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Research article

Halotolerant N-Fixing Bacteria Isolates for Increasing the Biochemical Activity, Total Bacteria Population, N-Uptake and Rice Seedling Growth

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Abstract.
Rice farming is hampered by saline soil, which contains a large amount of soluble salt. The high salt content inhibits plant growth and causes nitrogen nutrient deficiency, as well as sodium and chlorine ion poisoning. Beneficial microbes that have adapted to saline ecosystems (halotolerant) can reduce salinity's impact on rice growth. Microorganisms known as halotolerant bacteria can survive in high salt environments by maintaining an osmotic balance. The use of halotolerant N-fixing bacteria (HNB) as biofertilizers is an attempt to boost nitrogen nutrients and rice plant productivity in saline land. The goal of this study was to see how effective HNB isolates were at increasing rice plant growth. The experiment was conducted in the greenhouse of Universitas Padjadjaran's Faculty of Agriculture, using a randomized block design with 16 treatments (control, single, and consortia of inoculant) and was repeated three times. Rice seedlings were inoculated with HNB isolates and grown in a Fahraeus saline medium. The HNB consortium application increased plant height by 9.03 cm, root-shoot ratio by 0.92, IAA content by 0.475 g/mL, nitrogen content by 2.94%, and the total number of HNB isolates (*Azotobacter*, *Azospirillum*, *Bacillus*, and *Stenotrophomonas*) by 8.10x107 CFU/mL.

Keywords: Biofertilizer, consortia inoculum, N-fixing bacteria, rice seedling

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1. Introduction

Saline soils are soils that contain large amounts of soluble salts. Salinity problems arise when the salt concentration of NaCl, Na_2CO_3, Na_2SO_4 is present in excessive amounts of soil [1]. High salt concentrations can cause poisoning of plants with Na and Cl ions that can take place throughout cell membranes. In addition, salinity affects the water potential in plants which causes plants to physiological stress due to lack of water supply.

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Soil salinization is a global threat that causes a huge reduction of agricultural yield worldwide. More than 20% of all irrigated land on earth is affected by salinization. Rice is very sensitive to salinity stress. Height, root length, shoots and root dry matter of rice plant were affected significantly by salinity. Dissolved salt in high amounts can inhibit normal plant growth and development [2]. High salt content in the soil will affect the osmotic stress of plants so that it will cause morphological and physiological changes. High chloride and sodium sulfate salt content will affect plant growth due to changes related to plant character traits [3]. Soils containing high salt become infertile due to poor nutrient content, microbial activity, biomass, and organic matter [4].

High electrical conductivity is a characteristic of soils that are exposed to the effects of salt. These characteristics can be seen from the soil saturation extract (ECe) of more than 4 dS/m, the salt content of chloride, bicarbonate, sulfate, Na^+, Ca^{2+}, and Mg^{2+} cations are excessive. The presence of excessive cations can interfere the plant growth [4]; [5]. Due to the high salt content in the soil, it can cause an ionic imbalance due to the accumulation of Na^+ and C^{l-} in the cells, disrupting the assimilation of nutrients, interfering with carbon and nitrogen metabolism, and reducing the rate of photosynthesis [6]. Mitigation through improving the physical and chemical properties of saline soils such as washing and rinsing the soil with water, application of gypsum and lime takes a long time and also results in a decrease in the biodiversity of native plant species and microbes [7].

The problem of high salinity can be the main cause of decreased productivity of rice plants in saline land [8]. [9] stated that salinity reduces the ability of plants to absorb water, causing a decrease in growth. If the plant absorbs too much salt, it will cause poisoning in old leaves. This will cause early leaf aging and reduce leaf area which then has an impact on the disruption of the photosynthesis process. In addition, salinity soil fertility is also low due to nutrient deficiencies, one of which is nutrient N [10].

Plant N needs can be met by providing HNB which is able to fix N2 gas from the air. The results of the research [11] showed that the Azotobacter and Azospirillum consortium was able to reduce 25% of urea use and increase yields up to 8.59%. Halophilic bacteria are a type of microorganism that can survive at high salt levels by maintaining osmotic balance. Halophilic bacteria can be the key to the use of biofertilizer technology in saline land, saline tolerant isolates that have the potential as biofertilizer inoculants are one solution as an effort to use saline land in agriculture [12]. This study aimed to determine the effectiveness of halotolerant HNB isolates in increasing the growth of rice plants.

2. Methodology

The study was carried out from February to March 2020 at Soil Biology Laboratory Agriculture Faculty Universitas Padjadjaran, while bioassays were performed at a greenhouse belonging to the Faculty of Agriculture in an experimental station located 752 m above sea level.

The experiment was laid out in the Randomized Block Design with 16 treatments and three replicates. The treatments were single and combination of HNB that inoculated on saline (6 ds/m) Fahraeus liquid media planted with rice seeds:

b0 : Control (without HNB)

b1 : Single inoculant HNB *Stenotrophomonas* sp.

b2 : Single inoculant HNB *Bacillus* sp.

b3 : Single inoculant HNB *Azotobacter* sp.

b4 : Single inoculant HNB *Azospirillum* sp.

b5 : Consortium HNB *Stenotrophomonas* sp. dan *Bacillus* sp.

b6 : Consortium HNB *Stenotrophomonas* sp. dan *Azotobacter* sp.

b7 : Consortium HNB *Stenotrophomonas* sp. dan *Azospirillum* sp.

b8 : Consortium HNB *Bacillus* sp. dan *Azotobacter* sp.

b9 : Consortium HNB *Bacillus* sp. dan *Azospirillum* sp.

b10 : Consortium HNB *Azotobacter* sp. *Azospirillum* sp.

b11 : Consortium HNB *Stenotrophomonas* sp., *Bacillus* sp. dan *Azotobacter sp.*

b12 : Consortium HNB *Stenotrophomonas* sp.*,* *Bacillus* sp. dan Azospirillum sp.

b13 : Consortium HNB *Stenotrophomonas* sp., *Azotobacter sp.* dan Azospirillum sp.

b14 : Consortium HNB *Bacillus* sp., *Azotobacter* sp. dan *Azospirillum* sp.

b15 : Consortium HNB *Stenotrophomonas* sp., *Bacillus* sp., *Azotobacter* sp., dan *Azospirillum* sp.

2.1. Experimental establishment

Isolation of indigenous N2-fixing bacteria from saline soil was carried out at the Soil Biology Laboratory, Department of Soil Science, Faculty of Agriculture, Universitas Padjadjaran, West Java. The soil samples used were derived from the rhizosphere of rice, mangrove, and grass plants in a saline ecosystem in Karawang Regency, West Java. The saline soil sample used has an EC of 4-8 dS/m. Isolation of N2-fixing bacteria was carried out by diluting the soil sample using sterile aquadest. The saline soil solution at dilutions of 10-5 to 10-6 was put in 1 ml into a sterile empty petridish, then 1% TSA agar medium was poured at 45^{o}C. After being incubated for 48 hours, the isolate colonies were transferred by simple streak method on JNFb agar medium. At the end of incubation (48 hours) each isolate was cultured by streaking on JNFb agar.

Preparation of HNB inoculants was carried out by rejuvenating the stock of pure bacterial cultures. On the third day, each pure culture was suspended in 10 mL of NaCl 0.85% solution. Then 1% of the isolate suspension was put into 50 mL of liquid JNFb for each treatment and incubated for three days on a shaker at 115 rpm at room temperature (25^o-28^{o}C).

Preparation of rice plants was carried out by planting rice seeds of the Inpari 34 variety, on sterile straw paper. Rice seeds that had been sterilized using a 0.1% HgCl solution then rinsed with sterile distilled water three times then planted on the surface of the straw paper and covered with another straw paper and then stored in dark conditions for four days. The rooted rice seedling was then used for bioassays.

Planting of rice seedling on Fahraeus media salinization up to 6 dS/m was carried out in test tubes with a diameter of 20 mm and a height of 300 mm. Fahraeus liquid media was inoculated with HNB isolates according to the treatments. Rice seedling are grown by immersing the roots of rice plants into Fahraeus liquid medium and then stored in a greenhouse.

2.2. Parameters and statistical analysis

Plant height of the rice plant was measuring the height of the stem from the surface of the test tube to the highest shoot of the rice plant. Measurements were carried out at one and two weeks after planting. The root shoot ratio is the ratio of dry weight of root biomass divided by dry weight of shoot biomass. Measurements were made when the plant was two weeks after planting. The HNB population was calculated by the total plate count method using JNFb media when the plants were two weeks after planting. Analysis of the N content of rice plants was carried out using the Kjeldahl method [13]. The presence of phytohormones IAA of liquid medium rice plant was determined by spectrophotometer at 510 nm [14].

The data were analyzed using analysis of variance (Anova) at $p \leq 0.05$, to determine the average difference between treatments, the Scott Knott test was carried out at $p \leq 0.05$. The statistical analysis had been carried out using the DSAASTAT statistical program.

3. Results and Discussion

3.1. Plant Growth

The rice plants that inoculated by single and consortium HNB inoculants were grown on Fahraeus liquid media showed different plant heights. At 7 to 14 days after planting, the height of plants inoculated with single isolate b4 and consortium of HNB b14 and b15 was tends higher than control plants. The plant height of HNB b15 consortium increased the largest plant height compared to other treatments against controls (Figure 1)

At the end of the observation, other treatments showed lower plant height than the control, this was influenced by the effect of salinity on plants. Low plant height is caused by salinity stress so that plant growth is disrupted and plants cannot grow optimally [15]. Salinity can affect plants because of the high content of soluble salts. Symptoms of salinity in plants with a high enough salinity level lead to abnormal growth such as dry leaves at the tips and symptoms of chlorosis [16].

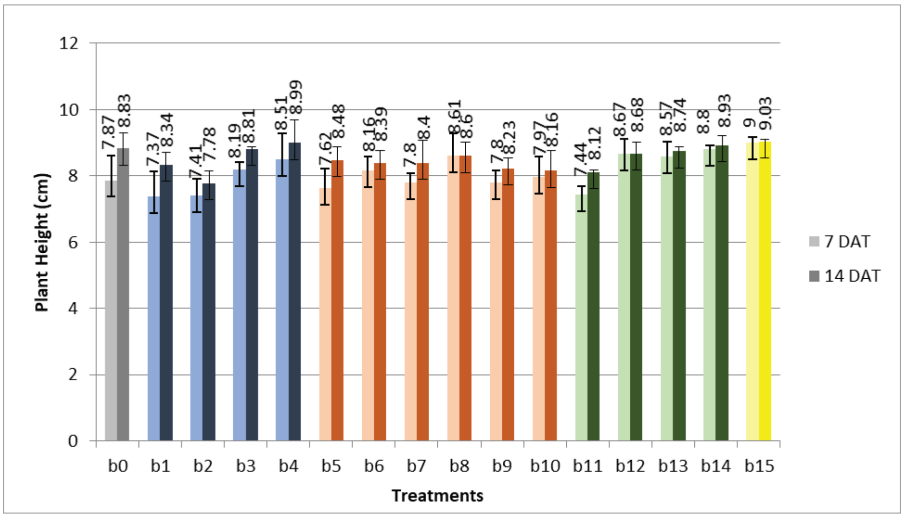

Figure 1: Change in plant height of 7 and 14 days old paddy plant after different single and consortium NHB application.

The Figure 2 showed that there was no different effect between the treatment of single and consortium HNB isolates on the root shoot ratio. However, there was a tendency for almost all consortiums of more than two HNB isolates (b12 to b15) to produce higher root shoot ratios than control plants. The higher the root soot ratio in rice showed that the HNB inoculants increased the shoot growth more than the roots. According to [17] salinity can reduce plant dry weight so that it affects the root loss ratio.

The root shoot ratio provides an overview of the partition flow of photosynthate and explain the efficiency of roots in the photosynthesis process [18]. A root shoot ratio that

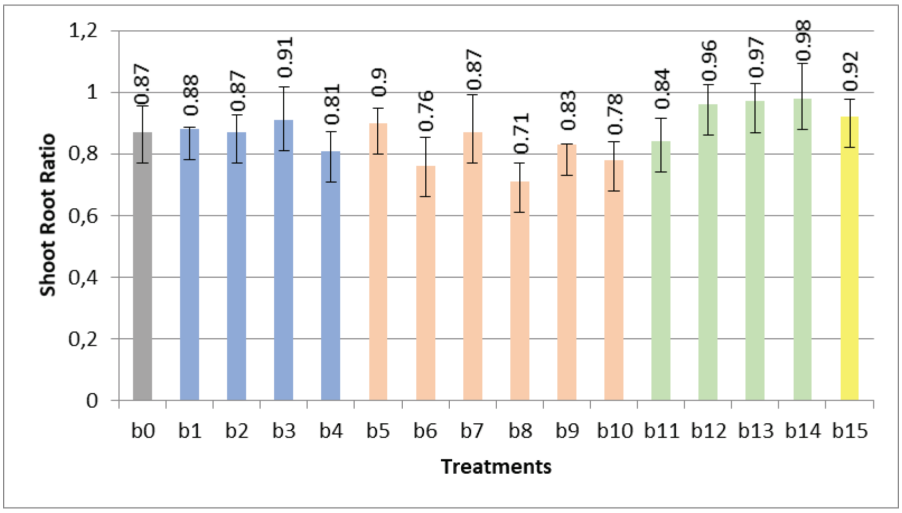

Figure 2: Root Shoot Rasio of paddy plant of different single and consortium NHB treatment.

is more than one indicates plant growth is more in the direction of extinction. A root shoot ratio equal to one indicates a balanced growth between root and shoot plants, while a root shoot ratio of less than one indicates that plant growth is more towards the roots [19]. The consortium microbes supports root growth indicated by higher R/S (Figure 2) due to available N released from *Azospirillum* and consortium bacteria.

3.2. IAA concentration of liquid medium growth rice plant

The all HNB applied singly or in a consortium produced various of Indole Acetic Acid (IAA) concentration (Figure 3). The IAA the concentration of single and consortium of the HNB were not different, but all of mixed HNB (b15) consortium showed a higher IAA concentration than the control and consortium of three species of HNB. Indole Acetic Acid has an effect on stimulating plant vegetative growth, increasing plant height and number of leaves. The increase in rice plant growth is thought to be influenced by the phytohormon IAA (Indole Acetic Acid) produced by HNB.

The content of phytohormones in HNB can increase plant growth and development. Plant hormones regulate several aspects of plant growth and development, such as the formation and maintenance of meristems [20]. Exogenous hormone administration which is usually done has the same effect on the response of plant cells to endogenous hormones synthesized by bacteria [21]. Plant growth hormone is produced by plants and is also obtained from HNB. From the results of research, [22] reported that saline

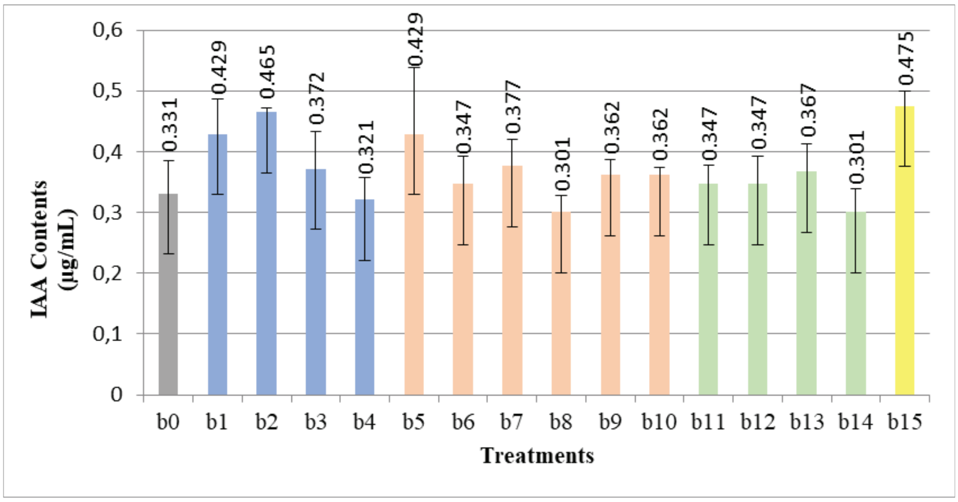

Figure 3: The effect of different single and consortium NHB on IAA concentration of liquid medium growth rice plant of two-week old paddy plant.

endemic bacteria are able to produce IAA hormones of 2.10 ppm to 10.08 ppm. The IAA hormone was able to inhibit the increase in the ethylene hormone when the plant was under stress.

3.3. N content of paddy plant shoots

The results of the analysis showed that there was no significant effect between single HNB and a consortium on the N content of plants. N is necessary for cell division. Plants that are given enough N cause optimal chlorophyll formation, so that the photosynthesis process will run well [23]. Elemental N is also used to build cell protoplasm and the formation of enzymes. N is also associated with photosynthetic activity which is very important in the process of metabolism and respiration [24].

Based on the data (Figure 4), the results showed that the N content of rice plants was included in the sufficient category except for control plants and b7. In control plants, the absence of N supply from HNB caused the plant N content to be low. According to [25] the N content in rice plants is in sufficient condition, which is 2.60% to 3.20% below from 2.40% N in rice plants which are included in the deficiency category.

In treatments b7 and b11 it is suspected that the supply of N provided by HNB is also used by microbes to form cell biomass. Each type of microbe requires different N for the constituent parts of cells and their activities. Deficiency of N elements is often found in saline soils so that N input is needed, one of which is through the activity of

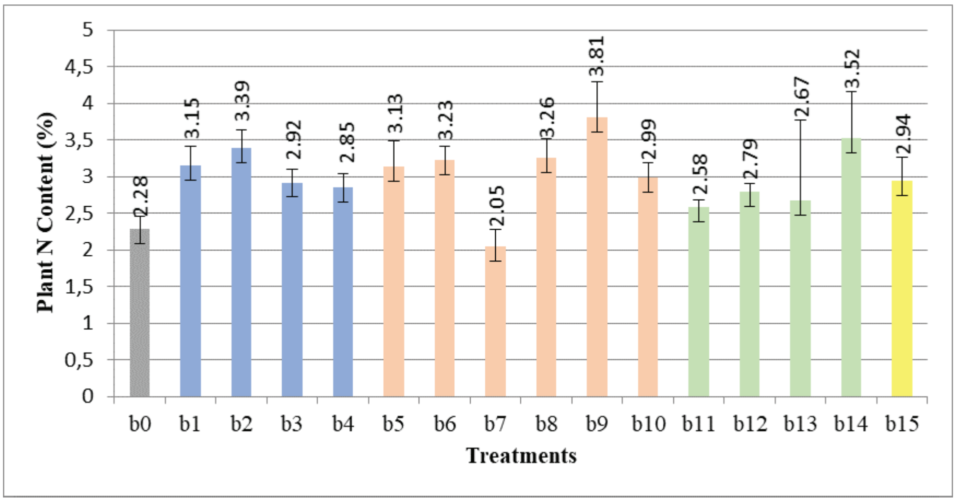

Figure 4: The effect of different single and consortium NHB on plant N content of 14-days old paddy plant shoots.

fixing N_2 from the air by HNB. In saline soil HNB can colonize plant rhizosphere due to root exudates [26]. These microbes are associated with plant roots and live in an intracellular symbiosis with the host plant. HNB is able to fix N_2 from the air, then with support of the nitrogenase enzyme, N_2 is converted to NO_3^- so that it can increase the N content of plants [27].

3.4. Total bacterial population of growth medium paddy plant.

Total bacterial population of the b15 HNB consortium showed a high population but it was not different from the b13 consortium and single HNB *Azotobacter* sp.(b3). HNB *Azotobacter* sp. which were single inoculated on rice plants (b3) or in a consortium with *Stenotrophomonas* sp. (b13 and b15) have a high population. Meanwhile, if *Azotobacter* sp. combined with other HNBs into a consortium, the total population does not increase. The existence of positive and synergistic interactions between several types of bacteria that live in the same growing medium can increase the total population. On the other hand, if there is a decrease in the total population, it can be caused by negative interactions or competing for the same nutrients for growth. In line with the findings of [28], at the environment in general the microbial density is limited by the scarcity of resources, so that microbes will compete for the same nutrients and are limited for their survival. Competition between microbes can occur for nutrients, sunlight, or the space in which they live.

According to [29] one of the important factors for microbial growth is the availability of nutrients. Microbial cell growth will be inhibited if there is a lack of nutrients. In

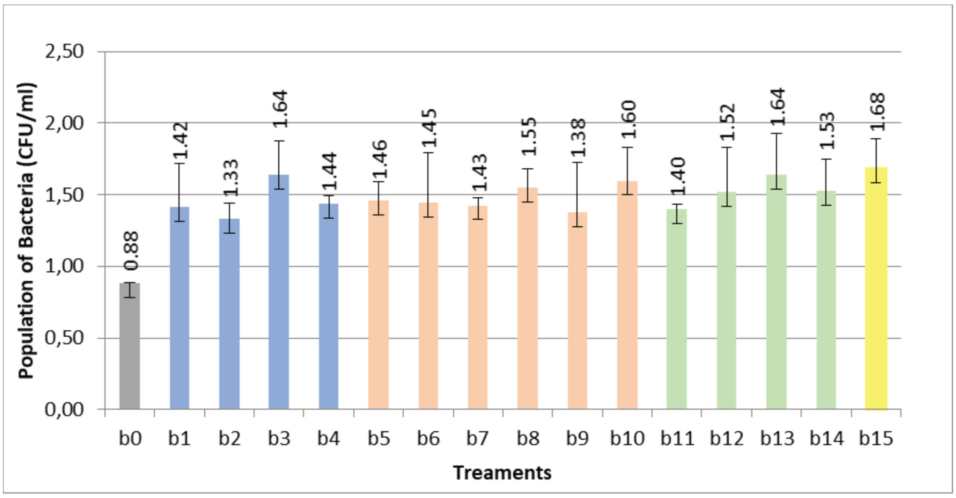

Figure 5: The effect of different single and consortium NHB on total bacterial population of growth medium 14-days old paddy plant.

the saline media, the osmotic pressure outside the bacterial cell is higher than inside the bacterial cell. Due to the high osmotic pressure, most of the bacteria that are not halophilic bacteria will have their growth disturbed. The resistance of halophilic bacteria to salinity depends on the adaptation and defense mechanism of these microbes to saline conditions. One of the defense mechanisms of halophilic bacteria is findings in rhizosphere bacterium *Rahnella aquatilis.* To cope with the high salt environment, the bacterium *R. aquatilis* JZ-GX1 can accumulate K^+ and increase the ratio of Na^+/K^+ [30]. Intracellular accumulation of the molar concentrations of chloride ions (Cl^-) and potassium ions (K^+) is a strategy to deal with high salinity. These activities need to be carried out because at high intracellular salt concentrations, proteins need to maintain their conformation and functional activity [31].

4. Conclusion

Plant height, root shoot ratio, IAA content, N content and total number of bacteria from plants that were given the HNB consortium tended to be higher than applied single one. The results of the experiment revealed that HNB consortium application was able to increase plant height 9.03 cm, root-shoot ratio of 0.92, IAA content at 0.475 µg/mL, N content of 2.94% and the total number of the consortia HNB isolates (*Azotobacer, Azospirillum, Bacillus* and *Stenotrophomonas*) 8.10x10^7 CFU/mL.

5. Acknowledgement

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