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RESEARCH ARTICLE

Potential of Indigenous Rhizosphere N_2-Fixing Bacteria in Improving Soil Properties, Growth and Yield of Maize on Alluvial Soils in the Dyke System

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Abstract

Nitrogen (N) deficiency hinders maize cultivation in diked alluvial soils (AS), and chemical N fertilizers can harm the environment. This study aimed to (i) select N_2-fixing bacteria from maize rhizospheres in AS; (ii) evaluate their effects on soil fertility, N uptake, and maize growth and yield. Thirty-six soil samples from maize fields in An Giang, Vietnam, were collected to isolate bacteria. A pot experiment followed with 9 treatments: (i) 100 % N of the recommended fertilizer dose (RFD), (ii) 85 % N of RFD, (iii) 70% N of RFD, (iv) 55% N of RFD, (v) 85% N of RFD with 2 N_2-fixing rhizospheric bacteria (RB) isolates, (vi) 70% N of RFD with RB, (vii) 55% N of RFD with RB, (viii) 0% N of RFD with RB, and (ix) 0% N of RFD. Out of 67 isolates, *Enterobacter asburiae* ASD-07 and *E. asburiae* ASD-28 were selected. The amount of available nitrogen in the soil was high in the treatments with low nitrogen fertilization, specifically 0% N + RB and 55% N + RB. However, the amount of nitrogen absorbed by the plants was highest in the treatments with 100% N and 85% N + RB. The highest grain yield was observed in the 85% N + RB treatment, which increased by 7.2% compared to 100% N fertilization and by 12.6% compared to the treatment receiving 85% N. These results indicate that *E. asburiae* ASD-07 and ASD-28 may have the potential to reduce the use of inorganic fertilizers and minimize environmental impact.

Keywords: *Enterobacter asburiae* ASD-07, *E. asburiae* ASD-28, Nitrogen fixation, Rhizospheric bacteria, Vietnam

Introduction

Nitrogen (N), one of the most abundant elements on Earth, contributes to 78.1% of the atmosphere and is a vital nutrient for all living organisms [1]. Nitrogen supplementation is extremely important for the intensive production of crops, which require large amounts of N fertilizer; however, there are more than 50% of N fertilizer left in the soil [2]. This leads to not only soil and air pollution but also a waste of resources if proper management is lacking [3-5]. Nitrogen fixed by bacteria can be used as an alternative source of N for sustainable agriculture [2]. Therein, rhizospheric bacteria can improve plant growth and yield [6]. Free-living and N_2-fixing microorganisms are found widely in some archaea and bacteria, including *Proteobacteria*, *Firmicutes*, *Cyanobacteria*, and green sulfur bacteria [7].

Maize, *Zea mays* L., is one of the most important annual crops, which can be used as a staple food and a source of income in many developing countries [8]. Maize requires enormous amounts of N fertilizer (approximately 200 kg N ha^{-1}) for optimal growth and yield [9]. The total N uptake of maize has been reported

to be approximately 177.0 - 331.1 kg ha^{-1} on different sandy soils and under various irrigation regimes [10]. The uptake at maturity has been reported to be higher than 400 kg N ha^{-1} and 200 kg ha^{-1} at silking [11,12]. Maize yield had a potential of 22.5 Mg ha^{-1} without stress from water and nutrients [13]. Thereby, the growth of maize goes up according to the application of N fertilizer. Because the N element plays a vital role in photosynthesis, N fertilization promotes leaf photosynthetic activities [14]. Consequently, in the study by Imran *et al.* [15], an increase in plant height and cob length of maize was observed when elevating N fertilizer from 100 to 400 kg N ha^{-1}. Apart from above-ground maize parts, maize roots can receive benefits from N fertilization [16]. Therefore, maize grain yield, dry weight, and grain protein content could be improved by the N fertilization [17]. Moreover, at 250 kg N ha^{-1}, maize performance can also be improved under drought stress by N fertilizer application [18]. Alluvial soils (ASs) are abundant in Vietnam, especially near large riverbanks. The soils receive minerals from river flows. However, the N content in the AS is insufficient for maize growth (approximately 0.71 kg ha^{-1}) and declines during plant growth [19]. In the Mekong Delta, especially An Giang province, enclosed dyke systems have been established to prevent annual floods. However, the average annual alluvial volume and total nitrogen content of alluvial sediment in the dykes have decreased significantly [20]. Furthermore, continuous cultivation of multiple crops throughout the year depletes the soil of nutrients, making fertilization necessary for more effective production. However, the use of fertilizers especially in excessive amounts can have a negative impact on the environment [21].

Moreover, several N_2-fixing endophytic bacteria were isolated and applied in diked ASs to improve N uptake, soil fertility, and crop yield by providing available N from N fixation from the atmosphere [22,23]. However, because bacteria use the nitrogen gas presenting in pore soil for their N_2-fixing activity, rhizospheric bacteria may possess a higher potential to perform N_2-fixation function than the endophytic ones. In this study, we purposed to identify an efficient and environmentally friendly biological N source for maize production in alluvial soil. The objective of this experiment was to identify N_2-fixing, rhizospheric bacterial isolates and determine the contribution of the selected N_2-fixing bacteria to soil fertility and growth and yield of maize cultivated on the diked AS in An Giang province, Vietnam. We also hypothesized that the application of the isolated N_2-fixing rhizospheric bacteria should improve some characteristics of soils and maize plants when combined with N fertilizer, which thereby reduced a certain amount of the chemical fertilizer used.

Materials and methods

Location: The experiment was performed at the greenhouse (10°01′51.1″N, 105°46′08.4″E), College of Agriculture, Can Tho University, Can Tho, Vietnam. The mean temperature was 42 °C, and the air moisture content was 62%. The initial soil properties were pH_{H2O} (5.46), pH_{KCl} (4.43), organic matter (1.96%), total nitrogen (0.172%), NH_4^+ (36.8 mg kg^{-1}) and NO_3^- (17.3 mg kg^{-1}).

Time

The experiment was conducted between September 2019 and March 2021.

Source of bacteria: The rhizosphere of fully fertilized hybrid maize was collected from 36 fields, each field with 5 plants to isolate bacteria at day 40 - 45th after planting. Thirty-six rhizosphere samples were derived from 36 maize fields in An Giang province, Vietnam at the end of 2018.

Source of plants

The hybrid maize variety CP888 was used in this experiment, which completed a growth cycle in approximately 100 days, with a 22 cm cob length, and yielded highly and stably roughly 10 - 12 t ha^{-1}.

Solid biofertilizer

We followed the method of Kantha *et al.* [24] to prepare the solid biofertilizer, with some modifications, where ash and maize leaves were used at a mass ratio of 1:4. Ash originated from rice husk burnt in the local factory. It was equally added for treatment applied to the selected RB. Maize leaves were collected at the harvest stage to dry until constant weight. Both were ground by a 2 mm sieve and separately sterilized into a mixture. The ground mixture was autoclaved at 121 °C, 1 atm. For a brief description, each isolate was cultured

separately at pH 4.5 for 48 h in the NFb medium [25]. To prepare an inoculant, a cell suspension was diluted in distilled water (DW) to a cell density of 10^9 cells mL^{-1}. To prepare the solid biofertilizer, 30 mL of the inoculant was added into 120 g of carrier to reach the final cell density of 10^8 cell g^{-1} approximately. The prepared biofertilizer was then stored in plastic bags for a month in the dark at room temperature before use. The cell density in the solid biofertilizer was measured before seed inoculation.

Fertilizers

The fertilizer formula was as follows: a urea fertilizer (46% N), a super phosphate fertilizer (16% P_2O_5), and a potassium chloride fertilizer (60% K_2O).

Isolation of rhizospheric bacteria

One gram of a soil sample was placed in a flask containing 99 mL of DW and shaken for 12 h at 200 rpm. The shaken solution was allowed to settle for 3 h. Then, 0.1 mL of the solution was spread on a petri dish with N-free Burk's medium [26]. The dish was left to dry and incubated at 30 °C. After 48 h of incubation, colonies appearing on the surface of the medium were inoculated into another medium until they were pure by repeated streaking. Pure colonies on N-free Burk's medium were used for the determination of N_2-fixing ability. We also used 57 bacterial strains isolated from rhizosphere maize in our previous research for this screening.

Selection of rhizospheric bacteria

Based on their ability to fix N_2 from the atmosphere, the isolated bacteria were cultured in N-free media. The growing colonies were selected and represented for their N_2-fixing ability. In brief, a 10% inoculum of each culture was transferred to a tube of 18 mL of N-free medium with pH 4.50. Then, the tubes were shaken at 120 rpm in the dark. After 2 d of being shaken, derived from the incubated culture, the supernatants were centrifuged at 10,000 rpm for 15 min to collect cells. The achieved suspension for NH_4^+ determination was detected using the salicylate method [27]**.** A blank medium served as the negative control**.** Rhizospheric bacterial isolates with the highest NH_4^+ productions among the isolates were chosen for further experiments.

Identification of rhizospheric bacteria

Isolates were selected based on their acid tolerance and N_2-fixing ability. The selected isolates were analyzed through the 16S rDNA sequencing. After 48 h incubation, 2 mL of liquid culture was centrifuged at 10,000 rpm for 5 min to obtain cells. Then, DNA was extracted from cell pellets using the Genomic DNA Prep Kit (BioFACT), following the manufacturer's instructions. Extracted DNA was visualized electrophoretically. For determining the concentration and purity of the DNA samples, DNA was resolved on 1.0% w/v agarose gel and underwent UV light exposure. Then, DNA was PCR amplified using the iProof High-Fidelity PCR Kit (BioRad, Hercules, CA) and the T100™ thermocycler (BioRad), according to the manufacturer's instructions. The primers pair used in this study consisted of 16S Forward Primer - 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 16S Reverse Primer - 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) [28]. The thermal reaction included: predenaturation for 5 min at 95 °C, 30 cycles for 90 min, and a final extension for 10 min at 72 °C. Each cycle consisted of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, and extension for 2 min at 72 °C. The PCR products were immobilized using DNA markers on 1.0% w/v agarose gel and 1× TAE buffer, then checked using a UV-trans illuminator and purified using the TIANquick Midi Purification Kit (Tiangen Biotech Ltd., Beijing, China) according to the manufacturer's instructions. Purified DNA was sequenced by an automated DNA sequencer at Macrogen DNA Sequencing Service (Macrogen, Seoul, Korea) and analyzed using BioEdit 7.0.5.3 for sequences and ChromasPro 1.7 for chromatograms. The results were compared with available sequences in the GenBank database using the Basic Local Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). Then, sequences were aligned using the ClustalW [29]. From the alignment output, the MEGA 6.0 [30] was used to build a neighbor-joining phylogenetic tree [31] and the Jukes–Cantor evolutionary distance matrix a 1,000-replicate bootstrap [32].

Experimental design

A completely randomized block design was utilized for the experiment. There were 9 treatments (4 replications) combined with the recommended fertilizer doses (RFD): 100% N (control), 85, 70 and 55% N and a mixture of N_2-fixing rhizospheric bacteria (RB). There was no treatment applied with both 100% N of the RFD and the RB because the study aimed to reduce the rates of chemical fertilizers in maize cultivation by beneficial bacteria. Each replication was a pot (31 cm top diameter ×27 cm bottom diameter ×25 cm height) and contained only one plant.

Soil preparation

The experimental soil was collected in An Giang province, Vietnam. The soil belonged to the 36 sampling sites. The soil was prepared by cleaning from residue materials, then mixing and drying in open air. Each pot had 10 kg of prepared dry soil.

Inorganic fertilizers

The recommended fertilizer dose for maize included 200 N, 90 P_2O_5 and 80 K_2O kg ha^{-1}.

Inoculation of rhizospheric N_2-fixing bacteria to maize grains

The maize seeds were prepared as in the study by Khuong *et al.* [23]. In brief, maize seeds were submerged sequentially in ethanol 70%, sodium hypochlorite 1%, and distilled water to sterilize. The seeds were incubated in dark conditions for a day to germinate. The germinated seeds were soaked in beakers containing 63 mL of rhizospheric bacteria suspension with a density of 10^8 cells mL^{-1}. The distilled water beaker was the negative control. The beakers with seeds-bacteria mixture were covered with aluminum foil, shaken at 60 rpm for 1 h, and let dry under a laminar airflow for 1 h at the end. The inoculated seeds were then sowed into the soil.

Solid biofertilizer of rhizospheric N_2-fixing bacteria

In each pot containing 10 kg of soil, only one seed of a maize plant was used and possessed an RB density of 4.2×10^3 cells g^{-1} dry soil weight (DSW) (6.3×10^6 cells $seed^{-1}$). Solid biofertilizer was applied at a weight of 5.0 g (10^8 cells mg^{-1}) on 10, 20, and 45 d after planting (DAP) to maintain an approximate cell density of 0.33×10^5 cells g^{-1} DSW for each stage of maize growth. The biofertilizers were spread dry on the soil surface in each pot. This resulted in a total bacterial density of 1.0×10^5 cells g^{-1} DSW of RB for one season from both solid rhizospheric bacterial biofertilizer and inoculated maize seeds [23].

Growth, N uptake, and yield parameters of maize

Those criteria were determined at the maize's physiologically mature stage (at 115 DAP).

Yield components

Cob length (cm): length from both ends of a cob; cob diameter (cm): Diameter at the center of the cob; number of rows/cob (rows): Rows in each cob; number of seeds/row (seeds): Seeds in each row; 100-seed weight (g): Weight of 100 seeds collected randomly in each replicate.

Maize yield (g per pot)

Both fresh weight and dry weight of seeds were considered. After the fresh weight of the seeds was measured, they were placed in bags with labelled with different codes for different treatments. The moisture value was measured and yield was calculated at 15.5% moisture.

Analysis of soil characteristics

Soil samples were collected and analyzed to evaluate the properties according to Sparks *et al.* [33].

Plant analysis

Maize straw of the stovers and seed samples were collected at the physiologically mature stage. The nitrogen concentrations uptakes, and biomass of maize and its components were determined similarly to the method of Thuy *et al.* [34].

Statistical analysis

The numeric data introduced in the Tables and Figures of the current study were the mean of each treatment. Data were processed using the SPSS version 13.0 with the one-way analysis of variance (ANOVA), and using Duncan's post-hoc test at the significance

level below 0.05 for significant differences between treatments.

Results and discussion

Isolation, selection, and identification of rhizospheric N_2-fixing bacteria to produce available nitrogen nutrients for plant

Isolation and selection of acid-resistant and N_2-fixing rhizospheric bacteria

A total of 67 isolates were isolated from rhizospheric soil cultivated in maize. Of these, 17 could survive under acidic conditions (pH 4.50). These isolates were screened for their ability to fix N_2 from the air. The evaluation was based on the amount of NH_4^+ produced by the bacteria in an N-free broth medium. The NH_4^+ production of acid-tolerant bacteria is shown in **Figure 1** and it ranged from 21 to 99.5 mgL^{-1}. The ASD-28 and ASD-07 isolates produced the highest content of NH_4^+, which was approximately 100 mg L^{-1}. The ASD-17 isolate was the 2nd best in fixing N_2 and its production of NH_4^+ (95.5 mgL^{-1}) was statistically equivalent to the 2 isolates mentioned previously and other isolates, including ASD-14, ASD-15, and ASD-19. Moreover, the ASD-50 isolates exhibited significantly lowest NH_4^+ content (**Figure 1**).

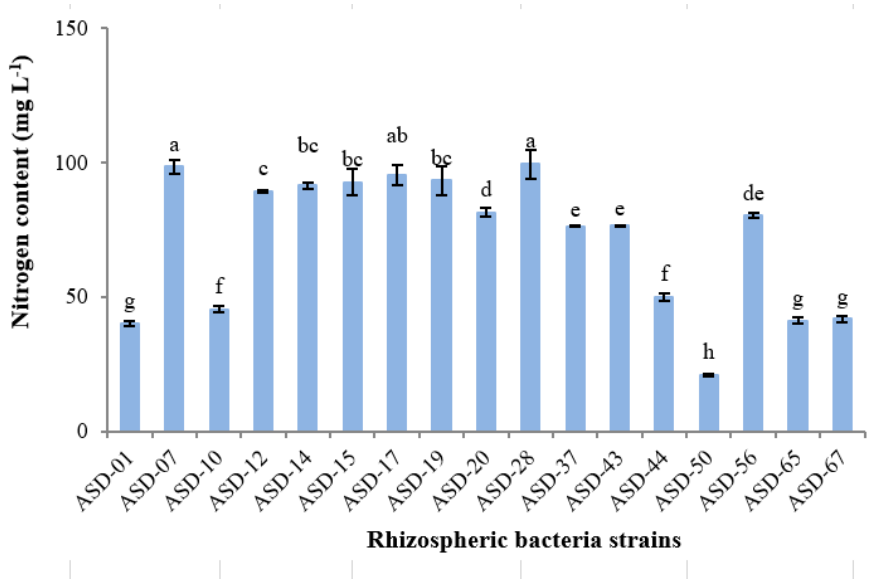

Figure 1 Ammonium content released by the potent N_2-fixing rhizospheric bacteria isolated from the rhizosphere of hybrid maize on diked alluvial soil.

Identification of the selected N_2-fixing rhizospheric bacteria

The phylogenetic tree clearly shows that both ASD-07 and ASD-28 cluster within the *Enterobacter asburiae* clade, closely related to known strains such as *E. asburiae* USMS9TH and SPM2, with high bootstrap support values (97 - 100%), indicating strong confidence in the grouping. This suggests that the 2 newly isolated strains are members of the *Enterobacter asburiae* species. The accession numbers of the isolates are ON326631 and ON326632, respectively (**Figure 2**)

Changes in the fertility of the diked alluvial soil supplied with the N_2-fixing rhizospheric bacteria

For pH values, in addition to a reduction in the N fertilizer level, the pH_{H2O} was reduced, and the pH_{KCl} fluctuated among treatments and peaked at the control treatment with 100% N of the RFD, whose value was 5.49, which is significantly different from those of other treatments. An improvement in the pH_{H2O} was seen under the influence of the bacterial application. In other words, the values were raised by the bacteria. Additionally, in the treatments with the bacteria plus 85 and 70% N of the RFD, the pH_{H2O} ranged from 6.36 to 6.55, which is significantly different from all treatments

without the bacterial supplementation. Moreover, the treatment with the bacteria (pH_{H2O} 6.21) outweighed the treatment with no fertilizer application (pH_{H2O} 6.05) for this soil property. However, the bacteria presented no significant effect on the pH_{KCl} value. No significant difference in the total N content was caused by the N fertilizer level and the bacterial application. By contrast, the available N content was influenced only by the bacterial factor. Notably, among bacterial treatments, the lower the N level in the fertilizer was, the more the available N in the soil was. Thus, the treatment with the bacteria plus either 0 or 55% N of the RFD had an available N content of 121.0 and 121.8 mg NH_4^+ kg^{-1}, respectively, which is significantly higher than that of other treatments at 5%. For the soil P content, the common trend for the soluble P was a decrease and increase influenced by the reduction in the inorganic N level, and by the bacterial supplementation, respectively. However, the total P content was equivalent in all treatments (Table 1). From 100 to 55 % N of the RFD, the total and available P concentration in the soil reduced from 0.16 to 0.14 and 78.0 to 65.6 mg P kg^{-1}, respectively. Furthermore, at both values, the treatment with the bacteria plus 85% N of the RFD (0.15% in the total and 78.2 mg P kg^{-1} in the available concentration) was statistically equal to the control treatment with 100% N of the RFD (**Table 1**).

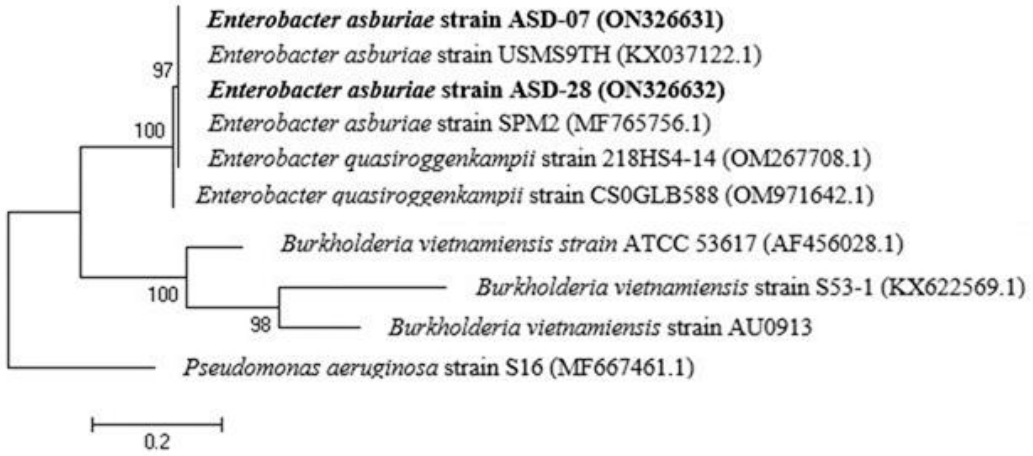

Figure 2 Neighbor-joining phylogenetic trees based on 16S rDNA sequences of 2 selected rhizospheric bacterial isolates ASD07 and ASD28 (in bold) and the 7 reference isolates (KX037122.1, MF765756.1, OM267708.1, OM971642.1, AF456028.1, KX622569.1 and DQ979872.1) included from the GenBank database and *Pseudomonas aeruginosa* strain S16 (MF667461.1) was treated as the outgroup. Bootstrap tests were performed with 1,000 replications.

Table 1 The maize soil fertility influenced by the N_2-fixing rhizospheric bacteria in the greenhouse condition.

Treatments	pH_{H2O} (1:2.5)	pH_{KCl} (1:2.5)	N_{total} (%)	$N_{available}$ (mg NH_4^+ kg^{-1})	P_{total} (%)	$P_{soluble}$ (mg P kg^{-1})
100% N	6.17^{e}	5.49^{a}	0.28	93.3^{cd}	0.16	78.0^{a}
85% N	6.29^{c}	5.35^{b}	0.25	90.1^{cd}	0.15	63.6^{e}
70% N	6.24^{d}	5.28^{bc}	0.23	91.1^{cd}	0.15	67.6^{d}
55% N	6.07^{f}	5.28^{bc}	0.25	91.4^{cd}	0.14	65.6^{de}
85% N + RB	6.55^{a}	5.33^{bc}	0.30	96.0^{bc}	0.15	78.2^{a}
70% N + RB	6.36^{b}	5.22^{cd}	0.30	100.8^{b}	0.15	70.7^{c}
55% N + RB	6.17^{e}	5.26^{bc}	0.25	121.0^{a}	0.13	74.5^{b}
0% N + RB	6.21^{de}	5.24^{bcd}	0.20	121.8^{a}	0.14	54.3^{f}
0% N	6.05^{f}	5.14^{d}	0.20	88.6^{d}	0.15	50.3^{g}
F-test	*	*	ns	*	ns	*
CV (%)	2.37	2.11	17.7	12.9	6.18	14.2

The values with the same following upper letter were not statistically different. *(*):* significantly different at 5%. RB*:* N_2-fixing rhizospheric bacteria.

Changes of the maize N uptake in maize supplied with the N_2-fixing rhizospheric on the diked alluvial soil

The N uptake in maize was also considered for determining the influence of both factors. The overall N uptake decreased along with a decline in the N fertilizer level applied. In addition, at the same N fertilizer level, the treatments with the bacteria always had higher N uptake content than treatments without the bacterial application, and the treatment with the bacteria plus 85% N of the RFD possessed the same result as the control treatment with 100% N of the RFD in the N uptake (1.04 g N per pot). For further evaluation, the biomass, N concentration, and uptake in stovers of maize, including grains, culms, leaves, and roots, were determined (**Table 2**). In grains, the parameters had a common pattern. A reduction in number was observed with a decrease in the inorganic N fertilizer content, i.e., when the N level was reduced from 100 to 55% N of the RFD, the N content in grains went down from 1.33 to 1.06%, their biomass reduced from 60.7 to 49.7 g per pot, and the N uptake dropped from 0.81 to 0.53 g N per pot. Furthermore, comparisons between treatments with the bacteria and treatments without the bacteria at the same N fertilizer level showed an outweighing. A statistically equivalent result was obtained upon comparing the treatment with the bacteria combined with 85% N of the RFD and the control treatment (100% N of the RFD), i.e., 1.33% compared to 1.31% for N concentration, 60.7 g per pot compared to 62.5 g per pot for biomass and 0.81 g N per pot compared to 0.82 g N per pot for N uptake. The results in other stovers showed an almost similar pattern (**Table 2**).

Changes in the growth, yield components, and yield of maize supplied with the N_2-fixing rhizospheric bacteria on the diked alluvial soil

On maize growth

The influence of the N fertilizer level and improvements due to the bacterial supplements appeared in the growth parameters of maize (**Table 3**). The leaf number and ear height shared a similar trend. A reduction was observed upon a reduction of the N fertilizer level. A dominance of treatments with the bacteria compared with treatments without the bacteria at the same N fertilizer level was seen. Simultaneously, a statistical equivalence was seen between the treatment with the bacteria plus 85% N of the RFD and the control treatment with 100% N of the RFD. Nevertheless, in these characteristics, the difference between the treatment with only bacteria and the treatment with no fertilization was insignificant. The size of maize plants was greatly affected by the bacteria applied in fertilizers. Surprisingly, the plant size in the treatment with the bacteria plus 85% N of the RFD was significantly higher and wider than that in the control treatment with 100% N of the RFD, at 175.3 cm compared with 171.5 cm in height and 1.12 cm compared with 1.04 cm in diameter. However, only in the plant height, the treatment with bacteria plus 0% N of the RFD was significantly higher than that in the treatment without the bacteria and the N fertilization, at 154.5 cm compared with 148.0 cm.

On the yield components and grain yield of maize

Better growth and nutrient uptake led to significant improvements in the yield components of maize, especially the cob size (**Table 4**). The cob size of maize in treatments with a combination of the bacteria and the inorganic N fertilizer was bigger than that in treatment with neither bacteria nor inorganic N. The mean cob size in the treatment with the bacteria plus 85% N of the RFD gave the highest results (11.9 cm in length and 4.10 cm in width), which are significantly bigger than that in other treatments at 5%, including the control treatments, excluding treatments with the bacteria plus 70 and 55% N of the RFD, where length values were 11.2 cm and 11.1 cm, respectively, and the treatment with 85% N of the RFD, where cob diameter was 3.98 cm. The number of rows per cob and number of grains per row showed no statistically significant difference among treatments with either only the inorganic N fertilizer or the combination of both factors. However, the characteristics in these treatments were significantly higher than those in both treatments with only the bacteria and no fertilization, i.e., 10.0 - 10.5 rows compared with 8.0 rows and 21.8 - 23.5 grains compared with 10.0 - 11.1 grains. There was no significant effect on 100-grain weight from both factors and the value ranged from 30.2 g to 34.0 g. Finally, the enhancement in maize's yield components increased grain yield dramatically. The yield reduced from 61.4 > 58.5 > 47.5 - 48.5 g per pot following the reduction of

the inorganic N fertilizer level from 100 > 85 > 70 > 55% N of the RFD. The differences were significant at 5%. At the same N fertilizer level, treatments with the bacteria possessed higher yields than the ones not treated with the bacteria. For instance, at the inorganic N fertilizer level of 70 and 55% N of the RFD, treatments with bacteria led to grain yields of 58.2 and 56.6 g per pot, which are significantly higher than 47.5 and 48.5 g per pot in groups untreated with bacteria. Notably, the treatment with the bacteria plus 85% N of the RFD (65.9 g per pot) exhibited the highest grain yield among treatments, which is even statistically equal to the control treatment (61.4 g per pot). Treatment with bacteria led to a remarkably higher grain yield than the treatment with no bacteria plus 0% N of the RFD, at 27.3 g per pot compared with 17.8 g per pot, respectively. This effect was visible in maize plants and cobs. At day 55 after planting, plants in the control treatment (100% N of the RFD) and the treatment with the bacteria plus 85% N of the RFD looked alike and noticeably higher than plants in the treatment with 85% N of the RFD.

Table 2 The nitrogen concentration, biomass, and uptake of maize influenced by the N_2-fixing rhizospheric bacteria in the greenhouse condition.

Treatments	N concentration (%)				N uptake (g pot^{-1})				Total N uptake (g pot^{-1})
	Grains	Culms	Leaves	Roots	Grains	Culms	Leaves	Roots	
100% N	1.33^{a}	0.40^{a}	1.80^{a}	0.65^{ab}	0.81^{a}	0.047^{a}	0.150^{a}	0.031^{a}	1.04^{a}
85% N	1.23^{b}	0.36^{b}	1.68^{c}	0.66^{a}	0.70^{b}	0.034^{c}	0.127^{c}	0.029^{b}	0.89^{b}
70% N	1.06^{d}	0.34^{c}	1.59^{d}	0.62^{c}	0.53^{d}	0.031^{d}	0.113^{e}	0.027^{cd}	0.70^{d}
55% N	1.06^{d}	0.32^{c}	1.72^{bc}	0.52^{f}	0.53^{d}	0.027^{e}	0.122^{d}	0.021^{e}	0.70^{d}
85% N+RB	1.31^{a}	0.38^{a}	1.75^{ab}	0.66^{a}	0.82^{a}	0.045^{a}	0.143^{b}	0.031^{a}	1.04^{a}
70% N+RB	1.31^{a}	0.39^{a}	1.36^{e}	0.64^{b}	0.75^{b}	0.038^{b}	0.107^{f}	0.027^{c}	0.92^{b}
55% N+RB	1.12^{cd}	0.32^{c}	1.26^{f}	0.60^{d}	0.62^{c}	0.030^{d}	0.092^{g}	0.026^{d}	0.77^{c}
0% N+RB	1.13^{c}	0.38^{a}	1.00^{g}	0.59^{de}	0.21^{e}	0.029^{de}	0.045^{h}	0.012^{f}	0.29^{e}
0% N	1.08^{cd}	0.33^{c}	1.01^{g}	0.58^{e}	0.10^{f}	0.022^{f}	0.041^{h}	0.009^{g}	0.17^{f}
F-test	*	*	*	*	*	*	*	*	*
CV (%)	9.61	8.91	10.7	7.33	4.42	4.22	5.93	3.24	4.10

The values with the same following upper letter were not statistically different. (*): significantly different at 5%. RB: N_2-fixing rhizospheric bacteria.

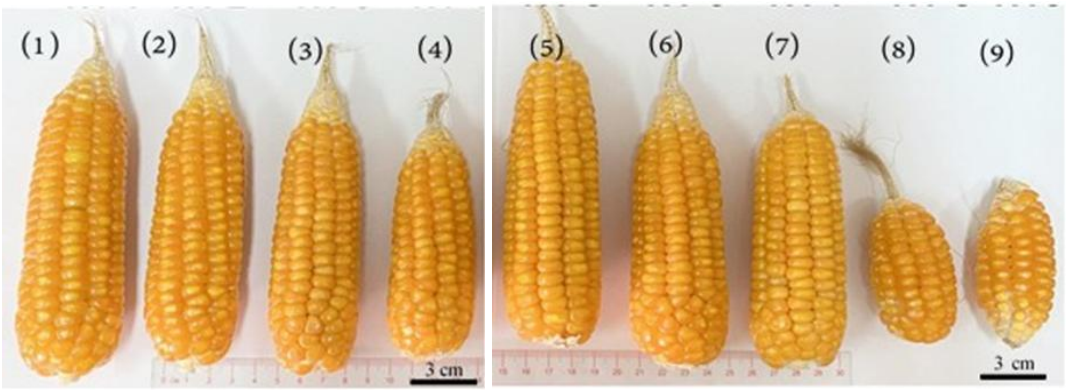

Figure 3 Maize cobs influenced by the N_2-fixing rhizospheric bacteria (RB) at harvest (1) 100% N, (2) 85% N, (3) 70% N, (4) 55% N, (5) 85% N + RB, (6) 70% N + RB, (7) 55% N + RB, (8) 0% N+ RB, and (9) 0% N. Cob length of 85% N + RB treatment was longest. scale bar = 3 cm.

Table 3 The maize growth influenced by the N_2-fixing rhizospheric bacteria in the greenhouse condition.

Treatments	Plant height (cm)	Height first cob appeared (cm)	Number of leaves (leaves)	Stem diameter (cm)	Biomass (g pot^{-1})		
					Culm	Leaves	Roots
100% N	171.5^{b}	69.3^{a}	11.0^{abc}	1.04^{bc}	11.9^{a}	8.38^{a}	4.79^{a}
85% N	166.5^{c}	65.0^{ab}	10.5^{bcd}	1.05^{b}	9.35^{b}	7.59^{d}	4.35^{b}
70% N	163.3^{d}	62.8^{b}	10.3^{cde}	1.04^{bc}	9.22^{b}	7.11^{ef}	4.28^{b}
55% N	157.8^{f}	62.3^{b}	10.5^{bcd}	0.98^{c}	8.50^{c}	7.05^{f}	3.96^{c}
85% N + RB	175.3^{a}	70.8^{a}	11.5^{a}	1.12^{a}	11.7^{a}	8.16^{b}	4.70^{a}
70% N + RB	167.3^{c}	69.8^{a}	11.0^{abc}	1.17^{a}	9.80^{b}	7.84^{c}	4.25^{b}
55% N + RB	160.8^{e}	65.8^{ab}	11.3^{ab}	1.16^{a}	8.50^{c}	7.27^{e}	4.26^{b}
0% N + RB	154.5^{g}	57.0^{c}	10.0^{de}	0.79^{d}	7.58^{d}	4.52^{g}	2.06^{d}
0% N	148.0^{h}	53.8^{c}	9.50^{e}	0.77^{d}	6.65^{e}	4.08^{h}	1.61^{e}
F-test	*	*	*	*	*	*	*
CV (%)	5.09	9.98	7.22	14.2	17.9	21.4	9.12

The values with the same following upper letter were not statistically different. (*): significantly different at 5%. RB: N_2-fixing rhizospheric bacteria.

Table 4 Yield and its components influenced by the N_2-fixing rhizospheric bacteria in the greenhouse condition.

Treatments	Cob length (cm)	Cob diameter (cm)	Number of rows per cob (rows)	Number of grains per row (grains)	100-grain weight (g)	Grain yield (g pot^{-1})
100% N	10.6^{b}	3.83^{bcd}	10.5^{a}	22.0^{a}	34.0	61.4^{ab}
85% N	10.9^{b}	3.98^{ab}	10.5^{a}	22.3^{a}	33.2	58.5^{b}
70% N	10.7^{b}	3.68^{d}	10.0^{a}	22.0^{a}	30.2	47.5^{c}
55% N	10.7^{b}	3.75^{cd}	10.0^{a}	21.8^{a}	31.7	48.5^{c}
85% N + RB	11.9^{a}	4.10^{a}	10.0^{a}	23.5^{a}	35.7	65.9^{a}
70% N + RB	11.2^{ab}	3.78^{cd}	10.0^{a}	22.8^{a}	34.0	58.2^{b}
55% N + RB	11.1^{ab}	3.88^{bc}	10.5^{a}	23.5^{a}	31.4	56.6^{b}
0% N + RB	6.70^{c}	3.28^{e}	8.0^{b}	11.1^{b}	33.7	27.3^{d}
0% N	6.28^{c}	3.15^{e}	8.0^{b}	10.0^{b}	30.6	17.8^{e}
F-test	*	*	*	*	ns	*
CV (%)	10.2	8.49	12.2	16.5	7.46	9.52

The values with the same following upper letter were not statistically different. (*): significantly different at 5%. RB: N_2-fixing rhizospheric bacteria.

As seen in **Figure 3**, cobs in the treatment with the bacteria plus 85% N of the RFD were a little bit bigger than those in other treatments, including the control with 100% N of the RFD. Furthermore, at the same levels of the inorganic N fertilizer, cobs from plants treated with the bacteria were longer and bigger than cobs from plants not treated with the bacteria.

The formation of dikes negatively affects soil fertility [35]. A nitrogen supplementation using biological approaches for maize is essential for sustainable agriculture, because the abuse of chemical fertilizer may lead to soil contamination [36], underground-water pollution [37], and climate change [38]. In this study, 17 out of 67 isolated RB from maize

rhizosphere survived under acidic conditions (pH 4.50), which is similar to soil properties in the Mekong Delta, Vietnam [39]. All acid-tolerant RB isolates produced NH_4^+ in the N-free medium (**Figure 1**), i.e., they had the potential to fix N_2 from the atmosphere into NH_4^+ compounds for plants under low pH conditions. However, the NH_4^+ concentrations varied among isolates. This is in accordance with the study by Ha and Chu [40], where 15 N_2-fixing bacterial isolates had NH_4^+ concentrations diversely ranging from 5.22 to 18.41 mg L^{-1}. Similarly, the NH_4^+ production in the study by Dat *et al.* [41] fluctuated from 16.2 to 104.1 mg L^{-1}. The RB isolates with the highest NH_4^+ production were the ASD-07 and ASD-28 isolates, with contents of approximately 100 mg L^{-1} NH_4^+, which is a statistically significant comparison with that of other isolates, excluding the ASD-17 isolate. These 2 isolates were later identified as *E. asburiae* (**Figure 2**). This result is consistent with that from the study by Toribio-Jiménez *et al.* [42], in which E. cloacae isolates were isolated from acidic conditions. Briefly, we screened potent isolates that could tolerate low pH conditions, fix N_2 from air, and apply them to the diked alluvial soil for maize. Moreover, the RB isolates of *E. asburiae* ASD-07 and ASD-28 released a greater concentration of NH_4^+ as compared to nitrogen-fixing endophytic bacteria (NFEB), whose highest NH_4^+-producing bacteria was E. cloacae ASD-21 with 40.0 mg L^{-1} [23]. This is the reason why the RB were performed in this research. Thus, in each soil condition, bacteria released different NH_4^+ concentrations.

The application of the selected RB isolates of ASD-07 and ASD-28 increased not only the soil fertility but also the maize growth and yield. Improvements in growth and yield by *Enterobacter* spp. have been widely reported in common crops, such as maize [43], potatoes [44], wheat [45], chickpea [46], rice [47], and okra [48]. The increase in soil fertility by the RB supplementation was significant in the pH_{H2O} and NH_4^+ concentrations (**Table 1**). The pH value significantly increased upon the RB application, whereas the addition of fertilizer reduced the soil pH, according to Roussos et al. [49]. This participated in balancing the soil pH. The most interesting result of this study is that the addition of the chemical N fertilizer did not help improve the NH_4^+ content and negatively affected the RB-mediated N_2 fixation, whereas the highest available N concentration belonged to the 2 bacterial treatments with less N levels. Thus, the less the N chemical fertilizer applied to the soil was, the more active the RB were. This is in accordance with the results of Li *et al.* [50]. However, the total N content in the soil was not affected by both N fertilizer and RB. The results of this study are not similar to some previous studies, possibly due to different experimental conditions such as experiments in soil microcosm conditions with *E. cloacae* AKS7 in India [51] and Andisol soil in Indonesia [52]. Furthermore, both of these experiments were conducted without the presence of plants on the soil. However, there are also studies showing that total N does not change under rice cultivation conditions on saline soil with different levels of chemical fertilizers and with nitrogen-fixing bacteria Rhodobacter sphaeroides S01 and S06 [53]. Another study by Thuc et al. [54] on nitrogen-fixing bacteria and fertilizer levels for sesame plants similar to our results may be due to the similarity in experimental conditions such as alluvial soil type and upland crops. Therefore, soil's total nitrogen content can be attributed by several factors such as the soil capacity, the nitrogen transformation processes, and the microflora in general and nitrogen-fixing bacteria in particular and the nutrient uptake by plants. The N uptake from the soil, N concentration, and biomass of maize changed positively (**Table 2**). Overall, the N uptake of maize in the treatment with the RB plus 85% N of the RFD was equal to the control treatment with 100% N of the RFD (1.04 g per pot), and higher than the treatment without the bacteria at the same N level. The result was in accordance with that of Sondang *et al.* [55]. Data on other characteristics in stovers, including grains, culms, leaves, and roots, showed similar outcomes. Having the higher N input, the growth of maize ameliorated remarkably, especially in plant size. Both the height and width of plant stem in the treatment with the bacteria plus 85% N of the RFD were significantly better than the control (**Table 3**). The increase in the maize growth by *Enterobacter* sp. was consistent with the results of Verma *et al.* [56]. For the yield components, the cob size was the longest and biggest in the treatment with the bacteria plus 85% N of the RFD. The difference between the outcome of this treatment and the control with 100% N of the RFD was remarkable. However, significant

differences between the treatment with only the RB mixture and with no fertilizer applied were seen only in the grain yield (**Table 3**). The grain yield peaked at 65.9 g per pot in the treatment with the bacteria plus 85% N of the RFD. Moreover, the value differed significantly from other treatments at 5%, excluding the control treatment with 100% N of the RFD. This increase in the maize grain yield in response to the bacterial treatment is consistent with the results of Di Salvo *et al.* [57]. Maize yield was also affected by the N fertilizer level. Lowering N fertilizer levels caused a reduction in all characteristics evaluated in this study, excluding the available N content in the soil, 100-grain weight, and number of rows and grains (**Table 4**). As per Gheith et al. [58], plant height, cob length, grain weight, number of grains/rows, number of grains/cob, and grain yields are influenced by rates of N fertilizer.

Thus, the mixture of the acid-tolerant RB ASD-07 and ASD-28 isolates can replace 15% of the chemical N fertilizer without reducing the N uptake, growth, grain yield, and NH_4^+ content in maize. This is similar to the studies by Wang *et al.* [59] where 25% of chemical fertilizer was replaced by a mixture of bacteria. Additionally, the mixture also raised the soil pH_{H2O}.

Conclusions

Seventeen of 67 isolates from the rhizosphere of maize growing on diked alluvial soil could live and produce NH_4^+ through N_2-fixation from the air under low pH conditions. The 2 highest N_2-fixing rhizospheric bacteria isolates were ASD-07 and ASD-28 isolates, which were then identified by the 16S rDNA sequencing as *E. asburiae.* The mixture of the indigenous N_2-fixing rhizospheric bacterial *E. asburiae* ASD-07 and ASD-28 isolates provided NH_4^+ to maize and replaced 15% of the recommended inorganic N fertilizer dose without negatively changing any traits related to the maize growth and yield in diked alluvial soil. Therefore, the *E. asburiae* ASD-07 and ASD-28 isolates should be further investigated for operation under field conditions. These 2 nitrogen-fixing bacteria have the potential to replace inorganic nitrogen fertilizers, leading to a reduced environmental impact and sustainable agricultural development.

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