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Isolating, selecting, and identifying Na^+, H^+, Al^{3+}, Fe^{2+}, Mn^{2+}-resistant purple non-sulfur bacteria solubilizing insoluble phosphorus compounds from salt-contaminated acid sulfate soil derived from rice-shrimp system

Le Tien Dat[1, 2], Ly Ngoc Thanh Xuan[3], Tran Chi Nhan[3], Le Thanh Quang[1], Nguyen Quoc Khuong[1]*

[1]Faculty of Crop Science, College of Agriculture, Can Tho University, Can Tho, Vietnam
[2]Vinh Long Department of Agriculture and Rural Development, Vinh Long, Vietnam
[3]An Giang University, Vietnam National University Ho Chi Minh City, An Giang, Vietnam

***Corresponding author: nqkhuong@ctu.edu.vn | ORCID ID: http://orcid.org/0000-0001-5654-8401**

Abstract

Salt intrusion has caused adverse effects on nutrient uptake, growth, and yield of rice plants in the Mekong Delta, Vietnam. Thus, (i) isolating, screening, and identifying phosphorus (P)-solubilizing purple non-sulfur bacteria (PNSB), and (ii) determining their ability to produce plant growth-promoting substances were aimed in the current study. Bacteria after isolation were investigated under microaerobic light and aerobic dark conditions for their ability to tolerate toxic cations and to produce available P from insoluble P compounds, such as Al-P, Fe-P, and Ca-P by spectrophotometry. Therefore, 46 isolates of PNSB were randomly collected from 21 soil samples and 21 water samples from a rice-shrimp paddy field in districts of Tran De, My Tu, and My Xuyen, Soc Trang province. Among them, four PNSB isolates could solubilize P and tolerate Na^+, H^+, Al^{3+}, $Fe^{2+,}$ and Mn^{2+}, which were identified by the 16S rDNA method as *Cereibacter sphaeroides* at 100% similarity. The four selected isolates (ST16, ST26, ST27, and ST32) dissolved 32.7 – 60.8 mg P L^{-1} from Al-P, 30.6 – 81.7 mg P L^{-1} from Fe-P, and 22.8 – 36.3 mg P L^{-1} from Ca-P in both conditions. Moreover, these bacterial isolates were potent in promoting plant growth by fixing nitrogen (16.2 – 104.1 mg NH_4^+ L^{-1}) and providing IAA (10.3 - 21.0 mg IAA L^{-1}), ALA (2.38 - 3.59 mg ALA L^{-1}), siderophores (8.53 - 55.3 %) and EPS (0.68 - 1.22 mg EPS L^{-1}). They should, thereby, be applied as a biofertilizer to increase crop yield in a pot experiment and subsequently a field trial.

Keywords: biological phosphorus solubilization; rice; soil contamination; saline condition.
Abbreviations: ALA_5-aminolevulinic acid; EPS_exopolymeric substances; IAA_indole-3-acetic acid; P_phosphorus; PNSB_purple nonsulfur bacteria; SCASS_salt-contaminated acid sulfate soil; TA_titratable acidity.

Introduction

Nowadays, salt intrusion has been happening in a wide area, and tends to grow in coastal regions in the Mekong Delta, Vietnam, which severely damages agriculture here, especially rice agriculture (Ministry of Agriculture and Rural Development, 2020). Consequently, to adapt to the salt intrusion, many farmers have utilized the rice-shrimp integrated cultivation. In particular, shrimps are farmed in dry seasons due to salt intrusion, and rice is grown in wet seasons because of the natural source of freshwater (Loc et al., 2021). Thus, the model has been widely utilized in coastal areas affected by the salinity to improve farmers' livelihood there (Loc et al., 2021). However, the rice shrimp system has a high concentration of salt (Kruse et al., 2020). This salinity adversely affects the soil microbial community (Zhang et al., 2019). To specify, high salinity reduces the diversity, activity, and biomass of microbes (Yan et al., 2015). As a result, salinity changes the chemical, physical, and biological properties of soil, which suppresses rice productivity (Zhang et al., 2022). Moreover, salinity also affects the presence of P forms, the contents of soil soluble P, and the growth and productivity of crops (Meena et al., 2018). High salinity indirectly results in a lack of soluble P, because P can form insoluble complexes with cations of Ca^{2+} and Mg^{2+}, which are supplied to reduce the salinity (Kruse et al., 2020). In addition, crops absorb only 10-20% of P applied in the first season, the rest is stored in soil and held by ions of Fe^{2+}, Al^{3+}, or Ca^{2+} (Tóth et al., 2014).

Regarding the traditional way to improve soil P deficiency, fertilizing with higher inorganic P than the amount that used to be applied is utilized, but this leads to higher cost and contaminations in the underground environment by heavy metals, especially Cadmium contamination after long-term use (Wiggenhauser et al., 2019). Applying to the soil at an amount of 4.0 - 8.0 Mg ha^{-1} of biochar made from straws and husks is efficient in improving the soluble P content in saline soil (Wu et al., 2021). Noticeably in acidic soils, and P-deficient soil, supplying biochar originating from manure at a dose of 10 t ha^{-1} results in increases in soluble P content in soil, and in P uptake capacity in plants (Tesfaye et al., 2021). Nevertheless, the mass use of biochar on alkaline soils or low-P-content soils can reduce the soil's fertility (Yang et al., 2021). Although organic fertilizers originated from legumes, straws, and manures ameliorate soil soluble P (Mengmeng et al., 2021), overuse of organic fertilizers for a long time increases erosion of P in soil (Burakova and Bakšiene, 2021). Therefore, thriving for a sustainable P source for rice plants on saline soils is demanded.

Table 1. The capacity of PNSB in solubilizing insoluble P forms in BIM containing NaCl (10‰), pH 5.

PNSB isolate	Al-P (mg L^{-1})		Fe-P (mg L^{-1})		Ca-P (mg L^{-1})	
	ML	AD	ML	AD	ML	AD
ST03	21.9^{d}±0.53	15.3^{c}±0.37	16.9^{gh}±0.93	36.7^{e}±0.92	9.83^{e}±0.11	21.4^{b}±0.02
ST05	20.7^{e}±0.25	14.2^{d}±0.70	24.4^{e}±0.50	29.4^{hi}±0.22	11.4^{bc}±0.14	15.3^{d}±0.63
ST09	21.7^{d}±0.16	14.2^{d}±0.38	17.0^{g}±0.72	32.5^{f}±0.32	5.92^{g}±0.02	17.6^{c}±0.02
ST15	18.9^{gh}±0.28	15.9^{c}±0.29	32.9^{d}±0.19	39.5^{c}±0.52	10.8^{cd}±0.46	20.7^{b}±0.14
ST16	19.5^{fg}±0.58	13.2^{ef}±0.59	41.2^{a}±1.15	40.5^{b}±0.18	13.1^{a}±0.54	23.2^{a} ±0.76
ST22	20.5^{ef}±0.17	12.3^{f}±0.63	33.9^{d}±0.98	36.0^{e}±0.80	6.38^{g}±0.19	9.18^{i}±0.29
ST25	21.1^{de}±0.45	17.2^{b}±0.77	39.8^{b}±0.16	31.0^{g}±0.32	11.3^{bc}±0.85	11.4^{h}±0.79
ST26	23.2^{c}±0.68	37.6^{a}±0.45	20.8^{f}±0.04	9.84^{k}±0.90	8.44^{f}±0.79	14.4^{ef}±0.46
ST27	24.6^{b}±0.96	13.8^{de}±0.21	35.6^{c}±0.14	45.2^{a}±0.04	11.6^{bc}±0.03	17.7^{c}±0.14
ST31	18.4^{h}±0.77	13.5^{de}±0.33	24.8^{e}±0.27	38.2^{d}±0.42	12.0^{b}±0.64	13.8^{fg}±0.61
ST32	25.8^{a}±0.47	15.3^{c}±0.58	39.1^{b}±1.55	30.1^{h}±0.39	11.3^{bc}±0.26	13.2^{g}±0.47
ST33	19.7^{fg}±0.55	7.70^{g}±0.62	15.7^{hi}±0.41	29.0^{i}±0.08	11.0^{cd}±0.24	15.1^{de}±0.57
ST34	19.8^{fg}±0.56	13.9^{de}±0.45	14.8^{i}±0.68	19.0^{j}±0.66	10.3^{de}±0.33	11.6^{h}±0.23

*Note: Different letters following numbers mean different statistically at $P < 0.05$ (*); PNSB: purple nonsulfur bacteria; ML: Microaerobic light; AD: Aerobic dark.

In the rice-shrimp system, SCASS in the Mekong Delta features high concentrations of Na^{+}, H^{+}, Fe^{2+}, Al^{3+}, and Mn^{2+} (Khuong et al., 2022), which form insoluble P compounds such as Ca-P, Al-P, and Fe-P (Kruse et al., 2020). In the soil, immobilized P can be solubilized by P-solubilizing bacteria such as *Pseudomonas* sp. and *Bacillus* sp. which carry out the P solubilization processes for plants via the production of organic acids and siderophores, chelation reactions, and cation exchange (Gomez-Ramirez and Uribe-Velez 2021). However, the capacity of solubilizing P of *Pseudomonas* strains is low under salt-contaminated soil conditions (Jiang et al., 2020). In the meantime, the group of PNSB can live under adverse conditions such as acidic and saline environments (Khuong et al., 2017, 2022). In particular, a group of P-solubilizing PNSB has been selected and successfully applied on acid sulfate soil for rice plants to improve the soil fertility and quality, and rice productivity (Khuong et al., 2022). Moreover, these bacterial strains can synthesize plant growth-promoting substances (IAA and siderophores) (Khuong et al., 2020a, 2022) and metabolites (ALA and EPS), which help rice plants overcome obstacles in saline soil (Nookongbut et al., 2019; Khuong et al., 2020b, 2021, 2022) and reduce toxic concentrations of Al^{3+}, Fe^{2+}, and Mn^{2+} (Khuong et al., 2018, 2020b). Therefore, the study proceeded to sort out PNSB strains, which can solubilize insoluble forms of soil P, isolated from salt-contaminated acid sulfate soil in the rice shrimp system.

Results

Chemical properties of salt-affected soil and water in rice-shrimp system

The soil analysis result revealed that soil pH_{KCl} and pH_{H2O} fluctuated roughly 2.91 - 4.05 and 2.90 - 4.56, and water pH was from 2.99 to 7.61. Total P content fluctuated from 0.02 to 0.03%. In addition, soluble P content was determined as 5.67 - 24.1 mg kg^{-1}. Al-P, Fe-P, and Ca-P contents were 348.8 - 352.7, 22.8 - 23.2, and 80.2 - 81.4 mg L^{-1}, respectively. Moreover, other basic soil characteristics are demonstrated in Supplementary Table 1.

Isolation of purple non-sulfur bacteria from rice-shrimp soil

There were 21 soil samples and 21 water samples collected in fields of rice-shrimp system in the Tran De, My Tu, and My Xuyen districts of Soc Trang province. From there, 46 PNSB isolates were obtained. Pure isolates of PNSB were detected to be Gram-negative. Therein, there were 8 colonies isolated from the soil samples and 38 colonies from the water ones.

Selection of PNSB that solubilized phosphorus under saline, acidic, and toxic conditions

Twenty isolates of PNSB were selected for living in a pH 5.0 environment with $OD_{660} > 0.50$ under at least one of the two incubating conditions (Supplementary Table 2). There were 15 isolates selected for being able to tolerate salinity at 5‰ with $OD_{660} > 0.5$. At 10 ‰ Na^{+}, under the microaerobic light condition, OD_{660} values fluctuated from 0.38 to 4.57 and averaged at 2.50. Under the aerobic dark condition, OD_{660} values were from 0.53 to 1.37 and 1.03 on average. Significantly, the ST05 isolate had the highest performance under both conditions (4.57 and 1.37, respectively). Isolates, which adapted the 10 ‰ Na^{+} concentrations with $OD_{660} > 0.7$ under at least one of the conditions were chosen for the experiment investigating their capacity in tolerating toxicity. Growth inhibition caused by Na^{+}, H^{+}, Al^{3+}, $Fe^{2+,}$ and Mn^{2+} toxins varied under both conditions. The lowest inhibition rate was in the ST32, ST26, and ST16 isolates (33.1%, 33.6%, and 35.0%) under the microaerobic light condition, while under the other condition, it belonged to the ST32, ST27, and ST26 isolates (22.6%, 22.7% and 24.3%) (Supplementary Table 2). Therefore, 13 PNSB isolates that can tolerate Na^{+}, H^{+}, Al^{3+}, $Fe^{2+,}$ and Mn^{2+}, with a growth inhibition rate lower than 50% were chosen for further experiment.

Selection for PNSB that provided phosphorus

The ST32 isolate had the highest capacity in solubilizing P from Al-P (25.8 mg P L^{-1}) under the microaerobic light condition, while the ST26 isolate solubilized Al-P best under the aerobic dark condition (Table 1). Therefore, the ST26 and ST32 isolates performed best in solubilizing Al-P under the investigating conditions. Under the microaerobic light condition, the capacity in solubilizing P from Fe-P appeared to be the highest at the ST16 isolate (41.2 mg P L^{-1}). Under the aerobic dark condition, the ST27 isolate solubilized Fe-P the best, while the ST16 isolate (40.5 mg P L^{-1}) came second (Table 1). The PNSB isolates were able to solubilize Ca-P from 5.92 to 13.1 mg P L^{-1} under the microaerobic light condition. On the other hand, the solubilized Ca-P contents were recorded as 9.18-23.2 mg P L^{-1} under the aerobic dark condition. Significantly, the ST16 isolate performed the best

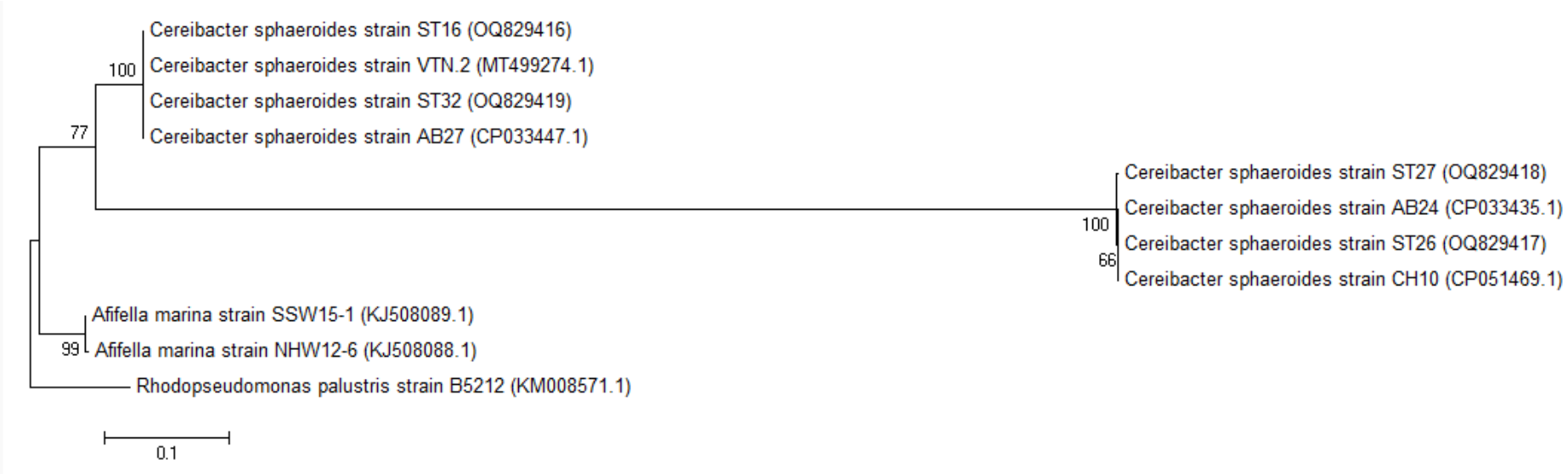

Fig 1. Neighbor-joining phylogenetic trees of four selected purple non-sulfur bacteria strains compared to the closely related strains.

Table 2. The capacity of PNSB in fixing N, and producing IAA, siderophores, EPS, and ALA.

PNSB isolate	N (mg L^{-1})		IAA (mg L^{-1})		EPS (mg L^{-1})		ALA (mg L^{-1})		Siderophores (%)	
	ML	AD	ML	AD	ML	AD	ML	AD	ML	AD
ST16	104.1^{a} ±0.77	50.6^{a}±0.48	13.3^{d}±0.59	10.3^{c}±0.16	0.68^{c}±0.05	0.83^{b}±0.05	2.38^{b}±0.14	2.77^{c}±0.09	8.53^{d}±0.46	51.9 ±2.50
ST26	21.9^{c} ±1.61	27.5^{d}±0.52	21.0^{a}±0.82	10.3^{c}±0.48	0.95^{b}±0.04	0.98^{a}±0.01	3.28^{a}±0.20	2.88^{c}±0.20	17.1^{b}±1.37	52.5±1.86
ST27	42.1^{b} ±0.33	41.7^{b}±0.70	12.2^{c}±0.20	19.4^{a}±0.95	1.22^{a}±0.17	1.03^{a}±0.08	3.34^{a}±0.14	3.59^{a}±0.23	26.8^{a}±0.84	55.3±2.46
ST32	16.2^{d} ±1.10	37.6^{c}±0.96	19.4^{b}±0.08	15.8^{b}±0.46	0.93^{b}±0.08	0.81^{b}±0.04	3.24^{a}±0.13	3.18^{b}±0.13	13.8^{c}±1.03	53.7±1.78
F	*	*	*	*	*	*	*	*	*	ns

*Note: Different letters following numbers mean different statistically at $P < 0.05$ (*); PNSB: purple nonsulfur bacteria, ML: Microaerobic light; AD: Aerobic dark; IAA: indole-3-acetic acid; EPS: exopolymeric substances; ALA: 5-aminolevulinic acid.

Ca-P solubilization under both conditions, with 13.1 and 23.2 mg P L^{-1}, respectively (Table 1).

Identification of phosphorus-solubilizing purple non-sulfur bacteria that tolerated saline, acidic, and toxic conditions

The four ST16, ST26, ST27, and ST32 bacterial strains have been identified by the 16S rRNA genetic sequences to be identical to *Cereibacter sphaeroides* with a similarity of 100% (Fig 1). Sequentially, the accession numbers of the isolates were OQ829416, OQ829417, OQ829418, and OQ829419, respectively.

Nitrogen providing capacity

The identified ST16, ST26, ST27, and ST32 isolates were examined for the ability to fix N_2. The results were 16.2 – 104.1 mg L^{-1} under the microaerobic light condition, and 27.5 – 50.6 mg L^{-1} under aerobic dark condition. Therein, the ST16 isolate fixed N the most (Table 2).

Plant growth-promoting substances providing capacity

The content of IAA synthesized by the identified isolates fluctuated from 10.3.2 to 21.0 mg L^{-1}. Among the isolates, the ST26 produced the most IAA. Under the microaerobic light and the aerobic dark conditions, the ST27 isolate produced the highest amount of EPS (1.22, 1.03 mg L^{-1}). According to the content of ALA secreted from the four PNSB isolates, the ST27 isolate produced the highest content under both conditions, with respective values of 3.34 and 3.59 mg L^{-1}. Siderophores produced from the four PNSB isolates fluctuated from 8.53 to 55.3%. Among them, the highest siderophores content under the microaerobic light condition was at the ST26 isolate (26.8%). On the other hand, under the aerobic dark condition, the differences were insignificant among isolates (Table 2).

Discussion

Chemical physical properties of soil and water in the rice-shrimp system

Soil characteristics at sites of My Tu, My Xuyen, and Tran De had pH_{KCl} and pH_{H2O} ranging from 2.91 to 4.05, and from 2.90 to 4.56, respectively, which represents a highly acid sulfate condition (Supplementary Table 1). According to the classification of Horneck et al., (2011), these soils were considered as high to moderate acidity due to pH < 5.1. The NH_4^+ content and the total N content fluctuated from 0.19 to 0.36 mg kg^{-1}, and from 0.30 to 0.32 % N. For the total P, the result was from 0.02 to 0.03% and considered as P-deficient soil (< 0.06% P_2O_5) (Cu et al., 2000). However, the available P content was from 5.67 to 24.1 mg kg^{-1}, which, according to the classification of Horneck et al. (2011), was medium due to being in a range of 20 – 40 mg kg^{-1}. Nevertheless, the Fe-P, Al-P, and Ca-P content peaked at 46.4, 423.8, and 187.7 mg kg^{-1}, respectively. The CEC value fluctuated from 11.4 to 19.8 meq 100 g^{-1} and was classified as moderate according to Landon (1984). The total C content fluctuated from 1.56 to 3.35% C and was also categorized as medium according to Metson (1961). Soil and water EC values were in ranges of 0.17 – 4.73 and 1.01 – 5.78 mS cm^{-1}. From the above, the surface soil featured high-moderate acidity, salt contamination, nutrient deficiency, and an abundance of insoluble P compounds. At above 3.0 dS m^{-1} salinity, the survival rate of rice at sowing reduces by 20% and its yield decreases by 50% when the salinity reaches 7.4 dS/m (Grattan et al., 2002). N is one of the three essential macronutrients for rice plants (Shrestha et al., 2020) because it can raise rice yield by 30% – 50% (Erisman et al., 2008). Therefore, to produce a high yield, farmers get used to fertilizing lots of N fertilizer (Ye et al., 2021). However, only 20 – 30% N in fertilizer is absorbed by crops (Wang et al., 2022). PNSB play a role as a biofertilizer, due to the capabilities of producing NH_4^+ and plant growth-promoting substances, and increasing productivity by 86.8% under saline conditions (Khuong et al., 2022).

PNSB that provided nutrients and plant growth-promoting substances under saline condition

The four P-solubilizing PNSB ST16, ST26, ST27, and ST32 have been selected from the rice-shrimp system, and identified as *C. sphaeroides*. They were tolerant to salinity, and acidity, and able to provide nutrients, such as P, and plant growth-promoting substances. The PNSB *C. sphaeroides* W01, W14, W22, and W32, *Rhodopseudomonas palustris* TN114, and *Rhodobium marinum* NW16 and KMS24 were also found in saline condition or rice-shrimp system (Khuong et al., 2022, Nunkaew et al., 2015a). This indicated the abundance of the *C. sphaeroides* on saline soils (Holguin et al., 2001).

The PNSB have produced plant growth-promoting substances (Table 2). The result was consistent with previous studies, where PNSB isolates including *R. palustris* and *R. pentothenatexigens* strains are all able to provide ALA, EPS, IAA, and siderophores (Nunkaew et al., 2015a, b; Khuong et al., 2020a, 2020b, 2023), which assist plants to overcome environmental stresses, such as acidity, salinity, heavy metal and metal toxicity. The EPS produced by PNSB bonds to Na^+ to limit the effect of high NaCl concentration; Moreover, high salinity stimulates more and more EPS production (Khuong et al., 2022; Nunkaew et al., 2015b). ALA has been proven to be able to minimize damages caused by saline stress on rice plants (Nunkaew et al., 2015). This is because ALA modified photosynthesis and anti-oxidation under saline conditions by increasing total Chl content and activating antioxidant enzymes (Rhaman et al., 2021). IAA and siderophores produced by bacteria can be utilized to support rice plant development under acidic and saline conditions (Khuong et al., 2022). The PNSB can fix N in saline environments, under the microaerobic light and the aerobic dark conditions (Table 2). Therefore, when PNSB fix free N, available NH_4^+ for rice increases, which facilitates better nutrient uptake and yield of rice (Khuong et al., 2021, 2022). In particular, utilizing PNSB with 50% or 75% of the recommended amount of N fertilizers obtained an equivalent N uptake when applying 100% N as recommended (Khuong et al., 2021). Regarding the P solubilizing capacity of PNSB in a saline environment, Al-P, Fe-P, and Ca-P were solubilized with concentrations of 7.7 – 37.6 mg L^{-1}, 9.8 – 45.2 mg L^{-1}, and 5.9 –23.2 mg L^{-1} (Table 1). This has shown that the PNSB can solubilize insoluble forms of P, which contributes to increasing soluble P content to ameliorate the growth and yield of rice plants. Thus, the PNSB have played a role as biofertilizers that not only produce plant growth-promoting substances but also provide nutrients for plants (Khuong et al., 2020). Therefore, the main functions of these *C. sphaeroides* ST16, ST26, ST27, and ST32 strains were providing P for plants, as well as producing some plant growth-promoting substances to help plants tolerate saline stress.

Materials and Methods

Soil and water samples

Soil and water samples were collected during the rice cultivation of a rice-shrimp system in communes of My Tu [9°38'31.9"N 105°45'42.3"E], My Xuyen [9°26'16.3"N 105°54'29.5"E] and Tran De [9°30'55.1"N 106°12'16.6"E], Soc Trang province. Each soil and water samples were collected at 7 different sites in the same location, and mixed into one representative sample for each soil and water. Particularly, 100 g sample^{-1} of soil was collected at a depth of 0-20 cm, and 100 mL sample^{-1} of water was collected from the bottom to 3 cm from the water surface of the ditch surrounding the paddy field. The samples were stored cold until experiments.

Chemical physical features of soil and water samples from the rice-shrimp system

The soil and water samples were analyzed according to Sparks et al. (1996). pH_{H2O} and pH_{KCl} were extracted with a ratio of soil: water (1: 5) and soil:1.0 M KCl (1: 5), and measured by a pH meter. The extract was reused to measure EC by an EC meter.

Total nitrogen (N_{tot}) was digested by a mixture of saturated H_2SO_4: $CuSO_4$: Se (100: 10: 1) and determined by the Kjeldahl distilling method. Available nitrogen (NH_4^+) was determined by the blue phenol method at the 640 nm wavelength.

Total phosphorus (P_{tot}) was turned into inorganic forms by a mixture of saturated H_2SO_4 -$HClO_4$, and measured at the 880 nm wavelength. Soluble P ($P_{soluble}$) was determined by the Bray II method, where soil was extracted with 0.1 N HCl, 0.03 N NH_4F (1: 7), colorized with phosphomolybdate reduced by acid ascorbic, and measured by a spectrometer. Insoluble P ($P_{insoluble}$) fractions were extracted with 0.1 M NaOH, 0.5 M NH_4F, and 0.25 M H_2SO_4 respectively for Fe-P, Al-P, and Ca-P, and then determined as the measurement of soluble P.

Titratable acidity in soil was determined according to the soil extraction method with 1.0 M KCl and titrated with 0.01 N NaOH. To determine the exchangeable aluminum, soil was extracted with 1.0 M KCl, and measured by a spectrometer at the 395 nm wavelength. Fe^{2+} was extracted with H_4EDTA and $Na_2S_2O_4$, measured by a spectrometer at the 248.3 nm wavelength. Organic matter was extracted according to the method of Walkley-Black, where the soil was oxidized by saturated H_2SO_4 - $K_2Cr_2O_7$ before being titrated with 0.5 N $FeSO_4$.

Cation exchange capacity (CEC) was extracted with 0.025 M $BaCl_2$ and 0.02 M $MgSO_4$ and titrated with 0.01 M EDTA. K^+, Na^+, $Ca^{2+,}$ and Mg^{2+} contents in the CEC extract were measured by an atomic absorption spectrometer at the 766, 589, 422.7, and 285.2 nm wavelengths, respectively.

Isolation, selection, and identification of PNSB from soil and water samples of rice-shrimp paddy fields

A total of 21 soil samples and 21 water samples collected from rice fields in the rice-shrimp system were utilized to isolate PNSB according to the method of Brown (2013) adjusted by Khuong et al. (2017). The basic isolation medium (BIM) was utilized for the isolation and made of 1.0 g $(NH_4)_2SO_4$, 0.5 g K_2HPO_4, 0.2 g $MgSO_4$, 2.0 g NaCl, 5.0 g $NaHCO_3$, 1.5 g yeast extract, 1.5 g glycerol and 0.03 g L-cysteine in 1 L of distilled water. The density of BIM was doubled when isolating PNSB in the water samples because the population of PNSB there was low (Kantachote et al., 2005). However, to adjust the isolation medium similar to the nature of the sampling sites, the pH of the liquid medium was adjusted to 5.0 by 1.0 M HCl (sterilized by filter papers 0.45 µm). A solid medium was prepared as the liquid one, but 1.5% agar was added and pH was adjusted to 5.0 after autoclaved.

*Selection for PNSB that lived under microaerobic light and aerobic conditions:*10 % extract of each isolate with an OD_{660} = 0.5 was transferred into a tube (15 x 150 mm) of 9.0 mL of BIM (pH = 7.0) and a flask of 18 mL of BIM (pH = 7.0) capped by a rubber plug and aluminum foil. Three replications were applied. The solution was incubated for 72 h under the microaerobic light condition with light intensity at 3,000 lux, in the aerobic dark condition at 30 °C, and shaken at 150 rounds min^{-1}. Subsequently, the bacterial growth was determined by a spectrometer at the 660 nm wavelength. Isolates whose OD_{660} was above 0.5 under both conditions were chosen for the following experiment.

Selection for PNSB that lived under acidic conditions: The experiment testing bacterial isolates' growth under acidic conditions was conducted similarly under the microaerobic light and aerobic dark conditions for 48h, but the pH of BIM was 5.0. Isolates whose OD_{660} was above 0.5 under one of the two conditions were selected to be evaluated for salt tolerance capacity.

Selection for PNSB that lived under saline conditions: All acid-tolerant bacterial isolates (pH 5.0) were cultured and screened on BIM (pH = 5.0) containing NaCl solution at 5 ‰ for 72h or 10 ‰ for 96h. The selection was similar to the above.

Selection for purple non-sulfur bacteria that tolerated Al^{3+}, $Fe^{2+,}$ and Mn^{2+} toxicity: The selected PNSB were cultured in BIM which contained 10 ‰ NaCl (pH = 5.0) and was supplied with Al^{3+}, Fe^{2+} and Mn^{2+} at the concentrations based on the threshold of toxicity for plants (70 mg Al^{3+} kg^{-1}, 250 mg Fe^{2+} kg^{-1} and 1,500 mg Mn^{2+} kg^{-1}) (Attanandana and Vacharotayan, 1986; Samaranayake et al., 2012; Upjohn et al., 2005). The Al^{3+}, $Fe^{2+,}$ and Mn^{2+} supplements were derived from $AlCl_3 \bullet 6H_2O$, $FeSO_4 \bullet 7H_2O$, and $MnCl_2 \bullet 4H_2O$ which were sterilized by 0.45 µm filters). After 4 days of incubation, growth-inhibiting rates of the bacteria were calculated based on development levels between bacteria cultured in BIM with Al^{3+}, Fe^{2+}, and Mn^{2+} and the control ones cultured in BIM without heavy metals (pH = 5.0).

Selection PNSB that solubilize phosphorus: Na^+, Al^{3+}, Fe^{2+}, and Mn^{2+}-tolerant bacterial isolates were utilized to assess the P solubilization. The PNSB were cultured in a P-free BIM (K_2HPO_4) containing 10 ‰ NaCl (pH = 5.0). Insoluble P compounds were added separately with 0.3 g $AlPO_4 \bullet 2H_2O$ L^{-1} or 1.0 g $FePO_4 \bullet 2H_2O$ L^{-1} or 0.5 g $Ca_3(PO_4)_2$ L^{-1}. The ratio between the bacteria and the medium was 1:9 with a total volume of 10 mL under the microaerobic light condition (3,000 lux) and at 20 mL under the aerobic dark condition (30 °C and 150 rpm). The BIM with P compounds was utilized as a negative control. After 72 h of incubation, the dissolved amounts of P were determined according to the ascorbic acid method (Murphy and Riley 1962). The P analysis was summarized as follows: the culture broth was centrifuged at 10,000 rpm in 15 min, from which 1.0 mL of bacterial extract was collected, colorized by ascorbic acid, and measured on a spectrometer at the 880 nm wavelength.

The capacity of PNSB to produce plant growth-promoting substances

All bacterial isolates were cultured in a BIM containing 10,000 mg L^{-1} NaCl (pH = 5.0) under the microaerobic light condition for 72 h.

***Nitrogen-fixing capacity*:** The bacteria were cultured under the condition of the P-solubilization experiment, but the BIM was N-free [$(NH_4)_2SO_4$]. After 72 h of incubation, the solution was centrifuged at 10,000 rpm for 15 min. The available nitrogen produced was determined by the blue phenol method (Nelson et al., 1983) on a spectrometer at the 640 nm wavelength. The N-free BIM without bacteria was utilized as the negative control.

***Exopolymeric substances producing capacity*:** The selected isolates were cultured under microaerobic light and aerobic dark conditions for 72 h, in 1,000 mL of BIM containing 10,000 mg L^{-1} NaCl (pH = 5.0). The culture was then centrifuged at 8,000 rpm for 15 min to collect cells and extract them. The extract was utilized to analyze EPS. The procedure for analyzing EPS was modified from the method of Eboigbodin and Biggs (2008). The extract and cold ethanol (4 $^{\circ}C$) were mixed with a ratio of 1:2.2 in volume, and incubated at -20 °C in 24 h to precipitate EPS. Subsequently, the solution was centrifuged at 8,000 rpm for 15 min at 4 °C to collect EPS. The dry weight of EPS was determined by the method of Ferreira et al. (2017).

***5-aminolevulinic acid producing capacity*:** A BIM containing 10,000 mg L^{-1} of NaCl (pH = 5.0) and ALA precursors, including glycine (0.563 g L^{-1}) and sodium acetate (5.44 g L^{-1}), was utilized to determine ALA after 4 days of incubation according to the method of Burnham (1970). In particular, 2 mL of sodium acetate (1.0 M, pH 4.7) and 0.05 mL of acetylacetone were added into a tube containing 1 mL of the centrifuged bacterial solution. The mixture was heated for 15 min at 99 °C and cooled. Then, 3.5 mL of reagents modified according to Ehrlich was added. After 20 min, the solution was measured on a spectrometer at the 553 nm wavelength. The modified reagents were prepared at every time of measurement (no prior preparation) by adding 1 g of p-dimethylaminobenzaldehyde to 30 mL of glacial acetic acid, then supplied with 8 mL of 70% perchloric acid, and finally adjusting to a volume of 50 mL with glacial acetic acid.

Indole 3-acetic acid producing capacity: A BIM containing 10,000 mg L^{-1} of NaCl (pH = 5.0) was utilized, and added with tryptophan (100 mg L^{-1}) as a precursor of IAA. After 72 h of incubation, IAA content was analyzed according to the colorimetric method of Salkowski at the wavelength of 535 nm.

***Siderophores producing capacity*:** A BIM was modified by adding precursors of siderophores, including 1 g L^{-1} of succinate and 0,5 µM of $FeCl_3 \bullet 6H_2O$ glycine. After 96 of incubation, 2.0 mL of culture was centrifuged at 10,000 rpm in 5 min, then 0.5 mL of the centrifuge solution was mixed with 0.5 mL of reagents, and measured by a spectrometer at the wavelength of 630 nm. The reagents consisted of 6 mL of HDTMA 10 mM, 1.5 mL of $FeCl_3$ 1 mM (in HCl 10 mM), and 7.5 mL of CAS 2 mM. Subsequently, the mixture was added with 4.307 g anhydrous piperazine and adjusted to pH = 5.6. The mixture was added with distilled water, added with exact 0.1017 g 5-sulfosalicylic acid, well shaken, and added with distilled water until the volume of 100 mL.

Identification of purple non-sulfur bacteria that lived under saline, acidic, and toxic conditions

The four selected bacterial isolates were cultured in 48 h in BIM. After that, 2 mL of colonies were collected and centrifuged at 10,000 rpm in 5 min, to obtain cells for DNA extraction by the Genomic DNA Prep Kit (BioFACT™) following the manufacturer's instructions. The DNA products were amplified at their 16S rRNA coding regions by the PCR technique with the forward primer 8 F (5'-AGA GTT TGA TCC TGG CTC AG-3') and the reverse one 1492 R (5'- GGT TAC CTT GTT ACG ACT T-3') (Suzuki et al., 2003) as described in iProof™ High-Fidelity PCR Kit – Bio-Rad (BioRad, Hercules, CA) in T100^{TM} hermos cycler (BioRad). The PCR amplicons were purified by the TIANquick Midi Purification Kit (Tiangen Biotech Ltd. Beijing, China) following the manufacturer's instructions. The PCR amplicons were sequenced by an automatic sequencing machine at Macrogen DNA Sequencing Service (Macrogen, Seoul, Korea). The sequencing result was analyzed by the BioEdit software, version 7.0.5.3 (Hall, 1999) and the ChromasPro software version 1.7 (http://technelysium.com.au/wp/chromaspro). The sequences of the four selected bacteria isolates were compared to available sequences in Genbank by the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) to determine for similarity.

Statistical analysis

Means were tested by analysis of variance (ANOVA) to compare differences by Duncan's post hoc test at $p < 0.05$ in the SPSS 13.0 software.

Conclusions

The four purple non-sulfur bacteria isolates were able to solubilize well insoluble P forms presenting in saline acidic soil with solubilized P amount of 32.7 – 60.8 mg L^{-1} from Al-P, 30.6 – 81.7 mg L^{-1} from Fe-P, and 22.8 – 36.3 mg L^{-1} from Ca-P under the microaerobic light and the aerobic conditions. These P-solubilizing bacterial isolates were identified as *Cereibacter sphaeroides*, ST16, ST26, ST27, and ST32 with 100% similarity. These isolates were potent in providing N and plant growth-promoting substances, such as 5-aminolevulinic acid, exopolymeric substances, indole-3-acetic acid, and siderophores.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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