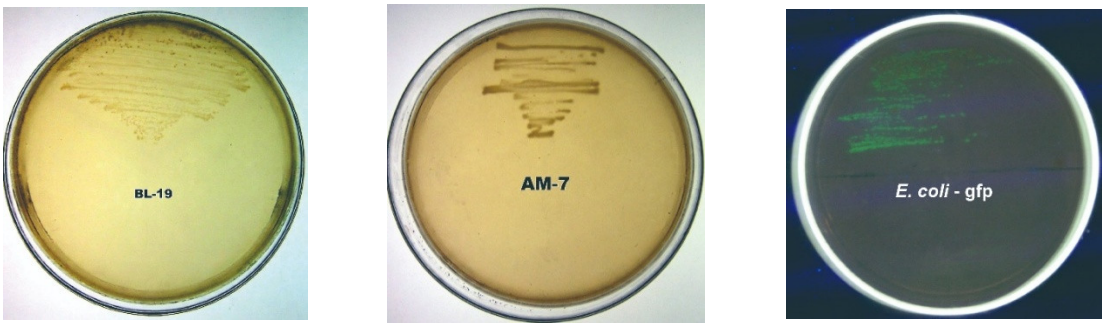
3. Results

3.1. GFP Gene Transformation

3.1.1. Subsubsection



a b c

Figure 1. Isolation of osmoprotective rhizobacteria isolates (a) Bl-19 parent, (b) Am-7 parent and (c) E. coli-gfp under UV light.

Isolate Bl-19-gfp exhibited green fluorescence under UV light, whereas Bl-19 did not show green fluorescence. E. coli-gfp also did not fluoresce because the cells were dead (Figure 2a). Similarly, isolate Am-7-gfp exhibited green fluorescence under UV light, while Am-7 did not show green fluorescence. E. coli-gfp did not fluoresce because the cells were also dead (Figure 2b). This indicates that the transformation of the GFP gene from E. coli to isolate Bl-19 and E. coli to isolate Am-7 was successful, and the medium used was a selective medium containing rifamycin.

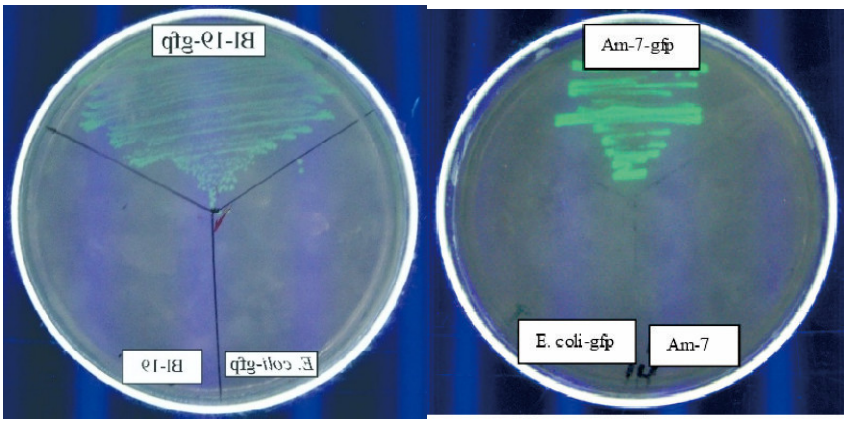


Figure 2. Colonies of Bl-19-gfp (a) and Am-7-gfp (b), their respective parental isolates (Bl-19 and Am-7), and *E. coli*-gfp grown on rifamycin-supplemented selective medium and observed under UV light. GFP-tagged isolates showed green fluorescence.

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3.2. The Presence of Osmoprotective Rhizobacteria in the Rhizosphere and on the Root Surface

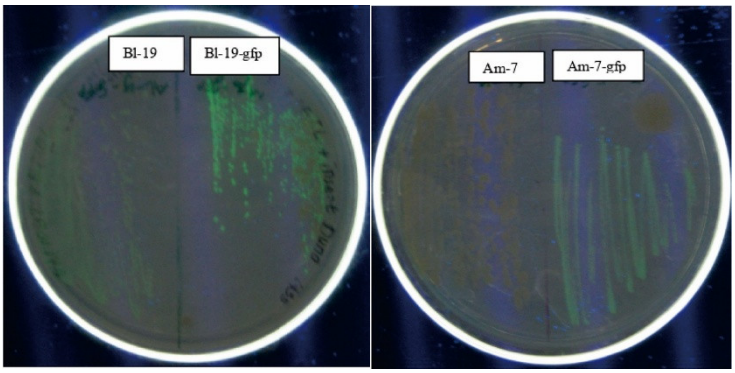

Figure 3. Re-isolation of Bl-19-gfp (a) and Am-7-gfp (b) isolates from the mung bean rhizosphere on selective medium and observation under UV light. The parental isolates (Bl-19 and Am-7) did not exhibit green fluorescence, whereas GFP-tagged isolates showed green fluorescence.

Re-isolation of Bl-19-gfp isolate from the rhizosphere of mung bean plants on selective medium, and observation under UV light showed green fluorescence (a). Similarly, re-isolation of Am-7-gfp isolate from the rhizosphere of mung bean plants on selective medium, and observation under UV light also showed green fluorescence (b). These results showed that both Bl-19-gfp and Am7-gfp isolates were present in the rhizosphere of the inoculated mung bean plants.

Inoculation of Bl-19-gfp at an initial density of 10^6 CFU mL^{-1} resulted in a decrease in bacterial density to 1.4 x 10^5 CFU 1 week after inoculation. After this initial decline, the density of the Bl-19-gfp isolate remained stable up to 4 weeks. Similarly, inoculation of the Am-7-gfp isolate through mung bean seeds and the rhizosphere at an initial density of 10^6 CFU decreased to 1.7 x 10^5 CFU one week after inoculation. Afterward, the density of the Am-7-gfp isolate remained stable for up to 4 weeks.

3.3. Visualization of the Formation of Osmoprotectant Rhizobacterial Colonies on the Surface of Mung Bean Roots Using a Fluorescence Microscope

The presence of Bl-19-gfp and Am-7-gfp isolates on the surface of mung bean roots was observed by examining root sections using a fluorescence microscope at 100x magnification (Figure 4). Observations using a fluorescence microscope showed that the Bl-19-gfp and Am-7-gfp isolates had rough root surfaces, while the non-inoculated isolates had smooth root surfaces. In addition, the density of the isolates on the root surface was observed.

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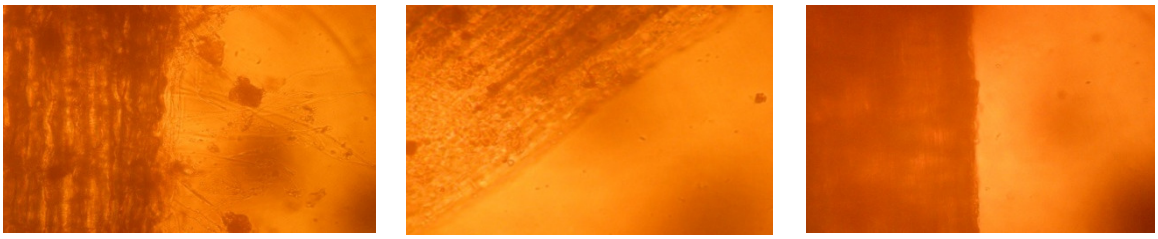


(a) (b) (c)

Figure 4. Mung bean roots grown in sterile growing media under three treatments: (a) inoculation with Bl-19-gfp, (b) inoculation with Am-7-gfp, and (c) non-inoculated control.

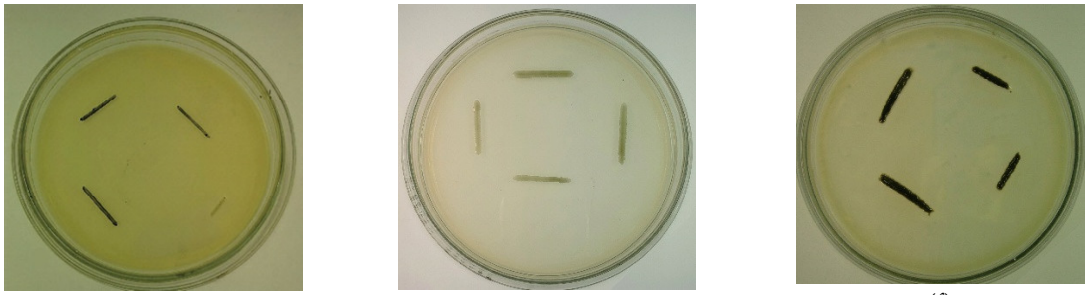
Re-isolation of Bl-19-gfp and Am-7-gfp isolates from the root surface of mung bean plants showed that Bl-19-gfp had a density of 3.0 x 102 CFU, while Am-7-gfp exhibited a density of 2.0 x 101. The density of both isolates on the root surface lower than the density detected in the rhizosphere soil.

3.4. The Presence of Osmoprotectant Rhizobacteria in the Mung Bean Plant Tissue (Endophytic)

Visualization of the search for the endophytic properties of osmoprotectant rhizobacteria in mung bean plant tissue grown in sterile media is presented in Figures 5, 6, and 7.

(a) *(b)* *(c)*

Figure 5. Re-isolation of osmoprotective rhizobacteria isolates Bl-19-gfp and Am-7-gfp in mung bean leaf tissue under UV light. (a) Control, (b) Bl-19-gfp, (c) Am-7-gfp.



(d) (e) (f)

Figure 6. Re-isolation of osmoprotective rhizobacteria isolates Bl-19-gfp and Am-7-gfp in mung bean stem tissue under UV light. (d) Control, (e) Bl-19-gfp, (f) Am-7-gfp.

(g) (h) (i)

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Figure 7. Re-isolation of osmoprotective rhizobacteria isolates Bl-19-gfp and Am-7-gfp in mung bean root tissue under UV light. (g) Control, (h) Bl-19-gfp, (i) Am-7-gfp.

Re-isolation of Bl-19-gfp and Am-7-gfp isolates from leaf, stem, and root tissues on selective medium and observation under UV light showed no green fluorescence. These results showed that neither Bl-19-gfp nor Am-7-gfp isolates were present in the leaf, stem, and root tissues of mung bean plants.

3.5. Mung Bean Growth

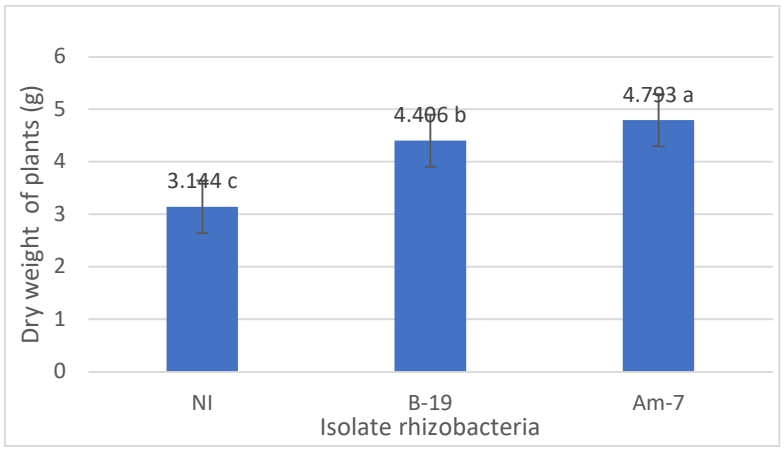

Figure 8. The relationship between rhizobacteria isolates and the dry weight mung bean plants.

Inoculation of Bl-19-gfp isolate and Am-7-gfp isolate on mung bean plants was carried out through seeds and the rhizosphere under drought stress conditions of 60% field capacity and sterile media. The non-inoculation treatment resulted in a dry weight of 4-week-old mung bean plants of 3.144 g, inoculation of the Bl-19-gfp isolate of 4.406 g, and isolate Am-7-gfp of 4.793 g. The Shapiro-Wilk and Levene's tests confirmed that the data were normally distributed and homogenous. The ANOVA showed a significant effect of the treatments, and Duncan's test revealed that the Am7 inoculation treatment gave the highest dry weight, followed by the Bl-19 isolate, and the lowest dry weight of the non-inoculation treatment. The dry weight of mung bean plants in soil moisture conditions of 60% of field capacity using Bl-19 and Am-7 isolates increased 40.13% and 52.44%, respectively.

4. Discussion

4.1. GFP Gene Transformation

The transformation of the GFP gene from E. coli to the Am-7 isolate was successful, and the medium used served as a selective medium containing rifamycin. Both the Am-7-gfp isolate and the Am-7 isolate were resistant to rifamycin, while neither E. coli-gfp nor E. coli was resistant to rifamycin. This is also shown in Figure 1 (b), where the Am-7-gfp isolate exhibited green fluorescence under UV light. However, Am-7 and E. coli-gfp did not fluoresce green, because the cells were dead.

The GFP gene can be used as a fluorescent marker in cells that have been transformed with the gene. As a marker, the GFP gene provided a clear identity for the tagged strain within a natural microbial community [24] (Ohba and Fujioka 2016). Modified GFP plasmids show high levels of GFP expression in several types of studies, making it a very useful tool in molecular biology and biotechnology studies [12].

4.2. The Presence of Osmoprotectant Rhizobacteria in the Rhizosphere and Root Surface of Mung Beans

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The green fluorescence in the Bl-19-gfp and Am-7-gfp isolates also indicates that the GFP gene remained stable under the experimental conditions for four weeks of a genetic marker for tracing the presence of bacterial isolates. GFP fluorescence was detected throughout the four-week observation period under the tested conditions. This supports the statement that chromosomal insertion of the GFP gene can remain stable for a relatively long time [24].

The presence of Bl-19-gfp and isolate Am-7-gfp isolates in the mung bean rhizosphere is further supported by the population density data presented in Table 2. Inoculation of Bl-19-gfp and Am-7-gfp isolates through seeds and rhizosphere at an initial density 106 CFU showed a decrease in population density one week after inoculation, followed by stabilization of the population for up to four weeks. Rood exudates of mung bean grown in sterile media contained glucose and fructose (Table 1), which serve as nutrient sources for microorganisms, including the osmoprotectant rhizobacteria Bl-19-gfp and Am-7-gfp isolates. The initial decrease in bacterial density one week after inoculation may be related to the adaptation process of the introduced isolates to the rhizosphere environment. Particularly to the availability of mung bean root exudates as a nutritional source. Mung bean root exudates contain simple carbohydrates such as glucose and fructose, which serve as nutrients for bacteria. The dominant compounds consistently present in root exudates include simple carbohydrates, amino acids, and organic acids [25,16]. Root exudates function as an important source of nutrition for microbial communities in the rhizosphere [25,26,27].

Table 1. Density of osmoprotective rhizobacteria in the rhizosphere of mung bean plants (CFU/g soil).

Week	Density (CFU/g soil)	
	Bl-19-gfp	**Am-7-gfp**
1	$1.4 \times 10^5 \pm 0.12 \times 10^5$	$1.7 \times 10^5 \pm 0.14 \times 10^5$
2	$1.6 \times 10^5 \pm 0.14 \times 10^5$	$1.1 \times 10^5 \pm 0.16 \times 10^5$
3	$1.3 \times 10^5 \pm 0.17 \times 10^5$	$1.3 \times 10^5 \pm 0.13 \times 10^5$
4	$1.6 \times 10^5 \pm 0.16 \times 10^5$	$1.4 \times 10^5 \pm 0.15 \times 10^5$

Table 2. Density of osmoprotective rhizobacteria on the root surface of mung bean plants (CFU/ g root).

Osmoprotectant Rhizobacteria	Density (CFU/g root)
Control	0
Bl-19	$2.8 \times 10^2 \pm 0.12 \times 10^2$
Am-7	$2.0 \times 10^1 \pm 0.14 \times 10^1$

4.3. The Presence of Osmoprotectant Rhizobacteria on the Root Surface

The results indicated that the Bl-19-gfp and Am-7-gfp isolates form biofilm on the root surface. This finding is supported by data on the density of these isolates on the root surface, as presented in Table 2. Re-isolation of Bl-19-gfp and Am-7-gfp isolates from the surface of mung bean roots showed that the density of Bl-19-gfp was 3.0 x 102 and Am-7-gfp was 2.0 x 101 (Table 4). The densities of both isolates on the root surface were lower than those in the mung bean rhizosphere. This finding suggests that Bl-19-gfp and Am-7-gfp isolates adhere to the root surface through weak interactions. The initial stage was the primary attachment of rhizobacterial isolates to the soil surface, a weak bond that was easily detached because it did not yet include complex adhesive compounds such as EPS. This attachment was non-specific and reversible, mediated by hydrophobic and electrostatic interactions between the isolate cells and root surface molecules. Electrostatic repulsion occurred between the negatively charged bacterial cell sheath and the root surface. After this initial interaction, adhesins present on the cell surface mediate a tighter but still reversible association with the root [28]. The second phase of attachment was the strong and irreversible binding of bacteria to the root surface and was mediated by the synthesis of cellulose fibrils and species-specific secondary attachment factors. This process was induced after successful primary attachment. Secondary attachment will

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form bacterial microcolonies on the roots, and only a small proportion of inoculated isogenic bacteria, typically representing 0.4% to 3.5% of the population, attached to the roots [29].

4.4. The Presence of Osmoprotectant Rhizobacteria in Mung Bean Plant Tissue (Endophytic)

The mechanism of endophytic bacterial invasion into plant tissue can occur through several routes, such as lateral root growth points, growing radicles, and undifferentiated meristematic root tissues. Enzymatic degradation of the root hair cell walls also facilitates bacterial entry into the plant [12]. Figures 5, 6, and 7 showed that the Bl-19-gfp and Am-7-gfp isolates, which carried the GFP gene marker, did not exhibit green fluorescence under UV light. Reisolation from different parts of the mung bean plant, namely leaves, stems, and roots, also did not show green fluorescence under UV light, indicating the absence of Bl-19-gfp and Am-7-gfp isolates in these tissues. After attachment, microcolonies developed into mature biofilms on the root surface. Biofilm formation is a major determinant of successful root colonization and subsequent endophytic development. Isolates Bl-19 and Am-7 exhibited low colony-forming abilities on the root surface, at 3.0 x 102 and 2.0 x 101, respectively. These were unable to infect the root surface and penetrate the plant (endophytic). The ability to colonize the root surface determined the endophytic capacity. Secondary attachment culminated in the formation of bacterial microcolonies on the roots and ensured that the bacteria remained in the rhizoplane. For many bacteria, this was essential for subsequent colonization and endophytic development [30].

The different densities of Bl-19 and Am-7 have different effectiveness because each bacterial strain in the rhizosphere has specific properties and unique environmental carrying capacity. Isolates Bl-19 and AM-7 also have specific properties, both genetically and physiologically. Furthermore, isolate Bl-19 is more adaptive at certain densities to root exudates than isolate Am-7. According to [16], root exudates have the ability to attract or repel specific microbial populations. Plant root exudates contain carbon compounds, organic acids, and amino acids that function specifically as nutrients and chemical signals for microbes.

4.5. Mung Bean Growth

Isolates Bl-19 and Am-7 were osmoprotective rhizobacteria that produced the osmolyte glycine betaine and exhibited resistance to high osmotic stress, particularly drought stress. Under such conditions, both isolates synthesized glycine betaine to protect cells against osmotic imbalance, maintain cellular turgidity, allowing rhizosphere conditions to remain favorable for mung bean growth. Glycine betaine was a well-known osmolyte synthesized by osmoprotective rhizobacteria to mitigate osmotic stress, including high salinity and drought [15].

Am-7 isolate increased plant dry weight by 12.31% compared to Bl-19 isolate. The results showed that the density of both isolates in the same mung bean rhizosphere was 105. However, the glycine betaine-producing ability of Am-7 was 13.8463 mg g-1, which was higher compared to Bl-19 at 10.3212 mg g-1. Glycine betaine played a role in reducing water potential without disrupting metabolism. The decrease in water potential in the cell cytoplasm ensured the flow of water into the isolate cells under drought stress conditions. This condition also stabilized enzymes, protein structures, and turgor pressure, thereby maintaining the functional integrity of microbial cells and their host plants. Glycine betaine is one of the important osmoprotectants, accumulated by various organisms in osmotic stress conditions [15]. According to [6], glycine betaine is an essential osmolyte that helps in maintaining cell turgor pressure, stabilizing cell membranes, as well as protecting proteins and enzymes from denaturation. It also acts as a compatible solute for maintaining cellular osmotic potential that helps prevent water loss from cells and proper cell function under drought conditions. Isolate Am-7 with a higher ability to form glycine betaine, was able to increase the dry weight of mung bean plants under conditions of 60% field capacity.

The limitation of this study is in maintaining the sterile condition of the media for 4 weeks. The homogeneous microenvironmental conditions must be maintained to prevent the formation of a non-treatment environment. Observations on isolates Bl-19 and Am7 rhizosphere were carried out by re-

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isolation with a selective medium and GFP gene labeling, ensuring focus on both isolates. Limitations in isolate identification, molecular confirmation of GFP gene insertion, and physiological drought indicators were limited. Another limitation is the use of a single variety of green bean plants, which was performed to allow close observation of performance. Due to the nature of green beans as leguminous plants that naturally have a symbiosis with rhizobium, the interaction between rhizobium, both isolates, and the plants needs to be studied in depth. Further studies must also be conducted on other leguminous plants, such as soybeans.

Isolates Bl-19 and Am-7 were obtained from the isolation and screening of dryland plant rhizospheres in Bantul, Yogyakarta. Both isolates were observed for their ability to form glycine betaine, morphological tests, and antibiotic resistance tests. This research is a continuation of a novelty: the insertion of the GFP gene, which has a green fluorescence under UV light, to track osmoprotectant rhizobacteria isolates released into the mung bean rhizosphere and elicited the mung bean plant's drought response.

CFU counting for tracking the presence and density of isolates has a limitation: it only enumerates culturable cells. Furthermore, the use of antibiotic resistance for selection carries the risk of gene loss and affects the reliability of the tracking data.

In conclusion, GFP-tagged rhizobacterial isolates Al-19 and Am-7 survived in the rhizosphere. The density of rhizobacterial isolates Al-19 and Am-7 on the root surface was lower than the rhizosphere density. The rhizobacterial isolates Al-19 and Am-7 were not obtained from internal plant tissues and increased the dry weight of mung beans under sterile conditions of 60% FC.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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