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Potential of N_2-fixing endophytic bacteria isolated from maize roots as biofertiliser to enhance soil fertility, N uptake, and yield of *Zea mays* L. cultivated in alluvial soil in dykes

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Abstract

This study aimed to (i) select the endophytic bacteria from maize roots for their N_2-fixing ability and (ii) evaluate the efficacy of potent indigenous bacterial strains on soil fertility, nitrogen (N) uptake, and growth and yield of maize. A total of 31 maize root samples were collected from An Giang province in Vietnam to isolate the bacteria. The pot experiment was conducted in nine treatments: (i) 100% N of the recommended fertiliser formula (RFF), (ii) 85% N of RFF, (iii) 70% N of RFF, (iv) 55% N of RFF, (v) 85% N of RFF plus a mixture of two potent strains of nitrogen-fixing endophytic bacteria (NFEB), (vi) 70% N of RFF plus a mixture of two potent strains of NFEB, (vii) 25% N of RFF plus a mixture of two potent strains of NFEB, (viii) 0% N of RFF plus a mixture of two potent strains of NFEB, and (ix) 0% N of RFF. The experiment was conducted in the greenhouse to collect soil and plant samples at harvest and observe their growth and agronomic parameters. The results showed that two acid-resistant endophytic bacterial strains were selected and identified as *Enterobacter cloacae* ASD-21 and *E. cloacae* ASD-48. At 85% N level, a mixture of the two endophytic bacteria strains was applied as biofertilisers and proved their ability to significantly enhance NH_4^+ content and N uptake, with an increase of 14.8 mg NH_4^+ kg^{-1} and 0.26 g N pot^{-1}, respectively. A mixture of the two potent strains of NFEB produced higher values in plant height, stem diameter, cob length, and cob diameter compared to 100% N of RFF. It replaced 15% N of RFF but still maintained the maize grain yield.

Keywords: alluvial soil; dyke; endophytic bacteria; maize yield; N_2 fixation.
Abbreviations: DSW_Dry soil weight, EC_electrical conductivity, N_nitrogen, NFEB_nitrogen-fixing endophytic bacteria, P_phosphorus, RFF_recommended fertiliser formula.

Introduction

Nitrogen (N) plays an important role in the life of cultivars and microbes (Wakelin et al., 2010; Simon et al., 2014). It is very abundant in the atmosphere but not in soils (Greenwood and Earnshaw, 2012). However, N normally presents in the ecological system as a mineral in three ways: discharges from the atmosphere, biological N_2 fixation, and chemical fertilisers. Therefore, crops are supplied with a huge amount of N from chemical fertilisers every year to raise their yield (FAO, 2006). Nevertheless, overusing chemical fertilisers contaminates soil and groundwater and contributes to global climate changes (Zheng et al., 2021). As a result, the use of N_2-fixing microbes appears as an alternative, but the N_2-fixation ability is limited in the archaea and bacteria domains (Galloway et al., 2008). Bacteria capable of fixing N_2 from the atmosphere consists of endophytic bacteria, such as *Azoarcus* sp., *Gluconacetobacter diazotrophicus, Herbaspirillum seropedicae*, and *Serratia marcescens* (Gupta et al., 2012). Moreover, Puri et al. (2018) reported that endophytic bacteria with N_2-fixation capacity, such as *Bacillus* sp. and *Enterobacter* sp., can be isolated from maize and that 17%–56% of N in plants comes from fixed N_2 from the atmosphere by bacteria. Although endophytic bacteria are found in plants, indigenous bacteria may have higher adaptability to survive and perform their functions. The N requirement of maize is big. Maize usually receives larger N content from fertilisers (about 136 kg N ha^{-1} in average) than other cereal crops (FAO, 2006). Maize should be fertilised with N during the V6 and V8 stages (Ritchie et al., 1993), and delayed fertilisation can make the yield lose permanently. Therefore, the efficient use of N fertilisers for maize and the portion of N maize needs have been studied a lot. Dhital and Raun (2016) stated that the N requirement for maize varies by time and region. In the USA, the optimum N rate for maize fluctuates from 63 to 177 kg ha^{-1}, which has been reported in many studies from 1958 to 2020 Dhital and Raun (2016). However, in China, high N fertilisation at the rate of 180–240 kg ha^{-1} has been applied to reach the highest maize

yield (over 15 Mg ha^{-1}) (Guo et al., 2017). According to Cui et al. (2008) and Imran et al. (2015) to maximise the maize grain yield and N uptake, the N soil content must be 150 and 186 kg N ha^{-1}, respectively. In Africa, the N requirement for maize ranges from 48 to 209 kg ha^{-1} (Berge et al., 2019).

Alluvial soils are common in Vietnam, and undeposited alluvial soils are usually found in big river sites where dykes border the residential areas to prevent floods. As mentioned previously, maize growth needs tremendous N content, but in alluvial soils, the total N content is low, 0.71 kg ha^{-1} (Xiao-Tang et al., 2006). Moreover, the available N content in soil decreases during plantation (Pal et al., 1987). Thus, the necessity of N supplements has been raised in this study. Therefore, supplementing indigenous bacteria is a potential method to replace the chemical nitrogen fertiliser for maize. This work aimed to (i) select the potential acid-resistant endophytic bacteria capable of fixing nitrogen and (ii) determine the impacts of potent bacteria on soil fertility and the growth and yield of maize in undeposited alluvial soil.

Results

Isolation, selection, and identification of N_2-fixing endophytic bacteria from maize to produce available nutrients for plants

There were 72 strains of endophytic bacteria isolated from roots of hybrid maize in an acidic environment (pH 5.0). Among them, 26 endophytic bacteria stains had the OD_{660} value above 0.5. The highest values of OD_{660} belonged to strains ASD-02, ASD-13, and ASD-21, ranging from 0.884 to 0.986. Apart from being tolerant to low pH conditions, 20 endogenous bacterial strains of the isolated endophytic bacteria were able to fix nitrogen from air. Two bacteria strains ASD-21 and ASD-48 have high nitrogen fixation capability, with the produced NH_4^+ content of 40.0 and 34.8 mg L^{-1}, respectively, significantly different from other examined strains (Fig. 1). Therefore, the two strains were selected for further experiments. Based on rDNA 16S sequencing and the GenBank database, the two selected strains of endophytic bacteria from maize roots, ASD-21 and ASD-48, were identified to be a species of *Enterobacter cloacae*, with accession numbers MZ461951 and MZ461952, respectively (Fig. 2).

Effects of N_2-fixing endophytic bacteria on soil fertility, N uptake, and growth and yield of maize cultivated on alluvial soil in dykes in greenhouse conditions

Effects of N_2-fixing endophytic bacteria on alluvial soil fertility in dyke-cultivated maize: The soil pH properties lessened the effects of both inorganic N fertilisers and bacterial supplements. For pH_{H2O}, although there was an unremarkable fluctuation between treatments without bacterial supplements, soil pH_{H2O} after planting maize was significantly increased in the treatments with bacterial strains. The 85% N fertilisation treatment with strains of bacteria reached near-neutral pH_{H2O} (6.68) (Table 1). Moreover, at the treatments with and without bacteria only, the pH clearly increased while applying bacteria, 6.36 compared to 5.84. However, the adjustment in the levels of inorganic N and bacterial supplements did not cause any significant changes in the value of pH_{KCl} between treatments.

There was unclear difference in total N between the treatments with or without bacterial inoculation. However, the available ammonium content in the treatments of inorganic nitrogen fertilisers combined with endogenous bacteria was higher than those in which only inorganic fertilisers were applied according to the corresponding reduction of nitrogen fertiliser levels for hybrid maize. In particular, the available nitrogen content (128.0 mg NH_4^+ kg^{-1}) in the treatment of 85% N combined with endogenous bacteria was equivalent to the treatment with 100% N (123.7 mg NH_4^+ kg^{-1}) (Table 1). The same case applied to treatment with 75% N and treatment with 50% N combined with endogenous bacteria. Furthermore, for this parameter, the treatment with only bacteria was outstandingly higher than the treatment without fertilisers whose value was the smallest among the treatments (85.8 versus 67.4 mg NH_4^+ kg^{-1}).

The total phosphorus content in soil ranged from 0.143% to 0.160%, and there was a statistically significant difference between treatments with different levels of nitrogen fertiliser ($p < 0.05$). In the treatment without inorganic fertiliser and without adding endogenous bacteria, the total phosphorus content was 0.158% higher than the treatment without fertiliser (0.148%). The amount of available phosphorus in all treatments ranged from 48.7 to 84.5 mg P kg^{-1}, with the highest value in the treatment of 85% N combined with additional bacteria application (84.5 mg P kg^{-1}). There were significant differences in the amount of available phosphorus between treatments and control (100% N), except for treatment with 85% N and a mixture of *E. cloacae* ASD-21 and ASD-48. At the same nitrogen fertiliser levels in pairs, the available phosphorus in the treatments with bacteria was significantly higher than in the treatments without bacteria, with the exception of the pair of treatment with 50% N only and treatment with 50% N combined with bacteria (Table 1). Moreover, sharing the same trend as the available N content, there was a significant increase between treatment with only bacteria and treatment without fertiliser.

Effects of N_2-fixing endophytic bacteria on maize growth: Plant height and stem diameter were increased by bacterial inoculation (Mixed *E. cloacae* ASD-21 and ASD-48). For plant height, at the same N fertiliser level, treatments with bacteria inoculation resulted in higher values or equal to those without bacteria. For a clearer argument, at the treatment with only bacterial supplements, the value was 162.0 cm, significantly higher than without any supplement (154.3 cm). Besides, treatment with 100% N and treatment with 85% N combined with bacterial supplement (ASD-21 and ASD-48) obtained the highest heights of appeared ear (70.3 and 70.7 cm, respectively) and were not significantly different from each other. The lowest value of height of appeared ear was recorded in treatment with only supplement from bacteria (51.7 cm), and it was significantly different from all the others (Table 2). Treatments with bacterial inoculation were insignificantly different from treatments with 15% N higher as 85% N plus mixture of *E. cloacae* ASD-21 and ASD-48 compared to 100% N, 70% N plus mixture of *E. cloacae* ASD-21 and ASD-48 compared to 85% N and 55% N plus mixture of *E. cloacae* ASD-21 and ASD-48 compared to 70% N. For the plant's stem diameter, there was a 20% increase at 85% N. The values were 10%, 12%, and 32% at 70%, 55%, and 0% N, respectively, comparing between treatments with bacteria and those without bacteria. Additionally, the stem diameter at 85% N with bacterial inoculation was significantly different from

the other treatments without bacterial inoculation (Table 2). Moreover, for the number of leaves, there was an unclear trend in terms of differences among treatments. Therefore, it was likely that treatments were insignificantly different from each other in terms of the number of plant leaves.

Effects of N_2-fixing endophytic bacteria on yield components and maize grain yields: Cob size was increased by bacterial inoculation in both length and width. These two indicators in treatment with 85% N combined with bacteria inoculation (11.6 cm in length and 3.7 cm in width) were significantly higher than from treatments without bacterial supplement, except for treatment with 100% N (11.0 cm in length and 3.7 in width) (Table 3). Moreover, the treatment with only bacterial supplements outweighed the treatment without fertilisers in terms of cob size (7.4 cm compared with 6.4 cm in length and 3.2 cm compared with 3.0 cm) (Figs. 3–4). The number of rows/cobs and of seeds/rows did not significantly affect the difference between with and without bacterial inoculation, the only difference between treatment with 100% N and other treatments. A 100-seed weight showed almost no significant difference between the treatments (Table 3). The treatment with 85% N combined with a mixture of *E. cloacae* ASD-21 and ASD-48 supplement (52.3 g pot^{-1}) and the treatment with 100% N (52.0 g pot^{-1}) were statistically similar and the highest in the yield of grain among others. In the treatment with 0% N plus bacterial strains, the yield was significantly higher than that in the treatment without fertilisers. Except for the control treatment (100% N), grain yield had statistically significant differences between the other treatments (Table 3). To be more specific, in the same N fertiliser level, grain yield in treatments without bacteria was not as high as that in treatments with bacteria.

Effects of endophytic N_2-fixing bacteria on N content, biomass, and N uptake in grain and maize stover: The biomass of seeds, stems, leaves, and roots was all high in the treatments with bacterial inoculation at the same N level, especially in the treatment with 100% N. Without bacterial inoculation, the biomass was equivalent to the treatment with 85% N combined with bacterial inoculation (Table 4). In detail, the values in the treatment with 100% N and in the treatment with 85% N plus bacteria were, respectively, 40.3 and 43.0 g pot^{-1} in grain, 10.9 and 11.1 g pot^{-1} in stem, 11.7 and 11.4 g pot^{-1} in leaves, and 3.3 and 3.4 g pot^{-1} in root.

N concentration in seeds showed statistical differences among treatments with 70% N and 55% N compared to treatments with bacteria and inorganic N combination and the treatment without fertiliser. However, for N in stems, leaves, and roots, the treatments combined with bacterial inoculation were significantly different from the treatments that were not combined with bacterial inoculation for different levels of N. This occurred clearly while comparing the treatment with only bacterial supplements and the treatment without any fertiliser. Moreover, the similar situation appeared in N uptake (Table 4). Also, maize absorbed more nitrogen in treatments with bacterial inoculation than in those without (Table 4).

Discussion

Available ammonium content for maize cultivation on alluvial soil is not sufficient for the sustainable development of maize crop in terms of growth and yield because of low soil fertility being bordered by dykes (Huu, 2011). Thus, there are two common N supplement sources for this—chemical N fertiliser and N_2-fixing bacteria. Chemical fertilisers provide a large amount along with drawbacks, including soil contamination, underground water pollution, and greenhouse gas emission (Zheng et al., 2021). In this study, endophytic bacteria isolated from hybrid maize roots showed the potential to acidic resistance as well as plant growth promoters by fixing nitrogen from air. There were 72 isolates from maize roots, which could survive under pH 5.0 conditions. Among them, 20 strains showed the ability to fix free N_2 from the atmosphere (Fig. 1). In detail, ASD-21 was the strain with the highest NH_4^+ content (approximately 40.0 mg NH_4^+ L^{-1}). The second position belonged to strain ASD-48 with 34.8 mg NH_4^+ L^{-1}. Therefore, the two highest N_2 fixation strains were selected and identified as *Enterobacter cloacae* (Fig. 2), which is also reported in the study of Szilagyi-Zecchin et al. (2014) for a maize roots isolated *Enterobacter* spp. possessing nifH gene (nitrogen fixation ability). Ultimately, this research targeted potent strains that were able to provide N for maize and live in low pH conditions similar to Vietnam's soil property.

The key role of N in maize growth is undeniable. The levels of N supplement and maize yield components are positively correlated, which has been shown in many studies. For instance, the increased maize yield has been reported, associated with the levelling up of N fertilisers (Yan et al., 2021). This study has shown similar results, with a decrease in N levels, growth, biomass, and maize yield components (Tables 2–4). To be more specific, according to Table 2, although the number of leaves and the stem diameter did not receive a significant impact from N levels, plants were remarkably shorter, from 175.0 > 165.8 ~ 165.8 ~ 162.5 cm, corresponding to 100%, 85%, 70%, and 55% N. In Table 3, despite insignificant changes in 100-seed weight, cob size and grain yield showed a significant drop in values along with reduced N use. Following the decreasing order of N fertiliser levels (100%, 85%, 70%, and 55% N), the cob was smaller from 11.0 > 9.9 > 9.1 > 8.1 cm in length and from 3.7 > 3.4 ~ 3.5 ~ 3.4 in width, while grain yield dropped steadily from 52.0 > 46.4 > 42.7 > 36.0 g pot^{-1}. Regarding biomass, Table 4 shows a remarkable reduction in grain, stem, leaf, and root parts. Ultimately, N has shown vital effects on the growth and yield of maize.

However, rising N fertiliser levels could lead to a series of economic and environmental problems, that is, low efficiency in N use and environmental pollution in intensive agricultural systems (Hong et al., 2007). As a result, bacteria have been used as a biological source of N for maize. From the N produced by N_2-fixing endophytic bacteria, both the N uptake and the yield of crops increase. The increase has been recorded in the study of Szilagyi-Zecchin et al. (2014) for *Bacillus* sp. and *Enterobacter* sp. on maize; Puri et al. (2015) for *Paenibacillus polymyxa* on maize; and Puri et al. (2020) for *Caballeronia sordidicola* on hybrid white spruce. These claim the importance of endophytic bacteria in raising yield and N uptake. In this study, N uptake was always higher in treatments with bacteria inoculation than those without bacteria inoculation at the same levels of inorganic N fertilisers applied (Table 4). The same trend was recorded in maize growth values (Table 2) including plant height, stem diameter, grain yield, and cob size values (Table 3). Furthermore, treatments with 85% N plus bacteria strains *E. cloacae* ASD-21 and ASD-48 were not only equal to treatments with 100% N in the N uptake in grain and leaves and in the grain yield, plant height, and cob size parameters

Table 1. Effects of adding a mixture of N_2-fixing endophytic bacteria on soil alluvial fertility in dyke cultivated maize.

Treatment	pH (1:2.5~ Soil:H_2O)	pH (1:2.5~ Soil:KCl)	N_{total} (%)	$N_{available}$ (mg NH_4^+ kg^{-1})	P_{total} (%)	$P_{available}$ (mg P kg^{-1})
100% N	5.72^{f}	5.17	0.26^{ab}	123.7^{a}	0.143^{c}	84.0^{a}
85% N	5.64^{f}	5.33	0.24^{ab}	113.2^{b}	0.153^{ab}	72.4^{b}
70% N	5.64^{f}	5.30	0.24^{ab}	96.0^{d}	0.153^{a}	65.4^{d}
55% N	5.71^{f}	5.21	0.25^{ab}	90.5^{de}	0.148^{bc}	62.8^{d}
85% N + NFEB	6.68^{a}	5.37	0.28^{a}	128.0^{a}	0.158^{a}	84.5^{a}
70% N + NFEB	6.53^{b}	5.32	0.23^{ab}	105.8^{c}	0.160^{a}	69.4^{c}
55% N + NFEB	6.26^{d}	5.20	0.22^{b}	92.3^{d}	0.155^{a}	65.4^{d}
0% N + NFEB	6.36^{c}	5.38	0.23^{ab}	85.8^{e}	0.148^{bc}	54.3^{e}
0% N	5.84^{e}	5.37	0.23^{ab}	67.4^{f}	0.158^{a}	48.7^{f}
Significant differences	*	ns	*	*	*	*

NFEB: Nitrogen fixing endophytic bacteria. 100%N: Applied 100% N of recommendation fertilizer formula (RFF), 85% N: Applied 85% N of RFF, 70% N: Applied 70% N of RFF, 55% N: Applied 55% N of RFF, 85% N + NFEB: Applied 85% N of RFF and a mixture of *Enterobacter* spp. ASD-21 and ASD-48, 70% N + NFEB: Applied 70% N of RFF and a mixture of *E. cloacae* ASD-21 and ASD-48, 55% N + NFEB: Applied: 55% N of RFF and a mixture of *E. cloacae* ASD-21 and ASD-48, 0% N + NFEB: Applied 0% N of RFF and a mixture of *E. cloacae* ASD-21 and ASD-48 and 0% N: Applied 0% N of RFF. Within a column, numbers are followed with the same letter show no significant difference; (*) statistically significant difference at 5% level according to post hoc test; ns: not significant.

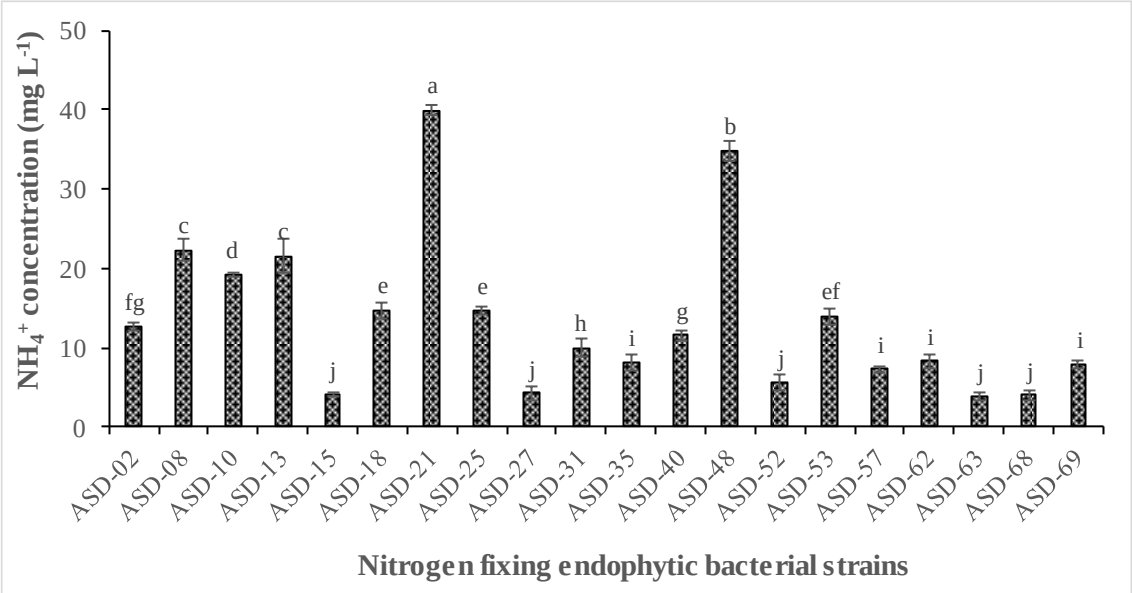

Fig 1. Ammonium concentration of acid-resistant N_2-fixing endophytic bacteria isolated from maize roots. Different lowercase letters indicate statistically significant difference at 5% level according to post hoc test between isolates.

Table 2. Effects of adding a mixture of N_2-fixing endophytic bacteria on maize growth.

Treatment	Plant height (cm)	Height of appeared ear (cm)	Number of leaves (leaf)	Stem diameter (cm)
100% N	175.0^{ab}	70.3^{a}	10.5^{ab}	0.97^{bc}
85% N	165.8^{cd}	65.3^{b}	10.8^{a}	0.92^{c}
70% N	165.8^{cd}	63.3^{b}	10.5^{ab}	0.95^{c}
55% N	162.5^{d}	62.0^{b}	10.0^{ab}	0.93^{c}
85% N + NFEB	178.8^{a}	70.7^{a}	10.5^{ab}	1.11^{a}
70% N + NFEB	171.5^{b}	65.3^{b}	10.5^{ab}	1.05^{ab}
55% N + NFEB	169.3^{bc}	64.3^{b}	10.3^{ab}	1.05^{ab}
0% N + NFEB	162.0^{d}	51.7^{d}	10.0^{ab}	0.94^{c}
0% N	154.3^{e}	57.5^{c}	9.8^{b}	0.71^{d}
Significant differences	*	*	*	*

Within a column, numbers are followed with the same letter show no significant difference; (*) statistically significant difference at 5% level according to post hoc test; ns: not significant; NFEB: Nitrogen endophytic fixing bacteria.

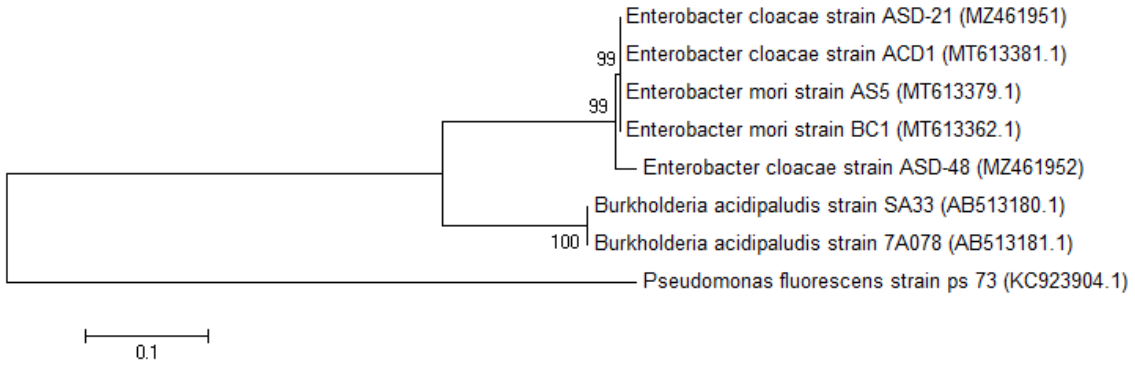

Fig 2. Neighbor-joining phylogenetic trees based on 16S rDNA sequences of two selected endophytic bacterial strains compared to the closely related strains in the GenBank database. The percentage levels of bootstrap analysis of 1000 replicates are indicated at each node. Bar, 0.1 substitutions per nucleotide position. *Pseudomonas fluorescens* strain ps 73 was used as the outgroup strain. Access numbers of GenBank sequences are implied in brackets.

Table 3. Effects of adding mixture of endophytic N_2-fixing bacteria on yield components and grain yield of maize.

Treatment	Cob length (cm)	Cob diameter (cm)	Number of row/ear (row)	Number of seed/ row (seed)	100-seed weight (g)	Grain yield (g pot^{-1})
100% N	11.0^{ab}	3.7^{a}	10.5^{a}	22.5^{a}	30.9	52.0^{a}
85% N	9.9^{c}	3.4^{c}	10.3^{a}	21.8^{ab}	28.5	46.4^{b}
70% N	9.1^{d}	3.5^{bc}	10.8^{a}	19.5^{bc}	29.1	42.7^{c}
55% N	8.1^{e}	3.4^{c}	10.5^{a}	17.0^{c}	28.9	36.0^{e}
85% N + NFEB	11.6^{a}	3.7^{a}	10.5^{a}	23.3^{a}	30.5	52.3^{a}
70% N + NFEB	10.6^{b}	3.7^{a}	10.3^{a}	19.3^{bc}	30.3	44.0^{c}
55% N + NFEB	9.5^{cd}	3.6^{ab}	10.5^{a}	19.0^{c}	30.9	37.6^{d}
0% N + NFEB	7.4^{f}	3.2^{d}	8.0^{b}	10.0^{d}	28.9	24.5^{f}
0% N	6.4^{g}	3.0^{e}	8.0^{b}	11.0^{d}	27.7	19.4^{g}
Significant differences	*	*	*	*	ns	*

Within a column, numbers are followed with the same letter show no significant difference; (*) statistically significant difference at 5% level according to post hoc test; ns: not significant; NFEB: Nitrogen endophytic fixing bacteria.

Fig 3. Growth of maize at 55 days after planting in treatments 85% N of RFF (left) and 85% N plus mixture of N_2-fixing endophytic bacteria (right).

Table 4. Effects of adding a mixture of N_2-fixing endophytic bacteria on N content, biomass and N uptake in grain and stover of maize.

Treatment	N concentration (%)				Biomass (g pot^{-1})				N uptake (g pot^{-1})				Total N uptake (g pot^{-1})
	Grain	Stem	Leaf	Root	Grain	Stem	Leaf	Root	Grain	Stem	Leaf	Root	
100% N	1.33^{a}	0.42^{c}	1.76^{b}	0.64^{cd}	40.3^{ab}	10.9^{a}	11.7^{a}	3.3^{b}	0.54^{ab}	0.046^{c}	0.21^{a}	0.021^{c}	0.81^{b}
85% N	1.24^{a}	0.37^{cd}	1.68^{bc}	0.66^{c}	32.2^{d}	10.0^{b}	10.1^{b}	2.9^{d}	0.40^{d}	0.037^{d}	0.17^{b}	0.020^{d}	0.62^{d}
70% N	1.10^{b}	0.34^{de}	1.59^{cd}	0.62^{e}	30.1^{de}	9.7^{c}	9.2^{c}	2.7^{ef}	0.33^{e}	0.032^{d}	0.15^{c}	0.017^{e}	0.53^{e}
55% N	1.09^{b}	0.32^{de}	1.53^{d}	0.63^{de}	28.0^{e}	7.3^{e}	8.2^{d}	2.6^{f}	0.30^{ef}	0.023^{efg}	0.13^{d}	0.016^{e}	0.47^{e}
85% N + NFEB	1.34^{a}	0.59^{a}	1.89^{a}	0.71^{b}	43.0^{a}	11.1^{a}	11.4^{a}	3.4^{a}	0.58^{a}	0.065^{a}	0.22^{a}	0.025^{a}	0.88^{a}
70% N + NFEB	1.32^{a}	0.55^{ab}	1.74^{b}	0.73^{ab}	36.7^{bc}	10.1^{b}	10.2^{b}	3.1^{c}	0.48^{bc}	0.055^{b}	0.18^{b}	0.023^{b}	0.74^{c}
55% N + NFEB	1.22^{a}	0.51^{b}	1.79^{ab}	0.74^{a}	36.3^{c}	8.8^{d}	8.9^{c}	2.7^{e}	0.44^{cd}	0.045^{c}	0.16^{bc}	0.021^{c}	0.67^{d}
0% N + NFEB	1.05^{bc}	0.38^{cd}	1.71^{b}	0.65^{c}	24.1^{f}	7.1^{e}	6.2^{e}	2.4^{g}	0.25^{f}	0.027^{ef}	0.11^{e}	0.016^{e}	0.40^{f}
0% N	0.93^{c}	0.30^{e}	1.03^{e}	0.57^{f}	19.6^{g}	6.6^{f}	5.1^{f}	1.9^{h}	0.18^{g}	0.020^{g}	0.05^{f}	0.011^{f}	0.26^{g}
Significant differences	*	*	*	*	*	*	*	*	*	*	*	*	*

Within a column, numbers are followed with the same letter show no significant difference; (*) statistically significant difference at 5% level according to post hoc test; ns: not significant; NFEB: Nitrogen fixing endophytic bacteria.

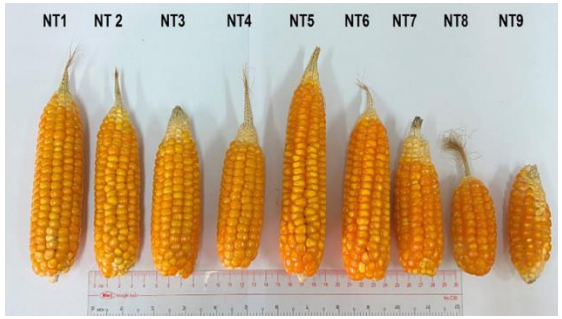

Fig 4. Effects of endophytic bacteria of nitrogen fixation on cob maize cultivated in alluvial soil in dyke.
NT1: Control, 100% N of recommendation fertilizers formula (RFF), NT2: 85% N of RFF, NT3: 70% N of RFF, NT4: 55% N of RFF, NT5: 85% N of RFF and a mixture of E. cloacae ASD-21 and ASD-48, NT6: 70% N of RFF and a mixture of E. cloacae ASD-21 and ASD-48, NT7: 55% N of RFF and a mixture of E. cloacae ASD-21 and ASD-48, NT8: 0% N of RFF and a mixture of E. cloacae ASD-21 and ASD-48, NT9: 0% N of RFF and no endophytic bacteria.

Table 5. Properties of initial soil for maize cultivation.

Soil properties	Value
pH_{H2O}	5.69 ± 0.07
pH_{KCl}	4.61 ± 0.04
OM (% C)	1.81 ± 0.13
Total N (%)	0.183 ± 0.03
NH_4^+ (mg NH_4^+ kg^{-1})	37.2 ± 1.35
NO_3^- (mg NO_3^- kg^{-1})	17.7 ± 0.76
Total P (%)	0.037 ± 0.003
Available P (mg P kg^{-1})	33.9 ± 0.16

Table 6. Parameters for soil analysis

Parameters	Description
pH_{KCl} and pH_{H2O}	For pH determination, soil pH_{KCl} and pH_{H2O} were extracted with 1 M KCl and deionized water at a soil, with solvent ratio of 1:5.
EC	From the extracted solution, electrical conductivity (EC) was also measured.
Total P	Total P was determined by ascorbic acid method in a spectrometer at a wavelength of 880 nm after being digested by perchloric acid and nitric acid mixture.
Available P	Available P was determined by the Bray II method.
Total N	Organic N was converted into inorganic N, resulting in total N (N_{total}) quantification by the Kjeldahl method. NH_4^+-N was extracted from soil by KCl 2 M and its concentration was detected by salicylate.
Total C	Organic C was converted to inorganic C, dichromate oxidized by a thermal conductivity technique in sulfuric acid and titrated with ferrous sulfate, resulting in total C (C_{total}) determination.

but also outweighed in terms of stem and root, overall N uptake, and stem diameter. Ultimately, the result has shown the possibility of endophytic bacteria isolated from hybrid maize roots in altering 15% of inorganic N fertilisers without lowering the growth and yield of maize.

Materials and methods

Plant material

Source of variety: CP 888, a hybrid maize, was used in pot experiment. Its growth cycle ranged from 95 to 100 days, its ear length was 22 cm, yielding highly and steadily from 10 to 12 ton ha^{-1}. It had small cob, orangish yellow firm seed, tolerant to drought, hard stem, green leaves and stable growth.

Source of bacterial strains

Source of bacteria*:* Roots derived from hybrid maize were used as the source to isolate bacteria. The collection samples took place in the day 40^{th}-45^{th} after planting. There were 31 root samples collected from maize fields at An Phu town, communes (Vinh Hau, Vinh Loc, Vinh Loi, Phu Huu, Quoc Thai, Khanh An and Da Phuoc) in An Phu district, An Giang province. We also used 48 bacterial strains isolated from maize in our previous research for this screening.

The N_2-fixing endophytic bacteria used were *E. cloacae* ASD-21 and ASD-48.

Soil source

Soil for pot experiment: local undeposited alluvial soil collected from An Giang province, with chemical properties (Table 5).

Isolation, selection, identification of N_2-fixing endophytic bacteria isolated from root maize

Sample preparation for bacteria isolation: Hybrid maize roots after collection were cleaned with tap water. Then, they were cut into small pieces and washed, then let dried at room temperature.

Bacterial isolation: A 250 ml Erlenmeyer flask was used to contained 1 cm long maize roots, about 10 g in each flask. Then, the flask with maize roots was added with 20 mL of alcohol 96% and shook by a machine at 100 rpm for 10 min. After that, the remaining alcohol was eliminated and replaced by 50 mL of distilled water. Next, the flask with maize roots was shook at 100 rpm for 5 min. This step repeated four times in order to achieve clean samples. Later on, calcium hypochloride 2% was added in with the amount of 20 mL and the flask was shaken at 100 rpm for 10 min. Finally, the samples were washed with distilled water four times as the step described above. Extracts from the liquid of the last wash were inoculated in the amount of 150 µL on petri dishes with TYGA medium, then the inoculated dishes were incubated at 30 °C. After 48 h of incubation, samples whose dishes had no colonies on the medium were accepted for following experiments. Then, the qualified samples were smashed by a sterilized mortar and pestle. The smashed mixture was added with 1.5 mL of distilled water and stirred. Extracts from the mixture of each sample was applied at 500 µL into three tubes containing N-free liquid NFb medium. Shaking incubation was applied for the tubes at 30 ˚C for three days. During incubation, a thin membrane on the surface pointed out the appearance of N_2-fixing bacteria in the sample extracts. The liquid media having N_2-fixing bacteria were inoculated into a N-free solid medium, incubating at 30 ˚C. Dilution was considered if there was high bacteria density. Forty-eight hours later, colonies appearing on the medium surface were spread on other media to achieve isolated colonies. The living bacteria drop method was used to examine the purity of isolated colonies via a microscope. After purity was ensured, the successful isolated bacteria were stored in tubes with appropriate media at 4 °C.

N_2-fixing capability of isolated endophytic bacteria from maize roots: An 10% inoculum of each sample culture was transferred an 18 mL tube of nitrogen-free medium at pH

4.50. The tube was shaken at 120 rpm under dark condition for two days. After 2 days of incubation, culture supernatants were centrifuged at 10,000 rpm in 15 min so as to separate the cells. Then, obtained suspension was measured by the salicylate method (Nelson, 1983) to determine the NH_4^+. The blank medium was considered to be the negative control. Each strain was performed by four replications. The endophytic strains with the highest NH_4^+ content would be chosen for further experiment.

Identification of the selected endophytic bacteria: From the result of previous experiments, there were two strains selected, consisting of ASD-21 and ASD-48 for their acid-resistant N_2-fixing. The application of 16S rDNA sequence was used to analyze sequences from selected strains. After 48 h of incubation in appropriate media, 2 mL of liquid culture was centrifuged at 10,000 rpm for 5 min for cells separation. From the extracted cell pellets, DNA extraction was proceeded via Genomic DNA Prep Kit (BioFACT™), following as the instruction of manufacturer. Then, the extracted DNA was visualized by electrophoresis technique. DNA was solved in 1.0% w/v agarose gel and exposed the bands under UV light to determine the concentration and purity of the DNA samples. Next, the correct DNA bands were amplified using PCR technique, using the iProof™ High-Fidelity PCR Kit - Bio-Rad (BioRad, Hercules, CA) with a T100™ thermo cycler (BioRad), as the manufacturer guide. The primers pair was 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') (Suzuki et al., 2003). The thermal reaction consisted of pre-denaturation for 5 min at 95 °C, 30 cycles in 90 min and the final extension for 10 min at 72 °C. Each cycle made up of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 55 °C and extension for 2 min at 72 °C. The PCR products, then, were fixed by DNA markers on 1.0% w/v agarose gel and 1X TAE buffer (Electrophoresis technique), checked by a UV-trans illuminator and purified by TIANquick Midi Purification Kit (Tiangen Biotech Ltd., Beijing, China) based on the guide of the manufacturer. The purified DNA sequences were determined by an automated DNA sequencer at Macrogen DNA Sequencing Service (Macrogen, Seoul, Korea) and analyzed BioEdit 7.0.5.3 and ChromasPro 1.7 (http://technelysium.com.au/wp/chromaspro) for sequencing results and chromatograms, respectively. The analyzed sequences were compared with available sequences in Genbank database by Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and aligned together by CLUSTALW. From the alignment result, a neighbor-joining phylogenetic tree and an Jukes–Cantor evolutionary distance matrix was constructed by MEGA 6.06, with bootstrap 1,000 replicates.

Potential of potent N_2-fixing endophytic bacteria for improvement of soil fertility and maize yield

Experimental design: The experiment was a completely randomized design, possessing nine treatments, four replicates. A replicate was a pot with one plant. The treatments were listed as follows (i) 100% N of recommendation fertilizer formula (RFF), (ii) 85% N of RFF, (iii) 70% N of RFF, (iv) 55% N of RFF, (v) 85% N of RFF and mixture of *Enterobacter* spp. ASD-21 and ASD-48, (vi) 70% N of RFF and mixture of *E. cloacae* ASD-21 and ASD-48, (vii) 55% N of RFF and mixture of *E. cloacae* ASD-21 and ASD-48, (viii) 0% N of RFF and mixture of *E. cloacae* ASD-21 and ASD-48 and (ix) 0% N of RFF. The experiment was carried out at the greenhouse, College of Agriculture, Can Tho University.

Soil and chemical fertilizer preparation: Soil for plantation in this study was collected from An Giang province. The soil was prepared by eliminating residue materials, mixing and drying in open air. Ten kg of prepared soil was put in a pot. The recommendation fertilizer formula for maize was 200 N, 90 P_2O_5 and 80 K_2O kg ha^{-1}, corresponding to urea (46% N), superphosphate fertilizer (16% P_2O_5), and potassium chloride (60% K_2O) chemical fertilizers.

Seeds preparation: Maize seeds were submerged respectively in ethanol 70% and sodium hypocloride 1% for 3 and 10 min for sterilization and then washed with distilled water. After that, the seeds were let germinate in dark condition for a day. There were approximately 1,000 seeds germinated. Then, they were all soaked in sterilized beakers containing 63 mL of mixed endophytic bacterial suspension (*E. cloacae* ASD-21 and ASD-48) with density of 10^8 cell mL^{-1}, and seeds submerged in distilled water played as a negative control. The beakers with seeds and liquid bacteria mixture were covered with aluminum foil, shaken at 60 rpm for 1 h and let dried under a laminar air flow for 1 h at the end. After dried, seeds with approximately 6.3 x 10^6 bacterial cells $seed^{-1}$ and seeds without bacterial supplement were planted in separate pots.

Solid biofertilizers*:* Solid biofertilizers were prepared following the method of Kantha et al. (2015) with a slight modification from ash and leaves maize at ratio of 1:4. In brief, each bacteria strain was separately cultured at pH 4.5 for 48 h. A cell suspension was then prepared in distilled water to obtain a cell density of 10^9 cells mL^{-1} for use as an inoculant. To prepare the solid formulae, 30 mL were added into 120 g of carrier to produce a final cell density of roughly 10^8 cell g^{-1}. The mixed biofertilizer was packed in plastic bags for 1 month in darkness at room temperature prior to use. The cell density of biofertilizer was counted before inoculation. One pot contained one seed of maize, which gave out a density of 4.2 x 10^3 cells g^{-1} dry soil weigh (DSW) (6.3 x 10^6 cells $seed^{-1}$, dry soil 10 kg pot^{-1}). Solid biofertilizer was used at the amount of 5.0 g (initial cell density 10^8 cells mL^{-1}, dry soil 10 kg pot^{-1}) so as to maintain roughly 0.33 x 10^5 cells g^{-1} DSW for each stage at 10, 20 and 45 days after cultivation, which led to a bacterial density of 1.0 x 10^5 cells g^{-1} DSW (5.0000 log cells) for three stages in one season. Therefore, each pot contained with 1.042 x 10^5 cells g^{-1} DSW (5.0179 log cells), or roughly 1 x 10^5 cells g^{-1} DSW from solid biofertilizer and cells inoculated in the maize seeds.

Parameters of survey

The growth and yield parameters of maize was determined at the stage of 100 days after plantation at physiological maturity to evaluate the efficacy of potent bacteria strains.

The agronomic parameters were determined as follows. Plant height (cm): the measurement took place at the segment between the ground and the top of the maize. Stem diameter (cm): the value was the average diameter inferring from the diameter measurement of the plant top, middle and bottom. Number of leaves (leaves/plant): Leaves were counted in each plant in each pot. Height of appeared first ear formation (cm): the measurement took place at the segment between the ground the first ear form.

The parameters of yield components were described as follows. Cob length (cm): measurement took place between both ends of a cob. Cob diameter (cm): the measurement took place at the middle of a cob. Number of rows/cob

(rows): at each cob, rows were counted. Number of seeds/row (seeds): at each row, seeds were counted. 100-seed weight (g): the calculation was proceeded based on 100 seeds collected randomly in a treatment by an electronic scale with 3 digits.

Maize yield (g pot^{-1}): Both dry weight and fresh weight of seeds from maize cobs collected from plants were measured. To be more specific, after being measured fresh weight, seeds were let dried naturally and put into labeled bags with codes representing for different treatments. The bags with seeds were measured moisture and calculated yield at 15.5% humidity.

Biomass (g pot^{-1}): fresh weight of grain, stem, leaves and root from each pot were scaled. Then, the parts were separately dried out at 70 °C in 72 h to have overall dried biomass weight.

Soil analysis: Soil samples were analyzed, following the standard methods described in Table 6 by Sparks et al. (1996).

Plant analysis: Stover straw and seed samples were collected at the harvest stage and dried up at 70 ^{o}C in 72 h. After that, the samples were cut, ground, and sieved through a 0.5 mm to determine total N contained above- and under-ground components of maize. In detail, total N content were detected by Kjeldahl distillation and the UV-VIS method (Walinga et al., 1989), respectively. N accumulation in the grain, stem, leaf and root were calculated from these concentrations.

Data analysis

The data shown in tables and figures of this study were means of four replications, which were processed by SPSS 13.0 software in subjecting to one-way analysis of variance (ANOVA). Significance of differences among means was calculated by Duncan's post-hoc test at $P < 0.05$.

Conclusions

There were 72 strains of endophytic bacteria isolated from hybrid maize roots under pH 5.0 condition. The 20 out of 72 strains are capable of fixing nitrogen from the atmosphere. Two acid-resistant endophytic bacterial strains were selected and identified as *Enterobacter cloacae* ASD-21 and ASD-48. In greenhouse, a mixture of two strains *E. cloacae* ASD-21 and ASD-48 improved plant height, stem diameter, cod length, and cod diameter compared to no bacterial addition at the same nitrogen fertiliser level. A mixture of the two endophytic strains was applied as biofertilisers and proved their capabilities of raising available ammonium content and replacing 15% of the recommended nitrogen fertiliser while maintaining hybrid maize growth, yield, and nitrogen uptake.

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