# The Ability of Endophytic Nitrogen-Fixing Bacteria and Azolla pinnata in Alleviating Salinity Stress on Rice Seedling

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# The Ability of Endophytic Nitrogen-Fixing Bacteria and *Azolla pinnata* in Alleviating Salinity Stress on Rice Seedling

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**Abstract:** *Nitrogen availability is one major constraint for rice growth due to salinity stress. The nitrogen substance used by rice plants is obtained during the fixation of $N_2$ gas by some endophytic bacteria. Azolla pinnata, as green manure, can providing a potential nitrogen source for rice plants. The research was conducted to investigate the performance of rice seedling inoculated endophytic N-fixing bacteria (ENB) and A. pinnata grown in different soil salinity. The experiment was set up as a randomized block design with 4 salinity levels combined with ENB and A. pinnata application. The selected ENB isolates were identified as Pseudomonas stutzeri. The application of P. stutzeri and A. pinnata enhanced populations of ENB, root length, dry weight, and N content of rice plant until 4 mmhos $cm^{-1}$ salinity levels. This finding reveals that ENB inoculation combined with A. pinnata was able to alleviate salinity stress of rice seedlings in saline soils.*

**Keywords:** *Azolla pinnata*; endophytic N-fixing bacteria; *Pseudomonas stutzeri*; rice plant; saline soil

## 1. INTRODUCTION

The intrusion of seawater in rice cultivation causes the accumulation of salts dominated by sodium chloride (NaCl) in the soils. High saline soils affect morphological responses and physiological and metabolic processes, inhibit respiration and photosynthesis due to changes in osmosis and ion concentration [1]. This has an impact on the disruption of plant growth such as decreasing length and thickness of leaves, leaf chlorophyll content, dry weight of plants, and yields have been declining due to increasing salt stress [2]. Rice crops are quite sensitive to salinity [3]. The absorption of excessive elements of salt leads to a decrease in water absorption and essential elements for plants, one of which N is indispensable in the vegetative phase as an element of photosynthesis support because it can increase the amount of chlorophyll in plants [4].

The biofertilizer application is expected to improve soil health and reduce the intensity of the major disease in rice. The application is also to increase the effeciency of fertilization and grain yield [5]. One type of microbial biofertilizer that can be given to rice plants is endophytic bacteria. Although, there is no external indication of infection or negatif effect on their host plant, the endophytic bacteria can be defined as bacteria that colonize the internal tissue of plant host without causing disease symptoms [6]. Gamalero [7] state that endophytes are microorganisms that appear to be harmless to the host plant and can be isolated from disinfected plant tissue either on the surface or inside of the plant. Several reports showed that endophytic microorganisms are capable of controlling plant pathogens and enhance seedling emergence and promote plant growth. Endophytic bacteria that live in plant tissues for a certain period and were able to live by forming colonies within plant tissues without endangering their host. Each high-level plant may contain several species of endophytic bacteria capable of producing secondary metabolites. In general, endophytic bacteria enter the plant tissue through the root, stomata, or wounded plant parts [8]. In the plants' vascular system, endophytic bacteria that can fix $N_2$ presents in the intracellular cavity. The bacteria take dissolved $N_2$ gas from the sap flow and then convert it into amines and ammonium nitrogen that are used by plant. In nonlegume plants, such as rice, these bacteria had been studied well. Inoculation with $N_2$-fixing bacteria has increased rice plants growth and its grain yield [9]. $N_2$-fixing endophytic bacteria isolated from various plant species can contribute up to 47% in $N_2$ fixing of air [10]. Selected Isolates of bacterial endophytes obtained from the roots, stems and leaves of rice showed nitrogenase activity and indole-3-acetic acid (IAA) phytohormone. These bacteria have also shown the ability to produce cytokinin (zeatin and kinetin) and gibberellin that contributes to enhance plant growth [11].

*Azolla pinnata* species of aquatic fern known by several common names, it is a symbiont of *Anabaena azollae* that is easily found in rice fields and can be utilized as an organic ameliorant to increase the availability of N elements in the soil [12]. Nitrogen (N) is an essential element that important for the growth and yield of plants such as rice. According to Setiawati [13], *A. pinnata* has a fairly high N content of 4-5 %. Fresh Azolla can be immersed into the soil, where its nitrogen content will be mineralized and released as ammonia then becomes available for rice plant [14]. Application of liquid biofertilizers of endophytic bacteria is expected to increase the absorption of N by plants from the air and ameliorate *A. pinnata* is expected to increase the availability of N in the soil for plants that can help the growth of rice grown on saline soil.

## 2. MATERIALS AND METHODS

### 2.1. Sampling and Isolation ENB

Sample collection (*Oryza sativa* L.) was carried from saline soil the central paddy field in Karawang District, West Java province from 06° 05,00' 8"S; 107° 25' 34,2"E to 06° 09' 26,5" S; 107° 28' 40,8" E and from 06° 12' 13,5"S; 107° 35' 52,7" E to 06° 12' 22,2"S; 107° 35' 42,9"E. Sample (rice root) was washed with tap water to remove the attached clay and then rice stem was separated from root by cutting. Subsequently, the stem and root were immersed in 70% ethanol for 3 min, then washed, consecutively, with solution of fresh sodium hypochlorite for 5 min and with hydrogen peroxide (3%) for 30 seconds, and finally they were rinsed five times with sterile distilled water. The stem and root were, respectively, cut by 1-2 cm, and macerated with a sterile mortar and pestle. The extracted tissue was, serially, diluted by tenfold with sterile water. As many as 200 µl-aliquot samples were for triplicate inoculation on Nitrogen-free semisolid JNFb agar plates. After 48-72 h incubation, bacteria grown in agar plates as single colonies were streaked JNFb agar plates to obtain pure single colonies. Bacterial colony was based on morphology and pigmentation. Each bacterial colony was then grown on the basic agar slant and incubated for 4 days at 30°C. Following, these cultures were stored at 4°C in the refrigerator.

### 2.2. N-fixing Endophytes Bacteria Selection by Acetylene Reduction Assay

The nitrogenase activity of 11 isolates of endophyte bacteria was determined by the method of an Acetylene Reduction Assay (ARA). Each bacterial isolate was grown in a 20 ml test tube containing 10 ml of N-free semi-solid medium and sealed with a rubber stopper, then incubated for 72 h at 30°C. Meanwhile, into the vial was added an equal volume by removing air with acetylene to achieve an ambient atmosphere containing 10% acetylene (v/v). Ethylene concentration was determined with the method of gas chromatography as described by [15]. At 24 hours intervals, 1 ml of the gaseous phase was injected into a gas chromatograph (GC) equipped with a flame ionization detector (FID) and a Porapak N column.

### 2.3. Preparation of Azolla Green Manure

Azolla production was carried out in nursery ponds in Jatinangor district, West Java province. With suitable bunds and irrigation channels, the field selected for Azolla cultivation with the size of 10 m × 2 m was prepared in the area covered with 10 cm-thickness paddy soil and soaked with at least 10 cm-depth waters. The cattle manure 50 kg was applied in the soil then inoculated with 4 kg Azolla. Single superphosphate (100 g) was applied in 2 split doses at an interval of 4 days to the ponds. 40-50 kg fresh Azolla have been harvested from the plot after 15 days. As green manure Azolla collected directly from ponds was applied in the pot with 7-ton/ha doses or 35 g/pot. Azolla incorporated as a basal manure 7 days before the rice was transplanted.

### 2.4. Soil Pots Preparation

The sandy silt loam soil (plow layer) with 2.55% organic-C, 6.27 pH, 0.28 mmhos $cm^{-1}$ electrical conductivity, 0.22% total-N was collected from Agriculture Experimental Farm of Universitas Padjadjaran, Jatinangor Sumedang, West Java. The soil was sieved through 0.2 cm and air-dried. The experiment was set up as a randomized block design with 8 treatments (0, 2, 4, and 6 mmhos $cm^{-1}$ combined with N-fixer bacteria and *A. pinnata* application) and provided with four replications. The salinity levels were adjusted by adding the NaCl into the soils. The quantity of NaCl required for the preparation of each saline soil treatment was calculated with the method suggested by [16]. The NaCl was directly mixed into the dry soil. For each treatment, the soil (1 kg/pot) was put in plastic pots without drainage holes in the bottom. The pots were then placed on the glasshouse. Subsequently, the pots were fertilized with nitrogen (urea 100 kg/ha), phosphate (SP-36 50 kg/ha), and potassium (KCl 50 kg/ha).

### 2.5. Preparation of Rice Seedlings

The seeds of the Inpari 34 rice plant variety (salinity tolerant) from the Indonesian Centre For Rice Research in Sukamandi, West Java, were sterilized by soaking in 0.1% HgCl solution for 1 minute, then soaking with 70% alcohol for 2 minutes, then rinsing with sterile aqua distillation 3 times. Sterile rice seeds were soaked for 48 hours in a solution containing 3% molasses as a carrier and $1 \times 10^7$ CFU $mL^{-1}$ endophytic biofertilizer. For treatment without biofertilizer, sterile rice seeds are immersed in a 3% molasses solution. After 48 hours, the seeds were germinated in a tray with soil and manure with a ratio of 1:1 and given water to inundate the soil. Rice seedlings are grown in trays for 10 days and transferred to plant pots.

### 2.6. Data Analyses

Statistical significance of all data obtained (in triplicates) with a completely randomized design were tested by using the Statistical Package for Social Science (SPSS) version 21.0. Data from the Population of Endophytic Bacteria, Nitrogen Content of Rice Plant, Content of Total N soil, Root Length of Rice Plant, and Dry Weight of Rice Plant were analyzed statistically. The different data of parameters were tested with one-way ANOVA. Means were separated using Duncan's Multiple Range Test (DMRT) at $P< 0.05$.

## 3. RESULTS AND DISCUSSION

### 3.1. Selection and Identification of Isolates

Eleven endophytic bacterial isolates were obtained from the tissues of roots, stems, and leaves of rice plants growing on saline rice fields in Karawang Regency, West Java. Three nitrogen-fixing endophytic bacterial isolates were selected from 11 endophytic bacterial isolates based on the Acetylene Reduction Assay analysis, they have nitrogenase enzyme capacity varied from 2.391 ppm until 7.197 ppm from roots, stems, and leaves tissues of rice plants (Table 1).

The isolate that has the highest nitrogenase enzyme capacity was used as selected endophytic bacteria biofertilizers, it was an endophytic bacterial isolate that is from paddy leaf tissue. The selected isolate has a nitrogenase enzyme capacity of about 7.197 ppm. Selected isolates produce gibberellins growth hormone at 16.001 ppm, although the gibberellins produced are not the highest but it is the second-highest at gibberellins produced by endophytic isolates.

Table 1. Nitrogenase activity and gibberellin of N-fixing endophytic bacteria from rice plant growth in saline soils

| No | Sample Code | Acetylene Reduction Assay (ppm) | Gibberellin (ppm) |
|---|---|---|---|
| 1 | K9P5 R1 | 5.070 | 29.015 |
| 2 | K10P4 R2 | 2.617 | 11.937 |
| 3 | K9P4 R1 | 3.808 | 12.445 |
| 4 | K10P5 R2 | 5.571 | 12.875 |
| 5 | K9P4 S2 | 5.335 | 13.498 |
| 6 | K9P5 S1 | 5.652 | 14.420 |
| 7 | K10P5 S1 | 2.391 | 15.635 |
| 8 | K10P5 L1 | 2.517 | 11.877 |
| 9 | K10P4 L1 | 7.197 | 16.001 |
| 10 | K9P4 L2 | 5.194 | 11.735 |
| 11 | K9P5 L1 | 4.944 | 15.420 |

Data from duplo sample R= root
K= Karawang (location sample) S= stem
P = Number sample L= leaf

Elbeltagy [17] said that endophytic bacteria can do colonization and nitrogen-fixing on Oryza species. The ability of nitrogen fixation in plants depends on rice species due to the existing various colonization of endophytic Herbaspirillum. This suggests the presence of host speciality or diazotrophic endophytes preference similar to that in microbe-plant mutualism, such as Rhizobium and legume. They studied the effect of inoculation of *Herbaspirillum* sp. strain B501gfp1. in rice seedling on Acetylene reduction activity (ARA). It is important to consider the possible emission of ethylene from plants. To verify nitrogen emission from plants of endophytes, the an indirect method of acetylene reduction assay can be used. The ethylene evolution from 7 day-old rice inoculated with and without endophytic bacteria and seeding with 10% acetylene was determined. The ARA of rice seed inoculated with *Herbaspirillum* sp. endophytic bacteria was 71.3 nmol $g^{-1}$ $hour^{-1}$ while non inoculated was 3.9 nmol $g^{-1}$ $hour^{-1}$.

The selected endophytic bacterial isolates from paddy leaf tissue were *Pseudomonas stutzeri* has nitrogenase enzyme capacity is 7.197 ppm that equals to 257.04 nmol $g^{-1}$ $hour^{-1}$. The amount of high ARA's *P. stutzeri* in this study compared to the results of Elbeltagy's research had an impact on the ability of these bacteria to fix $N_2$ and provide N in colonized rice plants. $N_2$ fixation by endophytic bacteria on rice plant tissue that grows on saline soil media will increase N uptake of the plant because the $N_2$ fixation process is carried out on plant tissue and is not affected by the effects of salinity stress.

Gibberellins, the hormones with a wide range of activities, are used for seed germination and cell elongation [18]. Although salinity stress causes a decrease in plant growth and yield the seed germination of plants promoted by gibberellin. It has been shown that the use of gibberellins can reduce the adverse effects of salinity on plant growth and yield. In treated soybean plants, increasing levels of bioactive gibberellin and jasmonates decreased levels of abscisic acid and salicylic acid. The reduction of stress by gibberellins on plant growth has not been fully elucidated, but it is likely that hormonal homeostasis is the most important factor contributing to reducing high salinity stress for plants [19].

The result of the identification 16S ribosomal RNA gene of selected endophytic K10P4L1 bacterial isolate from paddy leaf tissue was *Pseudomonas stutzeri* with a similarity percentage of 99% (Figure 1).

Result of identification

The sequence of nucleotide and type (% similarity)

CGCGCGTAGGTGGTTCGTTAAGTTGGATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCGAGCTAGAGTA
TGGCAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCT
GGGCTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT
CGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCATTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTT
AAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAG
GCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTAAGGTGGGCACTCTAAGG
AGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTAC
AATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCAGTCTGCAAC
TCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCATAATGTCACGGTGAATACGTTCCCGGGCCTTGTCGA

Partial sequence (99%) of ***Pseudomonas stutzeri*** 16S ribosomal RNA gene

Fig.1. The results identification and sequence of nucleotide and type (% similarity) of selected isolates

Identification of selected endophytic bacterial isolate was carried out using the 16S rRNA analysis method at Molecular Biology Laboratory BB Biogen in Bogor. Sequence variation in the 16S ribosomal RNA (rRNA) gene [20] and its hypervariable regions [21] is widely used to characterize taxonomic diversity present in microbial communities. The methode are available to download from multiple databases such as Greengene [21] and the Ribosomal Database Project [22]. Instead of the entire gene length, sequence individual hypervariable regions is sufficient for used in taxonomic classification. The result of 16S ribosomal RNA identification of selected endophytic bacterial isolates from paddy leaf tissue was *Pseudomonas stutzeri*. partial sequence (similarity percentage 99%). *Pseudomonas stutzeri* bacteria have been known in the results of previous studies that the ability of nitrogen fixation is determined by the characteristics of nif genes and nitrogenase enzymes. *Pseudomonas stutzeri* has the characteristics of genes and the enzyme nitrogenase [23]. These bacteria can increase the growth and fix of $N_2$ in upland rice [24]. In the research of [25] *Pseudomonas stutzeri* is used as a seed treatment. Nadeem [26] show that *Pseudomonas stutzeri* can produce indole-3-acetic acid, gibberellic acid, trans-zeatin riboside and abscisic acid. *Pseudomonas stutzeri* strain A15 can infect and survive within rice root and plant to provide fixed nitrogen and hence promote the plant growth. Besides that, the advantages of using *Pseudomonas* sp. which is one of the many bacteria known as the largest producer of

antibiotics in the rhizosphere [27].

### 3.2. Endophytic Bacteria Population and N Rice Plant Content

Biofertilizer and *Azolla pinnata* application increased significantly endophytic bacterial population (EB) of rice plants (Table 2). Compared to control, the population of ENB increased from 7.4 × $10^3$ CFU $g^{-1}$ to 13.4-20.1 ×$10^3$ CFU $g^{-1}$ at 2 and 4 mmhos $cm^{-1}$. Briefly, the inoculation and green manure application was able to increase the population of EB by more than 200%. In contrast, the ENB population of untreated pots decreased significantly. Under these experimental conditions, external colonization of root by the endophytic bacteria in 4-6 mmhos $cm^{-1}$ NaCl without *Azolla pinnata* was suppressed in soil saline medium.

Table 2. The effect of endophytic biofertilizer and *Azolla pinnata* application at different salinity on population of endophytic bacteria in root rice and N content of rice plant

| Treatments | | Endophytic Bacteria Population* ($10^3$ CFU $g^{-1}$) | N Content* (%) |
|---|---|---|---|
| A = | Non-saline soils (control) | 7.4 ab | 3.83 c |
| B = | Non-saline soils + biofertilizer + *Azolla pinnata* | 20.1 c | 4.04 d |
| C = | 2 mmhos $cm^{-1}$ NaCl soils | 6.9 ab | 3.36 b |
| D = | 2 mmhos $cm^{-1}$ NaCl + biofertilizer + *Azolla pinnata* | 17.7 c | 3.72 c |
| E = | 4 mmhos $cm^{-1}$ NaCl soils | 5.1 a | 3.22 ab |
| F = | 4 mmhos $cm^{-1}$ NaCl + biofertilizer + *Azolla pinnata* | 13.4 bc | 3.70 c |
| G = | 6 mmhos $cm^{-1}$ NaCl soils | 3.1 a | 3.04 a |
| H = | 6 mmhos $cm^{-1}$ NaCl+biofertilizer + *Azolla pinnata* | 4.5 a | 3.40 b |

*Number with different letters is significantly different at $p<0.05$, according to DMRT.

Endophytic bacteria populations at 7 days after planting have been to multiply rapidly to be able to provide a better effect on increased endophytic bacteria populations compare to in plants without biofertilizers and *Azolla pinnata* application up to 4 mmhos $cm^{-1}$ of salinity. Meanwhile, Rolfe and Weinman [28] stated that endophytic bacteria applied to rice leaves multiply 8 to 10 generations within 10-15 days after planting. Various studies on different methods have reported that endophytes bacteria could be isolated from different plant species as the bacteria are found distributed in several plant cells; roots, leaves, stems, fruits and seeds. Anyasi & Atagana [29] said that the root of the plant is the area with the highest density of the bacteria which could reach as much as $10^6$ colony-forming units per gram (CFU $g^{-1}$). Besides that microbial sources that colonize plant roots into endophytic microbes in plant tissues are derived from rhizosphere microbes. Mano [30] said that the total number of endophytic per gram of rice capable of forming colonies from $10^2$ to $10^4$ CFU $g^{-1}$. Etesami [31] told that size of colonization of the endophytic bacterial population in rice plants varied from $10^4$ to $10^7$ CFU $g^{-1}$ by wet weight basis. Etesami [31] stated that the endophytic bacterial population was not significantly affected by the N fertilization for rice cultivar and by isolates with different PGP traits. The colonization levels of population isolates in these studies were significant. The Inoculated rice roots appeared healthy with no the hypertrophies development like nodule nor obvious symptoms of the disease.

The density of endophytic bacteria population at 6 mmhos $cm^{-1}$ salinity level with or without biofertilizer and *Azolla pinnata* were not different that indicates we need to increase the density of endophytic bacteria population more than $10^7$ CFU $mL^{-1}$ in biofertilizer at of 6 mmhos $cm^{-1}$ salinity level. According to Setiawati [32], the increased concentration of liquid biofertilizer significantly increases the population of endophytic bacteria in rice roots due to increased bacterial density. The density of endophytic bacterial inoculants influenced the successful domination of bacterial colonization in rice roots plants.

Moreover, Table 2 revealed that the application of biofertilizer and green manure (*A. pinnata*) resulted in the highest content of N (4.04%) rice plants of non-saline soils compared to other treatments. In addition, the N contents at the higher salinity levels (2-4 mmhos $cm^{-1}$) were not significantly different from the control. These results showed that endophytic biofertilizer and *A. pinnata* can alleviate the adverse effects of salinity until 4 mmhos $cm^{-1}$.

Endophytic bacteria can directly fix $N_2$ from the air then through nitrogenase activity. $N_2$ converted to $NO_3^-$ in plant tissue so that the N plant content is the increase [33]. The significance of the increase in plant nitrogen content due to the high N content in Azolla was reported by [12] Bhuvaneshwari. Besides that, *Azolla pinnata* contains Ca as the third largest element that is 0.45%-1.70%. According to Wu & Wang [34] permeability of cell membranes damaged by high NaCl concentrations can be improved by $Ca^{2+}$ addition, this is because $Ca^{2+}$ can break the NaCl bond by replacing the $Na^+$ position so that the NaCl concentration in the soil is reduced.

N content in rice seedlings ranged from 3.0-4.0% [35]. In line with Setiawati [32] indicating that a high population of endophytic N-fixing bacteria isolated from roots and rice stems could increase N content in rice plants compared to controls. Sturz & Nowak [36] states that the inoculant of endophytic bacteria *Azospirillum* sp. in rice plants contributes to N up to 66%.

### 3.3. Content of Total N Soil and Growth of Rice Plant

The total N contents in soils were not affected by the application of endophytic biofertilizer and *A. pinnata* (Table 3). In contrast, the roots length and dry weight plants treated with ENB and green manure at 2-4 mmhos $cm^{-1}$ of salinity levels were significantly higher than untreated pots. Briefly, the addition of endophytic biofertilizer and *Azolla pinnata* was able to increase the roots length and dry weight of rice plants until 4 mmhos $cm^{-1}$. In general, the growth of rice at the higher salinity levels (6 mmhos $cm^{-1}$) decreased significantly compared to other treatments.

Table 3. The effect of endophytic biofertilizer and green manure (*A. pinnata*) application at different salinity on soil total N, root length, and rice plant dry weight

| Treatments | | Total N* (%) | Root Length* (cm) | Dry Weight* (g) |
|---|---|---|---|---|
| A = | Non-saline soils (control) | 0.24 ± 0.017 | 6.45 c | 0.17 c |
| B = | Non-saline soils + biofertilizer + *Azolla pinnata* | 0.25 ± 0.009 | 7.38 c | 0.20 d |
| C = | 2 mmhos $cm^{-1}$ NaCl soils | 0.24 ± 0.011 | 3.93 ab | 0.13 b |
| D = | 2 mmhos $cm^{-1}$ NaCl + biofertilizer + *Azolla pinnata* | 0.27 ± 0.042 | 6.45 c | 0.17 c |
| E = | 4 mmhos $cm^{-1}$ NaCl soils | 0.23 ± 0.019 | 3.13 ab | 0.12 ab |
| F = | 4 mmhos $cm^{-1}$ NaCl + biofertilizer + *Azolla pinnata* | 0.25 ± 0.002 | 4.50 b | 0.16 c |
| G = | 6 mmhos $cm^{-1}$ NaCl soils | 0.25 ± 0.006 | 2.13 a | 0.10 a |
| H = | 6 mmhos $cm^{-1}$ NaCl+biofertilizer + *Azolla pinnata* | 0.27 ± 0.014 | 3.50 ab | 0.11 ab |

*Number with different letters is significantly different at $p<0.05$, according to DMRT.

The plant that infected by N-Fixing bacteria, $N_2$ fixation and the process of converting the $N_2$ fixation to the nutrients available for the plants are all carried out in the plant tissue so as not to affect the increase in total N of the soil. The N content of the soil is affected by the amount of N input as well as the loss in cycle N. The low total N content in soil may occur because N has been absorbed by the plant or due to leaching and evaporation. *Azolla pinnata* application had no significant effect on N-total soil caused by rapidly mineralized Azolla and absorbed to rice plants so that N in soil did not differ in all treatments.

In lowland rice cultivation, the most important process is the conversion of organic nitrogen into ammonia. The C:N ratio affects the rate of mineralization, at low C:N the decomposition will be occure within 2 days while at high C:N its occurs in 5 days [37].The rate of N-organic mineralization of *Azolla pinnata* depends on environmental conditions such as soil acidity, temperature, C:N rasio, water content, and soil clay content [38]. In addition. the mineralization process is also influenced by the frequency of applying organic material or added green manure.

Prayoga. [39] said that *Azolla pinnata* can improve chemical soil properties (organic carbon and total nitrogen), plant height, number of tillers, and grain yield of rice plant. In rice fields, high clay content was causing aggregate soil to solidify so that the roots are difficult to grow and develop. Higher accumulation of salt on soil may inhibit root plant growth due to osmotic stress. This occurs because the water in the solution concentrates with the low soluble salt of the plant root cells moving toward a high salinity solution to the soil, so the plants lack water and have problems of division and expansion of cells at the ends of the roots [40]. According to Munif [41], biopriming of upland rice seeds for 6 hours in endophytic bacterial suspension $10^9$ - $10^{10}$ CFU $mL^{-1}$ density was able to produce root length better than controls that were 91% longer. Endophytic bacteria are also capable of producing phytohormones of Indole-3-Acetic Acid (IAA) which play a role in primary root elongation, lateral and adventitious root formation [42].

The role of endophytic bacteria in decreasing the adverse effects of salinity on the dry weight of plants has been reported by Jha [43], which inoculated endophytic bacteria *Pseudomonas pseudoalcaligenes* in rice plants. resulting in higher dry weight and significantly different to controls in all different salinity treatments 40 days after planting. Application biofertilizers of the highest concentration have a higher chance of colonizing plant tissue. Plant tissue colonized by $N_2$-fixing endophytic bacteria could convert $N_2$ to $NH_4^+$ and is used to form amino acids that play a role in the formation of plant biomass. The more amino acids that are formed. the greater the dry weight of the resulting plant. The $Ca^{2+}$ element contained in *Azolla pinnata* can improve plant growth inhibition by $Na^+$ in most plants [44]. Inoculation *Pseudomonas stutzeri* endophytic bacteria with *Azolla pinnata* aplication in 4 mmhos $cm^{-1}$ saline soil can increase the dry weight and roots length of paddy plants compare without those treatments. The effect to dry weight of paddy plant was evidently the same as non saline soil, while the growth of roots became more better on the soil with the same salinity.These results concluded that the application of endophytic bacteria and green manure (*Azolla pinnata*) was able to alleviate the effect of salinity stress and increased the growth (roots length and dry weight) under saline conditions. The selected and identified ENB isolates (*Pseudomonas stutzeri)* combined with the green manure could be used to improve the rice growth on saline soils.

## 4. CONCLUSIONS

The selected endophytic N-fixing bacteria (ENB) isolates from paddy plants (*Oryza sativa* L.) was carried from saline soil in Karawang, West Java were identified as *Pseudomonas stutzeri*. The application of *P. stutzeri* and *A. pinnata* enhanced populations of ENB, root length, dry weight, and N content of rice plant until 4 mmhos $cm^{-1}$ salinity levels. The population of ENB increased by more than 200%, while the ENB population of untreated pots decreased. The application of ENB and *A. pinnata* was able to alleviate the effect of salinity stress and increased the growth roots length and dry weight under saline conditions.

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