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INDIGENOUS DIAZOTROPHS AND THEIR EFFECTIVE PROPERTIES FOR ORGANIC AGRICULTURE

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SUMMARY

Nitrogen-fixing microorganisms (diazotrophs) converting the atmospheric N_2 into usable form NH_4 are considered the key players in the nitrogen cycle, chiefly responsible for the enriching nitrogen content in the soils. Globally, biological fixation of N_2 greatly contributes to plant growth, lessens the need for chemical fertilizers, and thus contributes to the mitigation of greenhouse gases NOx. In this study, diazotrophic bacterial strains were isolated from rhizosphere soils and root nodules of legume and non-legume plants in Vietnam. Quantitative analyzes by the acetylene reduction assay showed that the isolates have high nitrogen fixation activity compared with that of reference strain *Azospirillum vinelandii* KCTC 2426. In addition, other effective capabilities of the isolated strains toward supporting agriculture were investigated, i.e. synthesizing IAA and siderophore for promoting plant growth, or producing exopolysaccharides for maintaining soil moisture. Taxonomic positions of the isolated strains were identified based on the comparative analyses of sequences of the 16S rDNA and gene related to nitrogen fixation (*nifH*), revealing a high taxonomic diversity among free-living and symbiotic diazotrophic isolates. Direct support of the selected isolates to plant growth was proven in experiments with mung beans under laboratory conditions. Thus, the native diazotrophic strains obtained in this study would be good microbial sources for application in organic agriculture and soil amendment.

Keywords: diazotroph, nitrogen-fixation, organic agriculture, plant promoting, soil amendment

INTRODUCTION

N is the utmost nutrient component to plant growth, presenting in nucleic acid, amino acid, chlorophyll, and ATP biosynthesis (Menéndez *et al*., 2017). Plants utilize N in the soluble forms of nitrate (NO_3^-) or ammonium (NH_4^+), but not the abundant atmospheric N_2. Under normal temperature and pressure conditions, the atmospheric N_2 can be converted into usable form ammonium by different species of microorganisms, called nitrogen-fixing or diazotrophs. Globally, the biological nitrogen fixation is estimated to support up to 60% of plant needs (Menéndez *et al*., 2017).

The diazotrophic microorganisms perform either symbiotic (endophytic) or free-living lifestyles. The mechanism of plant and diazotrophs interaction is complex, involving many genes and signal mediators from both the plants and bacteria (Mus *et al*., 2016). Generally, the symbiotic diazotrophs are considered more effective in supplying N sources for plants, thus

have been exploited in microbial formulas for use in agriculture, such as *Gluconacetobacter diazotrophicus* (Azotic Technologies, UK), *Azorhizobium caulinodans* and *Bradyrhizobium* sp. (Vejan *et al*., 2016). The symbiotic diazotrophs can remain in soils for seasons after the host plants die, thus fertile the soils with usable N content (Herridge, 2013). Further, some diazotrophs also possess useful properties supporting plants such as enhancing nutrient uptake (via solubilizing K, P, Fe in soil), producing phytohormones and/or compounds against phytopathogens (Choudhary *et al*., 2011; Garcia-Faile *et al*., 2015).

In this study, 8 diazotrophic strains were isolated from rhizosphere soils and root nodules of legume and non-legume plants in Vietnam. Five of the eight isolates showed noticeable nitrogen fixation capability as determined via acetylene reduction assay, from 3.11 to 17.9 nmol/mL/h. In addition, the isolates also possessed several remarkable traits beneficial to organic agriculture, i.e. producing IAA (3 strains), releasing siderophore (2 strains), and accumulating extracellular polysaccharide (EPS) (5 strains). The supportive effects on the growth of mung bean sprouts were evidenced by *in planta* experiments implemented with four selected strains. The application aspects of these strains having such beneficial properties to support organic agriculture are discussed.

MATERIALS AND METHODS

Sampling, isolation, and cultivation

Healthy soybean and peanut plants in the vegetative stage were collected from Thanh Tri and Soc Son, Hanoi. Soil from the sugarcane rhizosphere was taken from Hoa Binh. The nodule and root were thoroughly washed with water and air-dried. Dry root/nodule and soil were macerated in PBS buffer (NaCl 8 g/L; KCl 0.2 g/L; Na_2HPO_4 1.42 g/L; KH_2PO_4 0.24 g/L). The suspension was then diluted to 10^{-4} - 10^{-6} with PBS and spread on Mannit agar plates (mannitol 10 g/L, KH_2PO_4 1 g/L, $MgSO_4.7H_2O$ 0.5 g/L, NaCl 0.5 g/L, $CaCl_2.2H_2O$ 0.05 g/L, 1 mL micro-element solution, agar 20 g/L, pH 7.2 (Steinbüchel, Oppermann-Sanio, 2003), then incubated at 30^{o}C for 5 - 7 days. Mucoid colonies on the N-free Mannit plates were selected and transferred to new Mannit plates until obtaining pure cultures.

Taxonomic identification

Bacterial genomic DNA of each strain was extracted using E.Z.N.A Bacterial DNA kit (OMEGA D3350-01). The nearly full-length 16S rDNA gene sequence was amplified using primers (27F-AGAGTTTGATCCT-GGCTCAG and 1492R- GTTACCTTGTTA-CGACTT) (Weisburg *et al*., 1991) with the thermos cycles as following: 94^{o}C, 3 minutes; (94^{o}C, 30 sec; 55^{o}C, 45 sec; 72^{o}C, 1.5 min) × 35 cycles; 72^{o}C, 7 min, end at 4^{o}C. The *nifH* gene fragments were amplified using primer pair (nifHF-GGHAARGGHGGHATHGGNAARTC and nifHR-GGCATNGCRAANCCVCCRCANAC) with the following thermos cycles: 94^{o}C, 3 minutes; (94^{o}C, 1min; 61^{o}C, 1 min; 72^{o}C, 2 min) × 35 cycles; 72^{o}C, 3 min, end at 4^{o}C (Mehta *et al*., 2003). The obtained sequences were then compared with sequences available on the EZTAXON/GenBank databases by using the BLAST search tool. Phylogenetic trees were constructed with the neighbor-joining method (Saitou & Nei, 1987). The topography of the constructed trees was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985).

Acetylene reduction assay

The nitrogen-fixing activity of the isolates was quantified through the acetylene reduction assay (ARA) as described previously (Capone *et al*., 1993; Kifle, Laing, 2016). The bacteria were cultured for 24 h in TSB 1/5 (HiMedia, India) at 28^{o}C, 120 rpm, then transferred (10% v/v) into serum bottles containing N-free Mannit liquid medium, tightly sealed with rubber stopper and aluminum lid. 3 mL of the air in the headspace of each bottle was replaced with 3 mL of acetylene gas, except for the negative control. All bottles were incubated further for 24 h at 30^{o}C, 120 rpm. Ethylene formed in each bottle was measured

(twice) using a gas chromatography system (Agilent 7890A, USA) equipped with a flame ionization detector (FID). The GC conditions were: 250°C for the inlet and detector, 60°C for the oven, and the makeup gas was helium at 30 mL/min. The ethylene formation rate in experiments was calculated based on an ethylene standard curve.

Determine other agriculture beneficial traits

IAA production

Bacterial strains were cultured in TSB 1/5 medium supplemented with L-tryptophan (1 g/L) at 30°C, 160 rpm for 7 days. Culture suspension was centrifuged at 6000 rpm for 20 min to eliminate bacterial cells. The supernatant was then mixed with Salkowski reagent (perchloric acid $HClO_4$ 35% 98 mL, $FeCl_3$ 0.5 M 2 mL) in the ratio of 2:1 v/v and kept in the dark for 30 minutes. The absorbance at 530 nm was measured and the concentration of IAA was calculated based on an IAA standard curve with concentrations in the range of 10 - 100 µg/mL (Ji *et al*., 2014).

Siderophore production

Siderophore production was determined by using the method described by Lakshmanan *et al.* (2015). Briefly, 60.5 mg chrome azurol S was dissolved in 50 mL water and mixed with 10 mL iron (III) solution (1 mM $FeCl_3.6H_2O$, 10 mM HCl). The solution was then slowly added to the solution of 5 M hexadecyltrimethylammonium bromide (CTAB) and autoclaved. The mixture was then added to TSA medium (10%) (HiMedia, India) and poured into Petri dishes, and used to inoculate the isolates for 3 days at 30°C. The bacterial isolates that changed the color of the medium from blue to yellowish-orange were considered positive for the production of siderophore.

EPS production

The production of EPS in cultural broth was quantified as described previously (Castellane *et al*., 2015). A single colony of each isolate was incubated in 50 mL of YEM liquid medium (yeast extract 0.5 g, mannitol 4 g, distilled water 1 L, pH 7) at 30°C, 160 rpm for 3 days. The culture broth was then centrifuged at 6000 rpm for 15 minutes, and the supernatant was mixed with cold ethanol 100% (kept at -20°C for at least 24 h) at the ratio of 1:3 (v/v) and the mixture was then kept at -20°C for 1 hour to precipitate the EPS. Afterward, the mixture was centrifuged at 6000 rpm at 4°C for 15 minutes. The pellets were collected and dried at 60°C for 48 hours or until constant weight. Dried EPS was weighed and calculated as g/L.

***In planta* experiment**

Mung bean seeds were surface sterilized in 75% ethanol for 1 minute and 1% sodium hypochlorite for 5 minutes then rinsed 6 times with sterile distilled water (Igiehon *et al*., 2019). The selected isolates were cultivated in TSB 1/5 medium at 30°C, 160 rpm, for 2 days. Afterward, the culture broth was centrifuged at 4000 rpm for 10 minutes, the supernatant was discarded. The pellet was then washed 2 times with sodium chloride 0.9% and normalized to OD_{600} = 1 with TSB 1/5. The sterilized mung bean seeds were submerged into the bacterial suspension (or in TSB 1/5 for the control) for one hour. The seeds were then placed in Petri dishes on filter papers previously moisturized with distilled water (50 seeds/isolate) and let germinate at 30°C for 7 days in the dark. The effects of the bacterial strains were evaluated via stem and root development (the length) and fresh weight of the sprouts.

Statistical test and data analysis

All experiments were performed in triplicate unless otherwise stated. The collected experimental data were processed and graphed using Microsoft Excel and SigmaPlot software. The statistical test was performed by using the SAS software, and the $p \leq 0.05$ was accepted as statistically significant. Data were statistically analyzed by two-tailed T-test analysis using the Statistical Analysis System (S.A.S 9.4) program (*: P< 0.05; **: P< 0.01; ***: P<0.001; ****: P<0.0001).

RESULTS AND DISCUSSION

Bacterial isolation and identification

From the rhizosphere soil and root nodule samples collected at different agricultural areas in Northern Vietnam, 52 bacterial strains were obtained on the N-free Mannit agar plates. The isolates were then subjected to the first screening on the presence of the *nifH* gene, and the results showed that only 8 strains yielded fragments of this gene via the PCR with specific nif-primers. These 8 strains were then taxonomically identified based on the 16S rDNA sequence comparison. It revealed that of the 8 isolates three were symbiotic of two genera *Rhizobium* and *Ensifer*, and five were free-living of four genera *Azospirillum*, *Paraburkholderia*, *Pseud-acidovorax*, and *Kosakonia* (Table 1). In addition to the 16S rDNA taxonomy (Fig. 1A), a taxonomic study based on the *nifH* gene sequences was carried out, showing a good agreement (Fig 1B).

Table 1. Identification of nitrogen-fixing strains from legume and non-legume samples.

Strain	Source of isolation	Homology (% identity)	GenBank accession No
MTR1	Sugarcane rhizosphere, Hoa Binh	*Paraburkholderia unamae* (100%)	OM570608
SDT2.3	Soybean root nodule, Hanoi	*Ensifer sesbania* (100%)	OM570606
DL12	Peanut rhizosphere, Hanoi	*Rhizobium daejeonense* (99.85%)	OM570604
RRL12	Peanut root, Hanoi	*Pseudacidovorax intermedius* (100%)	OM570610
SRL12.2	Peanut root nodule, Hanoi	*Azospirillum humicireducens* (99.1%)	OM570603
RDT14	Soybean root, Hanoi	*Rhizobium daejeonense* (99.93%)	OM570605
DDT20	Soybean rhizosphere, Hanoi	*Paraburkholderia heleia* (99.88%)	OM570607
SRL12.1	Peanut root nodule, Hanoi	*Kosakonia radicincitans* (99.93%)	OM570609

Nitrogen fixation activity of the isolates

Quantitative analyses of the nitrogen-fixing activity via acetylene reduction assay (ARA) showed that all the 8 isolates exhibited the activity at different levels, depending on their taxonomic affiliation and lifestyles (symbiotic or free-living). Out of 8 isolates, four strains had acetylene reduction activity significantly higher than the control *Azotobacter vinelandii* KCTC 2426 (Fig. 2). Of special interest was strain DDT20, a free-living diazotroph that possessed an outstanding acetylene reduction activity of 17.9 nmol C_2H_4/mL/h, about four times higher than other isolates, and significantly higher than the positive control. Evidentially, the free-living isolates DDT20, DL12, RDT14, and MTR1 showed remarkably higher acetylene reduction rates than the symbiotic isolates SRL12.1, RL12.2, SDT2.3.

A similar phenomenon has been shown in several studies. Kifle and Laing (2016) reported acetylene reduction rate by some free-living diazotrophs isolated from leaves, roots, and rhizosphere of maize in South Africa (such as *Bacillus megaterium, Pseudomonas nitroreducens, Burkholderia* spp., *Pantoea ananatis*), in the range of 4.81 to 73.2 nmoles C_2H_4/culture/h (Kifle, Laing, 2016). On the other hand, the symbiotic diazotroph *Rhizobium tropici* CIAT899 showed significant acetylene reduction activity only when inoculated with legume plants, at 40±10 nmol ethylene/g root dry weight/h (Dardanelli *et al*., 2008). Thus, the results gave hints to the use of the free-living and symbiotic diazotrophs for soil amendment and enhancing specific crop yields, respectively.

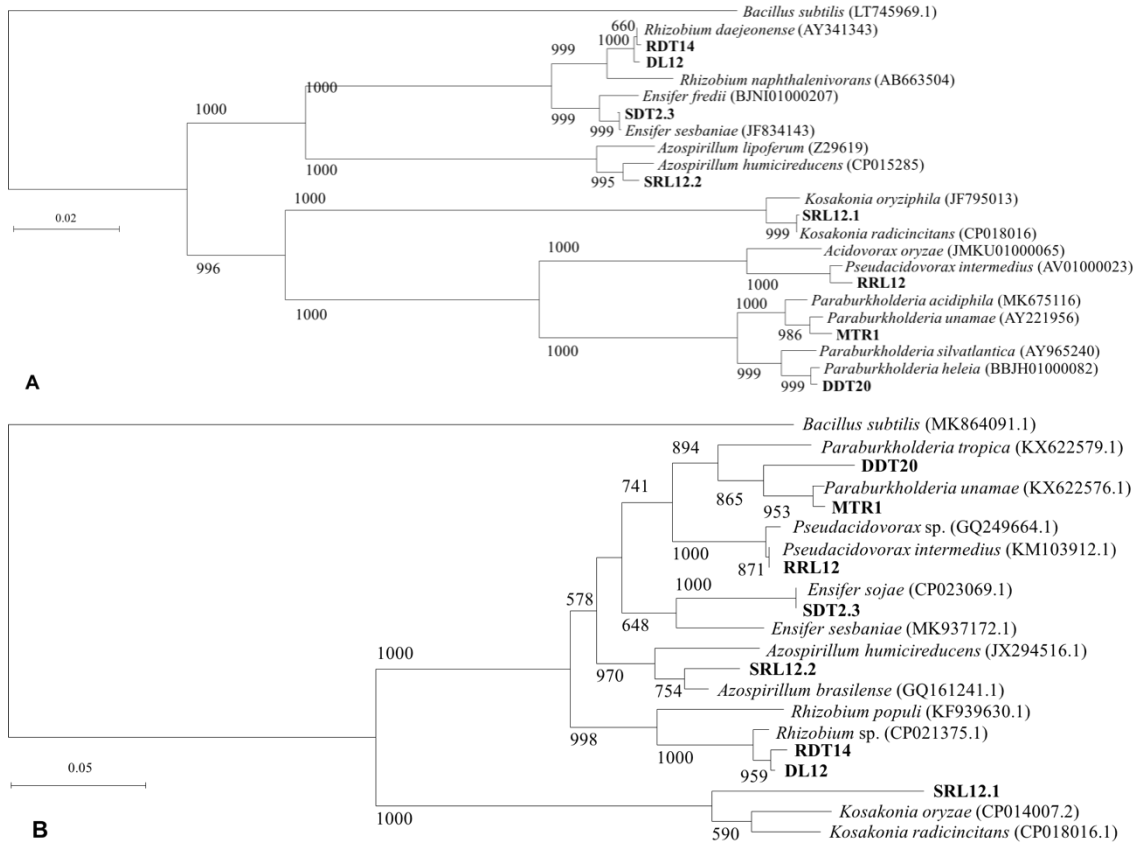

Figure 1. Phylogenetic trees showing taxonomic positions of the diazotrophic isolates within the related species based on the comparative analyses of 16S rDNA (A) and *nifH* (B) gene sequences.

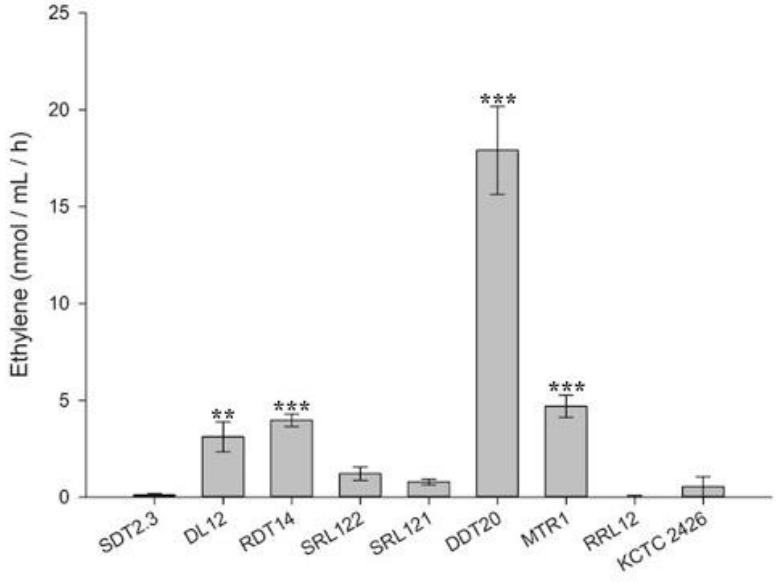

Figure 2. Quantification of acetylene reduction to ethylene by the diazotrophic isolates. Data obtain by SAS statistic program. Differences between treatment means were determined at a probability level of 0.05. (P=0.05).

Other beneficial traits of the isolates

Auxin or indole-3-acetic acid (IAA) is an important phytohormone, controlling not only plant growth but also their responses to various abiotic factors (Ghosh *et al*., 2011). The isolates were subjected to quantitatively analyze the IAA production in the presence of tryptophan at 2, 5, and 7 days after inoculation. It is shown that the IAA production was highest in *Rhizobium* spp. isolates (180 - 207.3 µg/mL after 5 days), followed by *Paraburkholderia heleia* DDT20 (89.4 µg/mL after 7 days) and *Ensifer sesbaniae* SDT2.3 (33.4 µg/mL after 7 days) (Fig. 3).

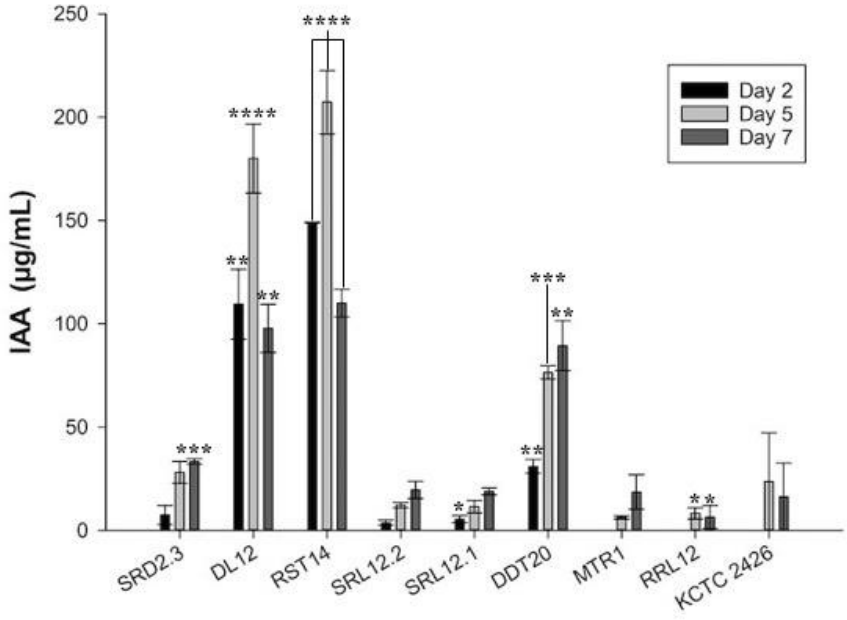

Figure 3. Comparison of IAA production by the nitrogen-fixing isolates in TSB ½ medium with tryptophan. Data obtained by SAS statistic program. Differences between treatment means were determined at a probability level of 0.05 (P=0.05).

Published data showed that diazotrophic species of the genus *Rhizobium* produce IAA at high to very high levels, from 47.1 to 309.7 µg/mL, whereas *Ensifer/Sinorhizobium* species produced IAA at much lower yields (Ghosh *et al*., 2011). Kaur *et al*. (2014) reported two *Ensifer* sp. strains LSER 7 and LSER isolated from soybean rhizosphere soil in India produced 28.58 - 30.90 µg/mL IAA (Kaur *et al*., 2014). In another study, *E. meliloti* strains isolated from alfalfa soil and roots in Chile were reported to produce IAA at low yields, 13.71 - 26.22 µg/mL (Cedeño-García *et al*., 2018). Thus, the ability to produce IAA of diazotrophs in our study was consistent with previous reports.

Rhizobia EPS has been suggested for several functions, e.g. protecting bacteria against environmental stresses, nodule formation, suppression of plant defense responses, and protection against plant antimicrobial compounds (Becker, Pühler, 1998). Recent studies have demonstrated an application aspect of EPS from rhizobia in promoting the adhesion of soil particles, thus enhancing soil moisture and supporting plant growth (Rossi *et al*., 2012). Among the 8 isolates, 5 strains actively produced EPS while growing on solid YEM medium (Fig. 4A), especially two strains SDT 2.3 and RDT14 produced 3.7-3.92 g/L EPS after 3 days of incubation (Fig. 4B), showing high potential for application in enhancing soil moisture.

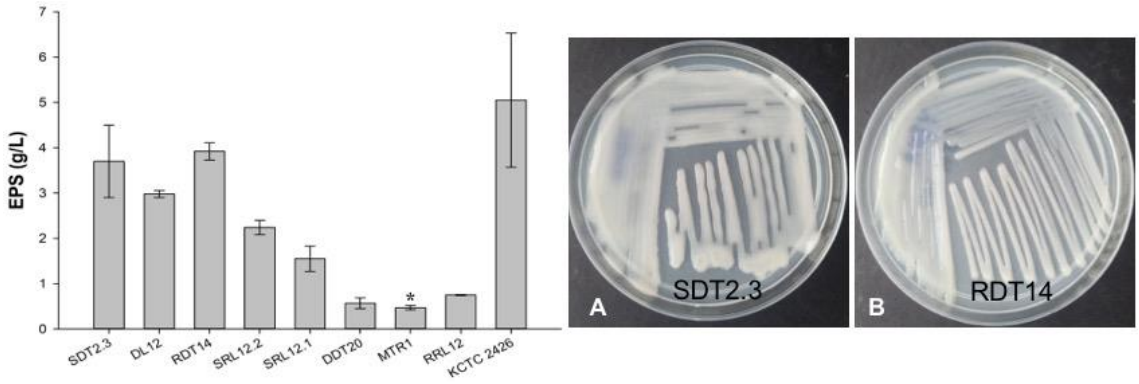

Figure 4. Comparison of EPS production by the bacterial isolates in YEM medium after 3 days of inoculation (A) and mucoid colony formation on YEM agar medium of nitrogen-fixing isolates after 3 days of incubation at 30^{o}C (B, C). Data obtained by SAS statistic program. Differences between treatment means were determined at a probability level of 0.05 (P=0.05).

Rhizobium species have been reported for EPS production at a wide range of 0.35 - 4.08 g/L (Ghosh, Maiti, 2016). *Ensifer* species such as *Ensifer* sp. LBMP-C03 and *E. meliloti* LBMP-C02 isolated from legume plants produced EPS at lower levels of 2.86 g/L and 2.75 g/L, respectively (Moretto *et al*., 2015). *Azospirillum brasilense* Ab-V5 strain isolated from maize in Brazil was reported to produce 1.9 g/L EPS after 24 h of culture (Oliveira *et al*., 2017). The EPS production activity of the diazotrophic isolates in this study, especially two strains SDT 2.3 and RDT14, was considered of high level compared with the reported strains.

The bacterial siderophore has been reported for several functions, such as influencing the plant uptake of various trace metals (Fe, Zn, and Cu), stimulating the biosynthesis of antimicrobial compounds against phytopathogens, and inducing host resistance (Ji *et al*., 2014). A strain of the species *Azospirillum brasilense* was reported to produce catechol-type siderophores that showed in vitro activity against the fungus *Colletotrichum acutatum*, causing agent of an important disease on strawberries (Pedraza, 2017). Among the 8 isolates, three strains (MTR1, SRL12.1, and SRL12.2) were able to produce siderophores, the highest activity was observed in strain SRL12.2 (Fig. 5). Of special interest is that two symbiotic strains SRL12.1 and SRL12.2 did present significantly higher siderophore production, compared to the free-living strain MTR1 and the control KCTC2426 as well.

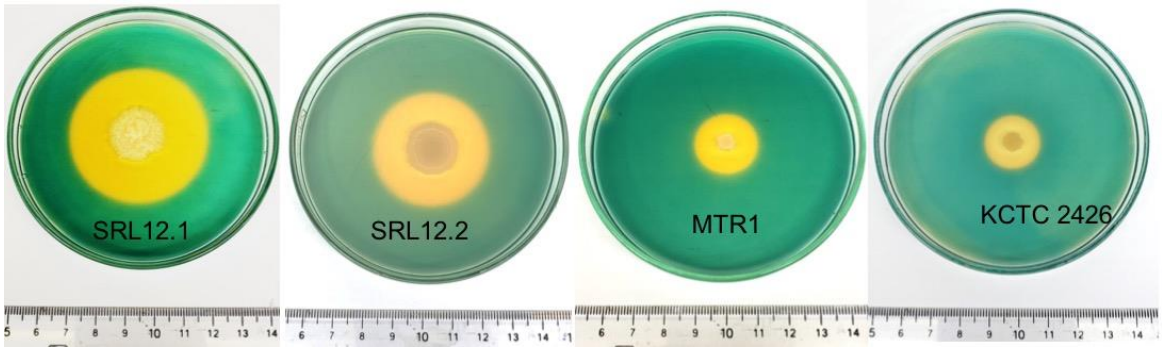

Figure 5. Confirmation of catechol siderophore activity by a color change from blue to orange on LB agar containing chrome azurol S *Azotobacter vinelandii* KCTC 2426 as a positive control.

In planta experiment on mung bean

The use of diazotrophs in promoting plant growth has been widely applied (Babalola, 2010). In this study, the *in planta* experiment was carried out with mung bean seeds. Four isolates that possessed a set of agriculture beneficial trains were selected, including three symbiotic strains SDT2.3, SRL12.1, and SRL12.2, and a free-living strain DL12 (Table 2). Mung bean seeds were treated with the selected strains and the effects were evaluated via root development and the fresh weight of sprouts in comparison to the control without bacterial treatment.

Table 2. Biological traits of the isolates that would be beneficial for organic agriculture.

Strain	Genera	Lifestyle	Agriculture-beneficial traits			
			Nitrogen fixation	IAA production	Siderophore production	EPS production
MTR1	*Paraburkholderia*	Free-living	++	+	+	+
SDT2.3	*Ensifer*	Symbiotic	+	+	ND	+++
DL12	*Rhizobium*	Free-living	++	+++	ND	++
RRL12	*Pseudacidovorax*	Free-living	+	+	ND	+
SRL12.2	*Azospirillum*	Symbiotic	+	+	+++	++
RDT14	*Rhizobium*	Symbiotic	++	+++	ND	+++
DDT20	*Paraburkholderia*	Free-living	+++	++	ND	+
SRL12.1	*Kosakonia*	Symbiotic	+	+	++	++
KTCC2426	*Azotobacter*		+	+	+	+++

(+++) High; (++) Middle; (+) Low; (ND) Not detected.

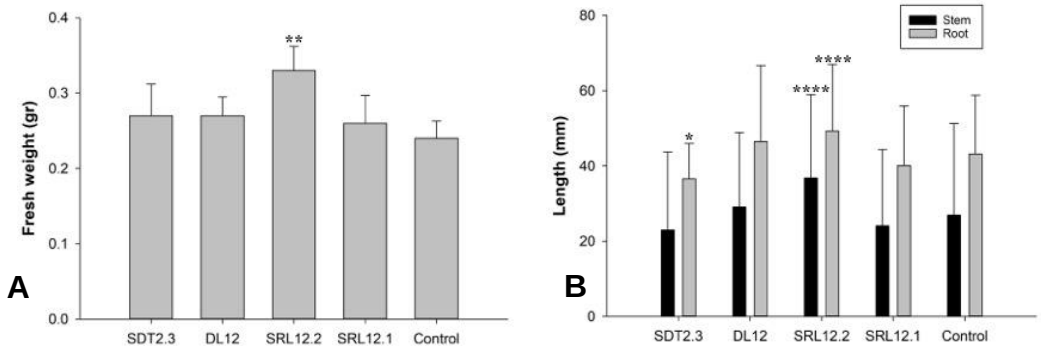

Figure 6. Effects of diazotrophic isolates SDT2.3, DL12, SRL12.2, SRL12.1 on fresh weight and the length of stem and root of mung bean sprouts. (A) The effect of diazotroph bacteria on fresh weight of mung bean sprout; (B) The effect of bacteria on stem and root length of mung bean sprout. Data obtain by SAS statistic program. Differences between treatment means were determined at a probability level of 0.05 (P=0.05).

The results showed that the four strains used in the experiment did enhance the seedling growth at different levels. The most special was the symbiotic strain SRL12.2, which increased the sprout fresh weight by 37%, the stem length by 36.7%, and the root length by 14.2% compared to the control (Fig. 6). Hungria *et al*. (2013) reported the efficacy of plant growth promotion of rhizobia on legumes at the field experiment level. Soybean seeds were inoculated with *Bradyrhizobium japonicum* (1.2×10^6 cell seed^{-1}) and co-inoculated with *Azotobacter*

brasilense in-furrow (2.5×10^5 cell seed^{-1}). The results showed that *B. japonicum* increased the soybean yield by an average of 222 kg ha^{-1} (8.4 %), and co-inoculation with *A. brasilense* in-furrow increased yield by 427 kg ha^{-1} (16.1 %) as compared to the non-inoculated control. Similar effects were observed in other legume plants when treating seeds with *Rhizobium tropici* and *A. brasilense* in-at the same cell concentration as described above. The common bean yield increased by 98 kg ha^{-1} (8.3 %) thanked to rhizobia alone and even resulted in higher yield improvement at 285 kg ha^{-1} (19.6 %) when co-inoculated with *A. brasilense* (Hungria *et al*., 2013). Thus, the diazotrophic strains in this study, especially SRL12.2 would be good bacterial sources for promoting plant growth and increasing crop yield.

CONCLUSION

Diazotrophic bacteria stand at the central position in agriculture sustainability owing to their contribution to promoting plant growth and enhancing soil fertility. Aiming to provide microbial resources for use in organic agriculture in Vietnam, the present study showed a range of indigenous diazotrophic strains (both free-living and symbiotic) that have significant nitrogen fixation activities. Of special interest, the presented strains also possess other physiological properties beneficial to the plant and soil, i.e. IAA production, EPS, and siderophore secretion, thus enhancing plant growth and soil fertility. Indeed, visible increases in the development of roots and seedlings were observed when the mung beans were treated with the isolates. The diazotrophic strains presented in this study would be potential candidates for the use for soil amendment and improving crop yield while reducing the dependency on chemical fertilizers.

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