Malaysian Journal of Microbiology, Vol 18(1) 2022, pp. 17-26
DOI: http://dx.doi.org/10.21161/mjm.211297

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

In vitro screening of siderophore-producing rice root endophytic bacteria from up-land paddies in north-western Vietnam for plant growth-promoting activities

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Received 15 September 2021; Received in revised form 27 September 2021; Accepted 26 November 2021

ABSTRACT

Aims: Endophytic bacteria (EB) living inside plant tissues possess different beneficial traits including siderophore production and other plant growth-promoting (PGP) activities. Siderophore-producing EB promote host plant growth by secreting ferrum in iron-deficient conditions. This study screened 19 siderophore producers *in vitro*, isolated from upland rice roots grown in mountain farms of Tung Village, Nậm Có Commune, Mù Cang Chải District, Yên Bái Province, Vietnam, for PGP traits, including phosphate solubilisation, indole-3-acetic acid (IAA), ammonia, gelatinase, amylase and catalase production.
Methodology and results: The bacteria were identified by Matrix assisted Laser Desorption Ionization Time of Flight mass spectrometry (MALDI-TOF MS). All 19 isolates were identified as genera *Pseudomonas*, *Enterobacter*, *Pantoe*, *Bacillus*, *Burkholderia*, *Staphylococcus*, *Ralstonia* and *Cronotacter*. The isolates produced catalase and ammonia. The amount of ammonia ranged from 60.74 ± 0.14 to 466.72 ± 0.18 mg/L. Out of the 19 siderophore producers, 17 (89.47%) were able to solubilise phosphate with solubilisation index (PSI) ranging from 1.12 ± 0.07 to 2.14 ± 0.15. The qualitative assays identified 12 isolates (63.15%) positive for IAA production with a tryptophan concentration of 5 mM, whereas 15 (78.94%) and 17 (89.47%) isolates were positive for gelatin and starch hydrolysis, respectively. Especially, 7 isolates were found to be positive for all tested assays *in vitro* including *Pseudomonas rhodesiae* (NC2), *Enterobacter asburiae* (NC50), *Pantoea ananatis* (NC63), *Bacillus cereus* (NC64), *Burkholderia cenocepacia* (NC110), *Staphylococcus sciuri* (NC112) and *Ralstonia pickettii* (NC122).
Conclusion, significance and impact of study: This study serves as crucial findings of multi-trait plant growth-promoting endophytic bacteria isolated from upland rice root in north-western Vietnam. The seven potential isolates positive for all tested assays could be effective PGP bacteria for bio-inoculants.

Keywords: Rice root endophytic bacteria, siderophore production, plant growth-promoting traits, upland rice, MALDI-TOF MS

INTRODUCTION

Rice (*Oryza sativa*) is among the most economically important crops in many countries including Vietnam (Khanh *et al.*, 2021). However, production of rice in Vietnam and other countries is threatened by pests and diseases causing huge losses (Khanh *et al.*, 2021). In modern society, farmers usually use agrochemicals such as chemical fertilisers, herbicides and pesticides to gain higher productivity and prevent pathogens and weeds (Elahi *et al.*, 2019). Applying agrochemicals in paddies is considered an easy solution to control diseases and maintain high yield; however, the overuse of these chemicals might cause tremendous impact on environment, public health and non-target organisms due to the high levels of toxic residues (Ali *et al.*, 2017). At the same time, prolonged agrochemical exposure of paddies also reduces their effectiveness and might increase the number of insecticide-resistant strains (Fitri *et al.*, 2020). In Vietnam, during the period of 1995-2018, the positive correlations between the amount of rice production and the use of pesticides indicated the intractable routines of farmers in using of pesticides for rice production. Moreover, the improper use of pesticides by farmers (e.g., too high dosages, cocktailing of pesticides, inadequate pre-harvest intervals etc.) leads to the heavy dependence

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ISSN (print): 1823-8262, ISSN (online): 2231-7538

of pesticides in rice production, especially in poorer areas where farmers have to largely rely on cheap but often with old and more toxic pesticides. This problem need to be urgently addressed because it causes significant adverse impacts on food safety, soil, water environment and human health (Pham *et al.*, 2013; Thanh and Tran, 2020). Thus, alternative solutions are being developed to alleviate the dependence of agrochemicals. Beneficial endophytic bacteria (EB) have been studied as an alternative measure to improve crop yield, grain quality and to prevent pathogens; at the same time, the use of endophytes does not cause serious damage to the crops and ecosystem (Moronta-Barrios *et al.*, 2018; Fitri *et al.*, 2020).

EB colonise internal plant tissues without causing infection or damage (Schulz and Boyle, 2006). EB are found in many plant crops including rice (Sen and Chandrasekhar, 2014; Shen *et al.*, 2019; Singh *et al.*, 2019) and their relationships with host plants have been widely studied (Reinhold-Hurek and Hurek, 2011). These interactions have been shown to benefit the hosts, namely, promoting growth via phytohormone biosynthesis, nitrogen fixation, nutrient solubilisation and phytopathogen suppression through antibiotic production and/or cell wall-degrading enzymes like matrix metalloproteinases (Inoue *et al.*, 2007; Olanrewaju *et al.*, 2017). These beneficial properties make EB promising candidates for biocontrol agents (Olanrewaju *et al.*, 2017).

Iron is a micronutrient needed for the development of plants and microorganisms. However, it is scarce in neutral to alkaline soils, which makes plants iron deficient (Rout and Sahoo, 2015). Siderophores are chelating agents with low molecular weights (200-2,000 Da) and high Fe3+ chelating affinity for facilitating iron uptake (Singh *et al.*, 2019). Plants and other microorganisms benefit from siderophore-producing EB that can solubilise and chelate iron from insoluble organic and inorganic sources in the soil (Singh *et al.*, 2019). Siderophore-producing bacterial genera include *Pseudomonas*, *Azotobacter*, *Bacillus*, *Rhizobium*, *Agrobacterium*, *Staphylococcus*, *Burkholderia*, *Erwinia*, *Serratia*, *Alcaligenes* and *Enterobacter* (Sayyed *et al.*, 2012; Gupta *et al.*, 2015). Thus, siderophores not only promote plant growth but also aid in plant defence against phytopathogens that are inhibited by iron starvation or competitive exclusion. Furthermore, EB have been widely known for their antagonistic activities such as competing for substrates or producing inhibitory compounds. The latter is critical for inducing systemic resistance in plants (Gupta *et al.*, 2015).

Phosphorus is one of the essential macronutrients required for plant growth and development (Dong *et al.*, 2019). Phosphorus normally exists in forms of organic and inorganic phosphates, which are either insoluble or very poorly soluble. The total available phosphates for plants in soil are very low since most occur as metal phosphates with iron, calcium and aluminium, which cannot be taken up by plants (Kirkby and Johnston, 2008). Therefore, phosphorus fertilisers are usually applied to croplands (Walia *et al.*, 2017). Different microorganisms, dominated by bacteria, can produce soluble phosphorus via acidification, chelation and exchange reactions. *Acinetobacter*, *Azotobacter*, *Enterobacter*, *Bacillus*, *Burkholderia*, *Paenibacillus*, *Pseudomonas*, *Rhizobium*, *Mesorhizobium*, *Flavobacterium*, *Klebsiella*, *Erwinia* and *Micrococcus* have been reported as efficient phosphate solubilisers (Villegas and Fortin, 2002; Paul and Sinha, 2013).

Indole-3-acetic acid (IAA), a plant growth hormone, functions as an important signalling molecule in regulating plant development (Mockaitis and Estelle, 2008). Different factors affect the IAA production from plant growth-promoting (PGP) rhizobacteria, such as types of species and strains, culture conditions, growth stage and substrate sources (Tan *et al.*, 2014). The inoculation of IAA-producing EB could be considered a promising way to enhance plant biomass, root length, root tip number and root surface area (Chen *et al.*, 2017).

Gelatine is a collagen-derived protein that comes from the connective tissues of vertebrates. The presence of gelatinases is detected by gelatine hydrolysis and some bacteria can produce extracellular gelatinases (Zhang *et al.*, 2015). These enzymes break down gelatine into polypeptides, which are then converted into amino acids that benefit bacteria in their metabolic processes (Inoue *et al.*, 2007). It has also been reported that the gelatinase B, a matrix metalloprotease, can rapidly digest extracellular matrix produced by *Magnaporthe oryzae*, a fungus causing blight disease of wheat. The production of gelatinase and other matrix metalloproteases by EB is greatly beneficial to the plant host in response to pathogenic fungi.

EB hydrolyse starch into simpler sugars through amylase production for energy and carbon capture. Thus, EB compete against other microorganisms for nutrients on the same host. Catalase is also known as an oxygen-scavenging enzyme that prevents cells from the negative impacts of hydrogen peroxide, and in certain situations during growth, highly toxic superoxide is converted into water and oxygen (Cappuccino and Welsh, 2017). Studies showed that catalase played a key role in reducing the detrimental effects of reactive oxygen species in plant cells under both abiotic and biotic stresses (Choodamani *et al.*, 2009; Sofo *et al.*, 2015).

In this study, we performed qualitative and quantitative *in vitro* screening for multi-trait plant growth promoting effects of 19 siderophore-producing EB from upland rice roots grown in Tung Village, Nậm Có Commune, Mù Cang Chải District, Yên Bái, Vietnam. This study of EB isolated from the roots of indigenous upland rice in north-western Vietnam will provide preliminary results for further investigation *in planta* to identify potential strains for developing bio-inoculants for rice farming in Vietnam in order to replace agrochemicals which are uncontrolled using.

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(1) 2022, pp. 17-26
DOI: http://dx.doi.org/10.21161/mjm.211297

MATERIALS AND METHODS

Bacterial strains

A collection of 19 best siderophore-producing endophytic bacteria isolated from upland rice roots of tillering stage grown in Tung Village, Nậm Có Commune, Mù Cang Chải District, Yên Bái Province, Vietnam (21°51'50.01" N; 104°16'25.38" E) with an elevation of more than 1,500 m was selected for all experiments of this study. These isolates showed the halo zone greater than 30 mm in diameter on the chrome azurol S medium (Ta *et al.*, 2020). The bacterial strain *Xanthomonas oryzae* pv. *oryzae* VX041, provided by the Agricultural Genetics Institute, Vietnam Academy of Agricultural Sciences (VAAS) was used as a negative control for starch hydrolysis assays.

Bacterial identification by MALDI-TOF mass spectrometry

The bacterial isolates were identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Briefly, a full loop of pure colonies was suspended in 300 µL ethanol 70%, then the cells were harvested by centrifugation at 10,000 rpm for 5 min. Cell pellets were incubated with a mix containing 50 µL of 70% formic acid and 50 µL of 100% acetonitrile for 3 min. After that, the cell suspension was centrifuged at 10,000 rpm for 5 min and then 1 µL of the clear supernatant is transferred onto 96-spot MALDI target plate and air-dried at room temperature. Before processing in Bruker MALDI-TOF MS, each spot was overlaid with 1 µL MALDI matrix and dehydrate for 15 min. The samples were then processed on Microflex MALDI-TOF MS system (Brucker, Germany). The protein spectrums obtained were analyzed by BioTyper 3.0 system software along with the searching on CDC database for bacterial identification. A log (score) of MALDI Biotypes which above 2.0 illustrates a valid identification while the score under 1.7 shows poor performance and represent for uncertain identification.

In vitro screening for plant growth-promoting traits

Qualitative assay of phosphate solubilisation

Siderophore producers were screened for their phosphate-solubilising activity on agar plate medium of National Botanical Research Institute's Phosphate (NBRIP) (Nautiyal, 1999) containing 5 g/L of insoluble tricalcium phosphate (Merk, Germany). A total of 5 µL of bacterial suspension (OD_{600} = 1) was spot-inoculated at the centre of agar plates aseptically. The plates were then incubated at 28 °C ± 2 °C for 5 days. A clear halo zone around the bacterial spot indicated phosphate solubilisation by the isolate. The phosphate solubilisation index (PSI) was estimated using the following formula (Edi-Premono *et al.*, 1996):

Phosphate Solubilisation Index (PSI)
= [Colony diameter + halo zone diameter (mm)]/Colony diameter (mm).

Indole-3-acetic acid production

The qualitative test for the ability of EB to produce IAA was implemented as described in a previous study by Nguyen *et al.* (2020). Briefly, 20 µL of the 24 h-old bacterial suspension (OD_{600} = 1) of each isolate was dropped at the middle of the agar plate containing 1/6 tryptic soy agar (TSA) medium (Merck KGaA, 64271 Damstadt, Germany) supplemented with 5 mM of L-tryptophan (Sigma-Aldrich, Germany). A sterile filter paper disc (diameter, 20 mm) was placed onto the bacterial drop to obtain a clear image of the positive result. The test plates were incubated at 28 °C ± 2 °C for 3 days. Then, the paper disc was harvested and stained with 100 µL of Salkowski reagent (50 mL of perchloric acid (35%) and 1 mL of $FeCl_3$ solution (0.5 M) for 30 min in the dark. The progression of pink to reddish colour on paper discs indicated a positive result for IAA production. The experiment was performed based on completely randomised design with three replications.

Ammonia production

Ammonia production was quantified by the nesslerisation reaction (Cappuccino and Welsh, 2017) with some modifications as follows. Briefly, isolates were initially grown in 1/6 tryptic soy broth (TSB) medium (Merck KGaA, 64271 Damstadt, Germany) at 160 rpm and 28 °C ± 2 °C for 24 h. Then, each isolate was subcultured in a 50 mL falcon tube containing 20 mL of 1/6 TSB for 3 days at the same conditions as mentioned above. The bacterial supernatant was collected using a centrifuge at 5,000 rpm for 10 min. Subsequently, 50 µL of Nessler's reagent [10% HgI_2, 7% KI, 50% aqueous solution of NaOH (32%)] was gently mixed with 1 mL of bacterial supernatant. Absorbance of the assay solution was determined at 430 nm with a UV-1800 UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan). The amount of ammonia was estimated using a standard curve with its linear regression line. The standard curve was generated using an NH_4Cl stock solution with concentrations of 0.1, 0.5, 1, 5, 10, 20, 40, 60, 80 and 100 mg/L. The experiment was repeated three times.

In vitro qualitative assays for hydrolytic enzyme production

Gelatinase production

Gelatinase production assays were carried out using the nutrient gelatine stab method (Cappuccino and Welsh, 2017) with some modifications. Briefly, a colony of 24 h-old EB was heavily stab-inoculated into cylinder glass tubes containing 4 mL of nutrient gelatine medium (peptone, 5 g/L; beef extract, 3 g/L; gelatine, 120 g/L).

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(1) 2022, pp. 17-26
DOI: http://dx.doi.org/10.21161/mjm.211297

This high gelatine concentration resulted in a solid medium and served as the substrate for gelatinase action. The inoculated tubes and an uninoculated control tube were incubated at 28 °C ± 2 °C for 7 days. Gelatine started to liquefy at 28 °C. The test tubes were then immersed in an ice bath for 30 min to assess the liquefaction in response to gelatinase activity. Afterward, the test tubes were kept in an inclined position to check the amount of degraded gelatine. The gelatine hydrolysis levels were qualitatively identified based on the amount of liquefied gelatine in the tube as follows: no gelatine hydrolysis, the medium was in a completely solid state as with the control tube; low, <1/3; moderate, 1/3-1/2; high, 1/2-2/3; very high, completely liquefied. The experiment was performed with a completely randomised design with three replications.

Amylase production

The assay for amylase production was adapted from Cappuccino and Welsh (2017). The experimental procedure was performed as follows: the EB isolates were streaked on the medium plates containing TSB (5 g/L), starch (20 g/L) and agar (15 g/L) (Sigma-Aldrich). After 24 h of incubation at 28 °C ± 2 °C, the test plates were completely filled with Lugol solution containing potassium iodide (KI) 1% (w/v) and iodine (I_2) 0.5% (w/v) (Sigma-Aldrich) for 10 min. Afterward, the Lugol solution was discarded and the plates were air-dried under a hood. To assess the presence of starch in the medium, the test plates were illuminated. With iodine, starch formed a dark-blue colour, suggesting lack of amylase and indicating a negative result for starch hydrolysis. A clear zone around colonies indicated the hydrolysis of starch and demonstrated a positive result. The strain *X. oryzae* pv. *oryzae* VX41 was used as the negative control. The experiment was performed with a completely randomised design with three replications.

Catalase production

The ability to produce catalase was screened following the plate method as described by Cappuccino and Welsh (2017). Bacterial isolates were streaked on the plates of 1/6 TSA medium. After 24 h of incubation at 28 °C ± 2 °C, one drop of 3% hydrogen peroxide (H_2O_2) solution was added to the tested bacterial colonies. The appearance of bubbles indicated a positive result for catalase production. The experiment was performed with a completely randomised design with three replications.

Statistical analysis

The difference between different parameters related to PSI and ammonia was statistically analysed using a one-way analysis of variance and the Student's t-test with R software v3.6 at a *p*-value of 0.05.

RESULTS AND DISCUSSION

Bacterial identification by MALDI-TOF MS

All 19 siderophore-producing EB isolates were identified by Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). All 19 isolates were successfully identified with the score value from 1.87 to 2.38. Only one isolate NC142 had score value below 2.0 and was identified as *Cronobacter* sp. Another 18 isolates showed score value more than 2.0 and belonged to two phyla Firmicutes (6 isolates) and Proteobacteria (12 isolates) (Table 1). Among 19 identified isolates, the taxonomic composition indicated a predominance of *Burkholderia* genus (7 isolates), followed by *Staphylococcus* (3 isolates), *Bacillus* (3 isolates), *Enterobacter* (2 isolates) and *Pseudomonas*, *Pantoea*, *Ralstonia* and *Cronobacter* with one isolate of each.

Table 1: Endophytic bacterial identification based on MALDI-TOF MS.

No	Strain code	Species (best match)	Score value
1	NC2	*Pseudomonas rhodesiae*	2.20
2	NC4	*Enterobacter asburiae*	2.20
3	NC50	*Enterobacter asburiae*	2.25
4	NC63	*Pantoea ananatis*	2.19
5	NC64	*Bacillus cereus*	2.15
6	NC83	*Burkholderia cepacia*	2.26
7	NC88	*Bacillus cereus*	2.15
8	NC89	*Bacillus cereus*	2.16
9	NC110	*Burkholderia cenocepacia*	2.25
10	NC112	*Staphylococcus sciuri*	2.17
11	NC113	*Burkholderia cenocepacia*	2.23
12	NC120	*Staphylococcus sciuri*	2.01
13	NC121	*Staphylococcus sciuri*	2.02
14	NC122	*Ralstonia pickettii*	2.30
15	NC124	*Burkholderia cepacia*	2.27
16	NC125	*Burkholderia cepacia*	2.38
17	NC140	*Burkholderia vietnamiensis*	2.05
18	NC141	*Burkholderia cenocepacia*	2.20
19	NC142	*Cronobacter* sp.	1.87

Characterisation of siderophore-producing endophytic bacteria

Qualitative measurement of phosphate solubilisation

Of 19 isolates, 17 (89.47%) were able to solubilise tricalcium phosphate with PSI ranging from 1.12 ± 0.07 to 2.14 ± 0.15. The maximum PSI was observed in isolate NC83 (*Burkholderia cepacia*) (Figure 1, Tables 1 and 2). It has been reported that the clear zone surrounding bacterial colonies can be due to the production of different compounds, including organic acids, polysaccharides, and phosphatase enzymes from phosphate-solubilising bacteria (Pande *et al.*, 2017). Interestingly, *B. cepacia*

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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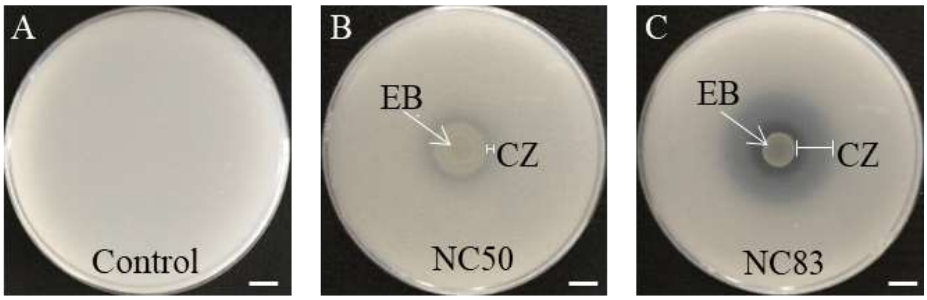

Figure 1: Qualitative assay for P-solubilisation of siderophore-producing endophytic bacteria. A, Negative control, sterile distilled water was spotted instead of bacteria. B and C, P-solubilisation by the isolates NC50 and NC83 on the NBRIP medium plates, respectively. The clear zone (CZ) around bacterial colonies (EB) were illustrated. Bar represents 0.5 cm.

Table 2: Plant growth promoting traits of 19 best siderophore-producing endophytic bacteria.

EB isolates	Plant growth promoting traits			
	Siderophore production	PSI	IAA production	Ammonia production (mg/L)
NC2	+++	1.45 ± 0.06	+	60.74 ± 0.14
NC4	+++	1.31 ± 0.14	++	131.75 ± 0.90
NC50	+++	1.12 ± 0.07	+++	298.68 ± 0.18
NC63	+++	1.47 ± 0.12	+++	393.06 ± 2.35
NC64	+++	1.47 ± 0.09	++	255.78 ± 0.30
NC83	+++	2.14 ± 0.15	++	395.42 ± 0.18
NC88	+++	1.7 ± 0.05	-	412.88 ± 0.46
NC89	+++	1.67 ± 0.10	-	398.08 ± 0.30
NC110	+++	1.47 ± 0.08	+	453.11 ± 0.18
NC112	+++	1.61 ± 0.06	+	435.07 ± 0.18
NC113	+++	1.61 ± 0.06	+	466.72 ± 0.18
NC120	+++	1.58 ± 0.03	-	396.61 ± 0.30
NC121	+++	0	-	170.87 ± 0.18
NC122	+++	1.61 ± 0.05	+	404.89 ± 0.18
NC124	+++	1.62 ± 0.07	-	430.04 ± 0.30
NC125	+++	1.62 ± 0.06	-	415.84 ± 0.18
NC140	+++	0	+++	200.16 ± 0.18
NC141	+++	1.38 ± 0.02	+	383.00 ± 0.18
NC142	+++	1.25 ± 0.03	-	356.66 ± 0.62
Total positive (Nos)		17	12	19
% Positive		89.47	63.15	100

*The signs -, +, ++ and +++ indicate no IAA production, low, moderate and high level of IAA production (based on intensity of pink color), respectively; IAA: Indole-3-acetic acid, PSI: Phosphate solubilisation index, Nos: Number of strains.

also had *in planta* phosphate-solubilising activity in *Lycopodium cernuum* L. (Ghosh *et al.*, 2016) and *Zea mays* (Zhao *et al.*, 2014). *Burkholderia gladioli*, another strain of *Burkholderia*, solubilised phosphate and enhanced plant growth in *Elaeis guineensis* and *Stevia rebaudiana* (Mamta *et al.*, 2010; Istina *et al.*, 2015). Moreover, in rice, when rice plants were inoculated with *Burkholderia* species, the plant biomass increased by 69% (Divan Baldani *et al.*, 2000). Van and Cao (2014) have reported that the strains *B. kukuriensis* and *B. vietnamiensis* isolated from rice in Phu Yen Province, Vietnam, possessed multiple traits of PGP including phosphorus solubilisation. However, the PSI observed in this study was lower than that in previous studies (Afzal *et al.*, 2017; Pande *et al.*, 2017). Nonetheless, these isolates could be further characterised along with the other PGP traits to identify promising candidates as inoculants for phosphorus-poor soils to maintain high yield in the highland areas of north-western Vietnam (Table 2).

Qualitative assay for IAA production

Of 19 isolates, 12 (63.15%) showed a pink colour with different intensities on the paper discs after staining with Salkowski reagent (Figure 2, Table 2). Based on the intensity of colourisation, IAA-producing isolates were qualitatively categorised into four groups: no production (7 isolates), low production (6 isolates), moderate production

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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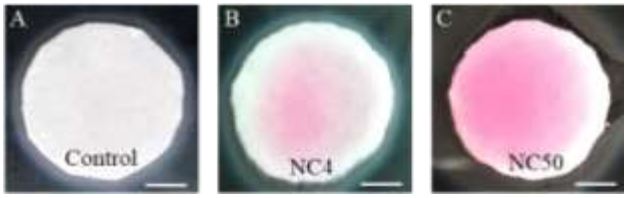

Figure 2: Qualitative assay for IAA production of siderophore-producing endophytic bacteria. A, Negative control, sterile filter paper disc on uninoculated plate was stained with Salkowski reagents following the same manner as for the tested plates. B and C, the pink color on the filter paper discs indicates the presence of IAA produced by corresponding isolates after staining with Salkowski reagent for 30 min in the dark. The intensity of the pink color is considered as the level of IAA production. Bar represents 0.5 cm.

(3 isolates) and high production (3 isolates) (Table 2). Notably, three isolates had the highest levels of IAA production including *Enterobacter asburiae* (NC50), *Pantoea ananatis* (NC63) and *Burkholderia cenocepacia* (NC140) (Table 2). The *Enterobacter* genera were also found to produce a high concentration of IAA in rice plants in Udon Thani, Thailand (Phetcharat and Duangpaeng, 2012). The IAA production levels for the *Pantoea* genera in our study were consistent with those in a previous study (Apine and Jadhav, 2011). Interestingly, the *P. ananatis* isolated from green onion can promote the growth of pepper (Kim *et al.*, 2012). These reported findings support our results, although further quantification is required to quantify the amount of IAA produced by these isolates.

Ammonia production

All 19 isolates produced ammonia. The ammonia concentration ranged from 60.74 ± 0.14 mg/L to 466.72 ± 0.18 mg/L (Table 2). Only one isolate NC2 produced ammonia at less than 100 mg/L, whereas 13 isolates produced ammonia at more than 300 mg/L. The maximum concentration of ammonia was observed in isolates NC113 (*B. cenocepacia*) (Table 2). Tagele *et al.* (2018) showed that *Burkholderia contaminans* isolated from rhizospheric soil of maize in South Korea can also produce ammonia (Tagele *et al.*, 2018). Different species of the *Burkholderia* genera isolated from rice, sugarcane, maize, and black gram also produce ammonia (Paungfoo-Lonhienne *et al.*, 2014; Van and Cao, 2014; Sandanakirouchenane *et al.*, 2017). These studies confirm the capacity for ammonia production among *Burkholderia* genera with respect to different plant species. Studies have also reported that ammonia-producing bacteria not only promote plant growth but also induce antagonistic activities against phytopathogens (Rana *et al.*, 2020).

Table 3: Hydrolytic enzyme production by 19 best siderophore-producing endophytic bacteria.

EB isolates	Hydrolytic enzyme production		
	Gelatinase[a]	Amylase[b]	Catalase[c]
NC2	++++	+++	+
NC4	-	++	+
NC50	+	+++	+
NC63	+	++++	+
NC64	+++	++++	+
NC83	+	-	+
NC88	++	++	+
NC89	+	++	+
NC110	++	++	+
NC112	++	++	+
NC113	-	++++	+
NC120	+++	+++	+
NC121	++	+++	+
NC122	+++	+++	+
NC124	+++	++	+
NC125	++	++	+
NC140	-	++++	+
NC141	-	+++	+
NC142	++	-	+
Total positive (Nos)	15	17	19
% Positive	78.94	89.47	100

[a]The signs -, +, ++, +++ and ++++ represent no gelatine liquefaction, low (<1/3), moderate (1/3-1/2), high (1/2-2/3) and very high (completely hydrolyzed) level, respectively.
[b]The signs -, +, ++, +++ and ++++ represent no starch hydrolysis, low, moderate, high and very high level of starch hydrolysis, respectively.
[c]The signs - and + represent negative and positive test, respectively; Nos: Number of strains.

Qualitative assays for hydrolytic enzyme production

Gelatinase production

All 19 siderophore producers were subjected to gelatine hydrolysis assay. At 7 days post-incubation, the gelatine liquefaction was observed with 15 isolates (78.94%). Different levels of liquefaction indicated corresponding levels of gelatine hydrolysis with 4, 6, 4 and 1 isolate exhibiting low, moderate, high and very high levels, respectively (Table 3, Figure 3). Notably, the isolate producing the highest level of gelatinase was NC2 (*Pseudomonas rhodesiae*) (Table 3). It has been reported that different strains of the genus *Pseudomonas* isolated from wetland rice were found to be positive for gelatine hydrolysis (Rath and Dangar, 2018). Other bacterial strains from *Bacillus cereus* and *Enterobacter cloacae* were found to have gelatinase activity in *Limoniastrum monopetalum* and *Zea mays*, respectively (Abedinzadeh *et al.*, 2019; Slama *et al.*, 2019). Moreover, gelatinase can rapidly digest extracellular matrix produced by *M. oryzae*. This activity detaches infection structures from membrane surfaces (Inoue *et al.*, 2007). A previous study reported that 24 EB isolated from submerged rice roots showed *in vitro* antifungal activity against *M. oryzae* strain 007-6,

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 18(1) 2022, pp. 17-26
DOI: http://dx.doi.org/10.21161/mjm.211297

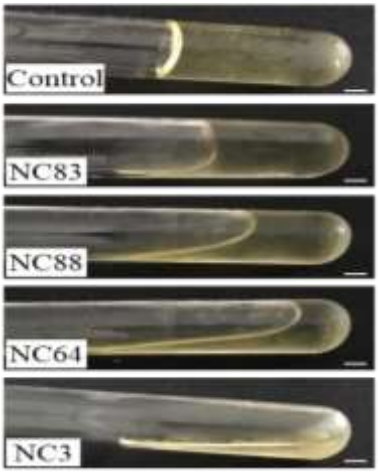

Figure 3: Qualitative assay for gelatin hydrolysis of siderophore-producing endophytic bacteria. From top to bottom, the control tube, uninoculated showed complete solid state of gelatin medium, whereas low, moderate, high and very high levels of gelatin liquefaction were observed in tested tubes inoculated by isolates NC83, NC88, NC64 and NC3, respectively. Bar represents 0.5 cm.

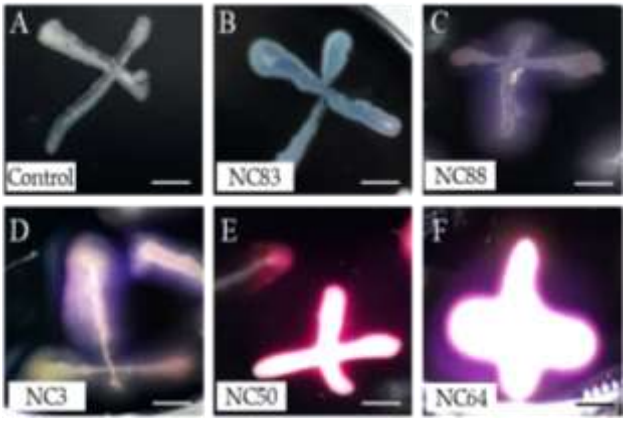

Figure 4: Qualitative assay for starch hydrolysis of siderophore-producing endophytic bacteria on 1/6 TSA medium plate containing 2% starch. Intensity of clear zone around colonies observing by light illumination indicated different levels of starch hydrolysis. A, the bacterial strain *Xoo* VX41 was used as negative control. B, isolate NC83 was negative for starch hydrolysis. C, D, E and F, isolates NC88, NC3, NC50 and NC64 represented low, moderate, high and very high levels of starch hydrolysis, respectively. Bar represents 0.5 cm.

with an inhibition ratio of more than 50% (Nguyen *et al.*, 2020). Gelatine hydrolysis by EB releases amino acids for their life processes for competition with and growth inhibition of other pathogens (Inoue *et al.*, 2007). Therefore, the ability of rice root EB to hydrolyse gelatine plays a critical role in finding beneficial strains to develop biocontrol agents.

Amylase production

Many bacteria can produce extracellular enzymes that participate in different catalytic chemical reactions outside the cell. Extracellular enzymes can break down large molecules, such as starch, for easier transportation into the cell. In amylase production assay, starch hydrolysis was indicated by a transparent zone around bacterial colony. Of 19 isolates, 17 (89.47%) were positive for starch hydrolysis (Table 3, Figure 4). Especially, four isolates (NC63, NC64, NC113 and NC140) showed very high level of starch hydrolysis. These isolates were identified as *Pantoea ananatis*, *Bacillus cereus*, *Burkholderia cenocepacia and Burkholderia vienamiensis*, respectively. A similar result was obtained by El-Deeb *et al.* (2012) in which *Bacillus* sp. isolated from rose plant showed high amylase activity (El-Deeb *et al.*, 2012). Moreover, EB from mangrove plants in Saudi Arabia, i.e., different strains of the genus *Bacillus* (EA154, EA157, EA161, EA171, EA195) had very high amylase activity, the diameter of clear zone was between 10 and 16 mm (Bibi *et al.*, 2017). Interestingly, these strains were also active against different pathogenic fungi, *Pythium ultimum* and *Phytophthora capsici*. Our data corroborate this evidence regarding the *Bacillus cereus* produced amylase and exhibited antifungal activity (data unpublished).

Catalase production

During aerobic respiration, many bacteria produce H_2O_2. Increased levels of H_2O_2 cause the death of cells unless it is enzymatically degraded. During the degradation of carbohydrates, EB use oxygen as the final electron acceptor leading to increased levels of H_2O_2 (Cappuccino and Welsh, 2017). Catalase produced by bacteria can rapidly degrade H_2O_2 into water and oxygen and appears to be important for the colonisation of EB in plant roots (Choodamani *et al.*, 2009; Sofo *et al.*, 2015). By adding one drop of 3% H_2O_2 solution on the 24 h-old bacterial colonies, the catalase producers were determined based on the appearance of oxygen bubbles. Results showed that all siderophore producers produced catalase. This catalase production activity of EB allows high resistance to different environmental stresses. In a similar study, all EB isolated from *Ophioglossum reticulatum* L. were found to also produce catalase enzyme (Mukherjee *et al.*, 2017). In rice, almost all EB isolated from the seed endosphere were shown to have catalase activity indicating its role for the survival and development of EB in the seed endosphere (Okunishi *et al.*, 2005; Walitang *et al.*, 2017).

CONCLUSION

In this study, we identified seven isolates that were positive for all tested assays *in vitro*, namely

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Pseudomonas rhodesiae (NC2), *Enterobacter asburiae* (NC50), *Pantoea ananatis* (NC63), *Bacillus cereus* (NC64), *Burkholderia cenocepacia* (NC110), *Staphylococcus sciuri* (NC112) and *Ralstonia pickettii* (NC122) with multiple PGP activities. These findings serve as guidance for further investigations of rice plants under conditions of the net-house and field trials to validate the beneficial activities of these EB in developing bioinoculants for upland rice farming in Vietnam.

ACKNOWLEDGEMENTS

This research was funded by The Postdoctoral Program from Graduate University of Science and Technology (GUST), Vietnam Academy of Science and Technology (VAST), Vietnam under grant number GUST.STS.ĐT2017-SH05. The authors acknowledge the support with laboratory facilities from the Institute of Biotechnology, VAST, University of Science and Technology of Hanoi (USTH), VAST, Mixed International Laboratory for Rice, Coffee and Environment (LMI-RICE2), and Agricultural Genetics Institute (AGI).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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