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Exploration of diazotrophic communities associated with *Solenostemon rotundifolius* and *Arachis hypogaea* in tropical agricultural land

Ahmad Arsyadi[1], Aris Tjahjoleksono[2] and Nisa Rachmania Mubarik[2]

[1]Faculty of Science and Engineering, Bangka Belitung University, Balunijuk 33172, Indonesia
[2]Department of Biology, Faculty of Mathematics and Natural Sciences, IPB University, Bogor 16680, Indonesia

Corresponding author: arsyadi@ubb.ac.id
ORCID ID: https://orcid.org/0000-0002-8917-5688

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Abstract: Land clearing through oil palm expansion potentially leads to several negative effects on farmland soil condition such as the deterioration of ammonium and nitrate. The native farmers in Bangka Islands, Indonesia, considerably cultivate black potatoes and groundnuts to elevate soil nutrient on the cleared land. Many studies have examined the ability of groundnuts to enhance soil quality through their specific association with diazotrophic bacteria, while there is still limited information about black potatoes. Therefore, the purpose of this research was to isolate and identify nitrogen-fixing bacteria associated with black potato and groundnut cultivated in this tropical farmed-land. In this study, a total 44 isolates were obtained using N-free and yeast extract mannitol agar medium. The isolates, BK1043 and BK1042, and AT1043 were able to produce ammonia and showed negative results in hemolysis, hypersensitivity, and antagonistic test. Based on *nifH* gene detection and acetylene reduction assay, BK1043 and AT1043 were recognized as diazotrophic bacteria. Furthermore all 3 isolates were able to produce indole acetic acid and hydrogen cyanide except BK1043. Identification of 16S rRNA gene showed that BK1043, BK1042, and AT1043 are belonged to *Paenibacillus alginolyticus*, *Burkholderia cepacia*, and *Arthrobacter enclensis,* respectively. This is the first study to report diazotrophic bacteria that are harbored in black potato rhizosphere.

Keywords: black potato; groundnut; nitrogen-fixing bacteria; *nifH*; 16S rRNA gene.

Introduction

Land clearing in Indonesia increased significantly since 1970 concomitantly with tremendous expansion of oil palm plantation. According to statistical data of plantation in Indonesia 2015, the areas of smallholder oil palm cultivation steadily accelerated to 4535400 hectares from 3125 hectares since 1979 (DGEC, 2016). Some studies have focused on the scarcity of N-soil minerals (NH_4^+ and NO_3^-) and their characteristics that easily leach and precipitate into groundwater systems (Jovanovic et al., 2008; Kabala et al., 2017). Several sustainable measurements were widely investigated to enhance N availability for plant growth such as application of N-fixing rhizobacteria as biofertilizer (Nabati et al., 2025) and their host plants including legume like *Mimosa tenuiflora*, *Acaciamangium*, *Pseudosamanea guachapele*, and non-legume involving *Parkinsonia aculeata*, *Morus* sp., *Azadirachta indica* (Chaer et al., 2017). Interestingly, diazotrophic bacteria also have been reportedly harbored in oil palm, potato, and sweet potato rhizosphere such as *Bradyrhizobium* (Henagemage et al., 2016), *Bacillus* (Calvo et al., 2010; Zhang et al., 2025), *Beijerinckia, Ensifer, Microbacterium, Caulobacter* (Harca et al., 2014), *Rhizobium* (Habibi et al., 2014), *Klebsiella, Erwinia* (Yasmin et al., 2009), *Azotobacter, Azospirillum* (Nafi'ah et al., 2025), and *Serratia* (Astriani et al., 2016).

Black potato (*Solenostemon rotundifolius* (Poir.) J. K. Morton) and groundnut (*Arachis hypogaea* L.) are recorded as plants capable of growing in various types of soil such as loose or dense soil under shade or direct sunlight (Anbuselvi and Priya, 2013). They have better tolerance to high temperature or rainfall intensity (NRCTNA, 2006), and well adapted to drought stress condition through an effective mechanism that enable them to maintain their root activity (Gohari, 2012). The farmers in Bangka Islands, Indonesia, considerably cultivate black potatoes and groundnuts to promote soil condition after land clearing. Many studies have examined the ability of groundnut to enhance soil quality through their specific association with N-fixing bacteria (Pena and Pueyo, 2012; Sengupta et al., 2024). However, there is still scarce information about *S. rotundifolius* as soil health promoting plant and soil microbes surrounding their roots. Therefore, according the best of knowledge, this is the first study to report diazotrophic bacteria as a potential biofertilizer agent that harbors with

S. rotundifolius rhizosphere. In addition, we also used groundnuts as well-studied leguminous plant to compare the abundance and N-fixing activity of rhizobacteria that we observed in black potatoes.

Results and discussion

Both A and B sites were characterized as podzolic, acidic, and sandy soils. However, the former site composed of higher nutrients including C-organic, and available P/K, while total-N and cation exchange capacity (CEC) were higher (two-time) in the latter site (Table 1). Podzolic soil has low level of moisture and pH as well as high aluminum Al^{3+} and iron Fe^{2+} ion levels (Saha et al., 2020), hence they limit the nutrient uptake and crop yield (Nikodem et al., 2013; Yamamoto, 2019). Consequently, most farmers in Kemuja intensively apply manure or other chemical fertilizers and lime to improve nutrient availability and soil pH, respectively. The higher content of C-organic, and available P/K in site A were as expected due to the accumulation of plant residues. On the other hand, the higher content of total-N and CEC of soil on site B can be explained by several reasons. Site A was composed of developed and repleted crops, in which the sunlight absorption is limited by black potatoes and groundnuts. This also limits their vegetative growth (as root activities) and production (Anbuselvi and Priya, 2013). This situation was corresponded to the slight growth of both plants in site A. Accordingly, N-fixing rate and deposition by diazotrophic bacteria was lower than site B which affected the total-N accumulation. The elevation of CEC was considered as the cause of incremental growth of black potatoes and groundnuts in site B that supplied more organic matter as green manure. A total 44 isolates were obtained in this study, of which 30 and 14 isolates were from N-free (soil samples) and YMA medium (organ samples), respectively. The characters of colonies were dominant by white color on N-free medium and varied from white to red colors on YMA medium. All isolates were classified into Gram (+) and (-) in an equal number while dominance of rod type cells was observed (Supplementary data 1).

Table 1. Soil edaphic properties of two farmlands in Kemuja village.

Soil properties	Site A[a]	Site B[b]
Type[c]	Podzolic	Podzolic
C-organic (%)	3.72	3.57
Total-N (%)	0.15	0.39
C:N ratio	24.8	9.15
Available P (ppm)	13.16	8.84
Available K (cmol (+) kg^{-1})	1.29	0.9
CEC (cmol (+) kg^{-1})	16.51	20.18
pH (H_2O)	5.46	4.81
Water content (%)	5.53	9.61
Sand (%)	43.46	61.88
Clay (%)	40.14	31.64
Dust (%)	16.4	6.48

[a]Site A was farmland having more than 4 years old after land clearing. [b]Site B was farmland having less than 1 year old after land clearing. [c]Podzolic soil was classified according to BPSB 2016.

Shannon's index (H'), Simpson's index (d'), richness (S), and evenness (J) of culturable isolates in rhizosphere soil were examined with regard to total plate count (TPC) (Table 2). In general, the average of total bacterial population between site A and B was almost similar while rhizosphere soils had higher bacterial count than bulk soil. Furthermore, bacterial diversity in plant rhizospheres measured by both Shannon and inverse Simpson index, was higher in groundnuts than black potatoes. The data demonstrated that the bacterial communities were impacted by plant species and ecological habitats. Plant species and ecological habitats can alter the structure of rhizobacterial populations through several mechanisms including organic acid exudation by plant roots, regulation of physico-chemical rhizosphere as pH, the effects of soil structure and nutrient, as well as other ecological conditions. Several studies have reported how each plant species and soil condition select a specific microbial population resulting in higher biomass and activities than in bulk soil or even, if compared to other plant species (Guo et al., 2019). Higher diversity of rhizobacteria in groundnuts than black potatoes can be as a result of groundnut's ability to provide more nutrients such as nitrate and soil organic matter as well as CEC in soils (Ajilogba et al., 2022).

In addition, there was a pattern, in which higher bacterial population in rhizosphere soil of groundnuts in site A (2.2×10^7 CFU g^{-1} weight soil) was not consistent with site B (1.4×10^7 CFU g^{-1} weight soil). On the other hand, less rhizobacterial count in black potatoes was observed in site A (1.1×10^7 CFU g^{-1} weight soil) while it was unexpectedly higher in site B (2.4×10^7 CFU g^{-1} weight soil) and two times higher in groundnuts. Despite their lower diversity and richness compared to groundnuts, the bacterial community evenness of black potatoes in site B was higher than site A. The higher and lower bacterial count in rhizosphere soil of groundnuts and black potatoes were respectively observed in site A, indicating the influence of ecological factor such as allowance of direct sunlight and more acidic condition. In site B the condition favored black potato's growth, hence increasing rhizobacterial population. The counts of putative N-fixing bacteria (10^6 CFU g^{-1}) in black potato and groundnut rhizosphere were higher than those reported in the wheat (10^4 CFU g^{-1}) rhizosphere on N-free medium (Rilling et al., 2018). This may be due to the difference in plant species and culture conditions used in each study.

Table 2. The information of bacterial numbers and diversities on each sample.

Plant[a]	Site[b]	Total bacterial count (CFU g^{-1} weight soil)	Shannon (H')	Inverse Simpson (1/D)	Richness (S)	Evennes (J)
Groundnut	A	2.2×10^7	0.92	1.95	6	0.51
Groundnut	B	1.4×10^7	0.74	1.59	5	0.46
Black potato	A	1.1×10^7	0.81	1.65	5	0.51
Black potato	B	2.4×10^7	0.75	1.59	4	0.54
n.p	A	1.7×10^6	1.30	3.46	4	0.94
n.p	B	1.1×10^6	1.21	2.58	6	0.67

[a] No plant (n.p) as negative control; [b] Site A and B were farmland having more than 4 years old and less than 1 year after land clearing, respectively

Deep insight into rhizobacterial population showed the dominance of AT1042, BT1042, AK1042, BK1042, BTK1042 among the isolates in rhizosphere soil of groundnuts in site A, groundnuts in site B, black potatoes in site A, black potatoes in site B, and bulk soil in site B respectively, while no significance difference was observed in bulk soil of site A (Fig 1). This is in accordance with a recent study that implied soil bacteria are influenced by various biotic and abiotic factors, including variety of plants (genotype) through a continuous excretion of organic materials as well as root exudates and soil edaphic properties such as CEC, pH, and other nutrient levels (Eo et al., 2021).

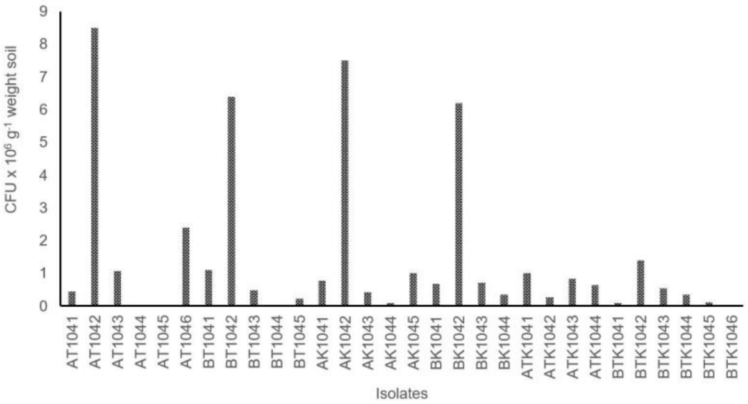

Fig 1. Population of different isolates grown on N-free medium. The total plate count was performed after 3 days incubation. CFU: colony forming unit

All 44 bacterial isolates were examined for their potential ability to produce ammonia (NH_3) in N-free medium through biological N fixation. The result showed that 9 of total isolates (AT1043, AT1044, BT1044, BK1042, BK1043, BTK1041, BTK1044, YAT1042, YBK1044) produced NH_3-N range from 0.001 to 0.046 ppm (0.03-1.3 µM) after 7 days of incubation (Fig 2). The highest and lowest activities of NH_3-N production were performed by isolate BT1044 and BTK1041, respectively.

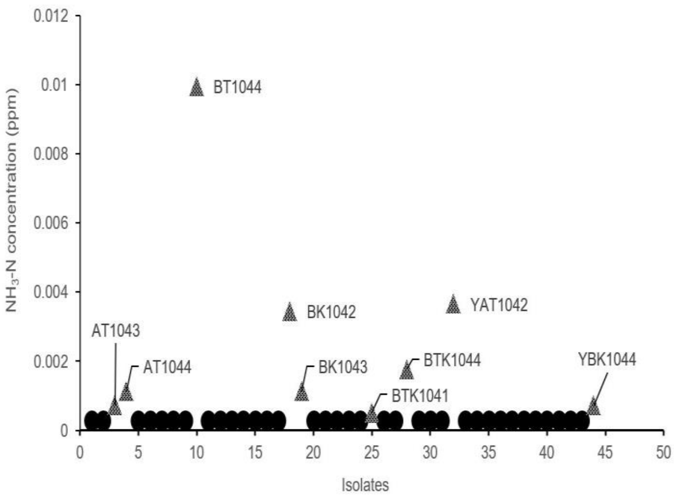

Fig 2. Ammonia-nitrogen producing activity of isolates. Dotted triangles and black circles indicate isolates that produce and lack of NH_3-N, respectively.

In other studies, diazotrophic bacteria such as *Azomonas agilis, Sthenotropphomonas* sp. MFC-C*, Flexibacter canadensis* ISSDS-428*, Rhizobium tropici* rif 200849, and *Azotobacter tropicalis were* able to excrete NH_3 in a range concentration 0.001-26.154 µM (Hartono et al., 2009), while other rhizobacteria including *Bacillus cereus, Enterobacter sichuanensis, Enterobacter asburiae, Enterobacter mori, Enterobacter sichuanensis, Klebsiella variicola,* and *Priestia aryabhattai* demonstrated the higher levels of ammonia production ranges 49.28-60.43 mM (Chhetri et al., 2025). This discrepancy may be due to the difference of bacterial species, measurement method, incubation time, and culture condition including carbon sources (Hartono et al., 2016).

Total 9 selected isolates were subjected to hemolysis assay in order to examine their potential risk on mammal and or human pathogenic bacteria. Isolates developing transparent (clear zone) or green-brown discoloration around colonies were considered to have beta- or alpha-hemolytic activities. Three bacterial isolates, AT1044 and BT1044 formed alpha-hemolysis while beta-hemolysis activity was observed on YAT1042 after 3 days incubation (Table 3). Therefore, only other 6 negative isolates were used for further analysis.

Table 3. The characteristics of bacterial isolates.

Isolates	Hemolysis Activity	Hypersensitivity Reaction[a]	*nifH*[b]	C_2H_4 concentration (nmol mg protein^{-1})[c]
AT1043	Negative	Negative	Negative	0.27
AT1044	Alpha	n.i	n.i	n.i
BK1042	Negative	Negative	Negative	0
BK1043	Negative	Negative	Positive	50.2
BT1044	Alpha	n.i	n.i	n.i
BTK1041	Negative	Positive	n.i	n.i
BTK1044	Negative	Negative	n.i	n.i
YAT1042	Beta	n.i	n.i	n.i
YBK1044	Negative	Positive	n.i	n.i

[a,b,c]not identified (n.i)

The hypersensitivity response (HR) test was carried out to investigate phytopathogenic traits of bacterial isolates through tobacco leaf infiltration. The necrotic lesion (yellowish to brownish leaves in color and dried) on the adjacent injected area was considered as positive HR. The result showed discoloration on the leaves with YBK1044 and BTK1041 infiltration as well as positive control bacteria, *Xanthomonas oryzae*. However, no HR symptoms were observed on BK1043, BK1042, AT1043, BTK1044, sterile NB medium, or sterile distilled water (Table 3).

All 6 bacterial isolates after hemolysis assay, were screened for their antagonistic activity. The presence of inhibition zones around the spotted cultures (applied bacteria) displayed inhibition activities towards tested isolates on the lawn (bacterial lawn). No visible inhibition zone was seen in 6 different applied isolates on each bacterial lawn after 48 h of incubation (data not shown). Recently, the application of beneficial bacteria has been widely reported to promote plant growth as a consortium rather than a single inoculation. For instance, a combination of *Serratia* sp. XY21, *Bacillus cereus* AR156, and *B. subtilis* SM21 significantly elevated the nutrient content and yield, soil fertility, as well as reduced plant disease in pepper fruit (Zhang et al*.*, 2019). Accordingly, it is necessary to understand the ability of each putative N-fixing bacterial isolate to interact or compete (as for limiting space and nutrient) with other isolates as a consortium.

A total 4 isolates were selected as potential diazotrophic bacteria involving BK1043, BK1042, AT1043, and BTK1044. However, within the isolates, BTK1044 was excluded from further analysis due to several reasons such as low-producing ammonia activity and the most slow-growth of bacteria (especially in liquid medium by forming aggregates). Therefore, to examine the ability of other 3 isolates, *nifH* gene PCR-based detection and acetylene (C_2H_2) reduction assay (ARA) were carried out in this study. The amplification result showed a 360 bp *nifH* amplicon using polF/polR primer pair in isolate BK1043 while no amplicon was observed in BK1042 and AT1043 (Table 3). Consistently with *nifH* gene detection, after 2 h incubation under C_2H_2 gas injection, the highest activity of ethylene (C_2H_4) production by isolate BK1043 was observed (50.2 nmol mg protein^{-1}). On the other hand, AT1043 surprisingly showed an activity of C_2H_2 reduction (0.27 nmol mg protein^{-1}) (Table 3). The negative result of PCR amplification in AT1043 may be due to high number of mis-match on conserved region, high GC-content of the targeted gene (Laursen et al., 2017), or other factors such as length of primer, degree of encoded protein conservation, PCR condition, and or PCR mix composition (Housley et al., 2006). Compare to other studies, the nitrogenase activities of both isolates were lower than *Azospirillum amazonense* BR 11877 (4.5 nmol mg protein^{-1} h^{-1}) (Rodrigues et al*.*, 2008), *Bacillus* spp. MK9 (402 nmol mg protein^{-1} h^{-1}) (Kaushal and Kaushal, 2015), Staphylococcus xylosus (227.3 nmol/h/vial), Bacillus paranthracis (167.2 nmol/h/vial), Bacillus cereus (91.1 nmol/h/vial), Bacillus thuringiensis (82.6 nmol/h/vial) (Renugadevi et al., 2024), and higher than *Bradhyrhizobium* sp*.* TM122 (64.7 pmol tube^{-1} h^{-1}) (Hara et al*.*, 2019), *Paenibacillus bracinonensis* AF91 (0.3 nmol h^{-1} 10^{-6} cells) (Habibi et al*.*, 2019). The differences of these nitrogenase activities are based on different species, incubation time, carbon (nutrient) source, and the presence of inhibitory molecules as NH_3 or NO_3^- accumulation (Suleiman et al., 2019).

All 3 isolates were subjected to plant growth promoting assay including phosphate solubilization, indole acetic acid (IAA) production, and hydrogen cyanide (HCN) production. The phosphate solubilizing activity of isolates indicated by clear zone that formed around the colony. This assay used Pikovskaya (PKV) medium which contains insoluble tricalcium phosphate. However, no halo zone formation was observed in all 3 spotted cultures after 5 days of incubation (Table 4). The detection of auxin production was performed through colorimetric assay using Salkowski reagent. The results showed that all 3 isolates were able to produce IAA after 5 days of incubation, ranged from 2.02 to 5.46 ppm. The highest and lowest auxin production was recorded for isolate BK1042 and AT1043, respectively (Table 4). The filter paper treated with picric acid was used to examine the HCN production of isolates while discoloration of filter paper from yellow to orange or brown indicates the presence of HCN product. In brief, after 5 days of incubation, the orange (3 in scale) and brick like color (5 in scale) were observed on culture of isolate BK1042 and AT1043, respectively, while no color change (orange or 1 in scale) was seen on isolate BK1043 (Table 4).

Table 4. Plant growth promoting features of bacterial isolates.

Isolates	Phosphate Solubilization (halo/colony ratio)[a]	IAA Production (ppm)	HCN Production (color change)[b]
BK1043	-	3.45	1
BK1042	-	2.02	3
AT1043	-	5.46	5

[a]No halo zone was observed (-); [b]HCN scale was determined by the color change (yellow: 1, cream: 2, orange: 3, light brown: 4, brick like color: 5).

Attempts to identify the growth-promoting activities of selected isolates were important to understand their ecological functions. The phosphate solubilizing activity offers beneficial effects in most soils, which contain low amount of the soluble P because of its non-renewable characteristics. In addition, the biosynthesis of auxin by PGPR has been reported to play a major role on water and nutrient acquisition by plants through the formation of lateral roots (Zuluaga et al*.,* 2020). Another important trait from PGPR to enhance plant growth is the production of HCN which plays an effective role in inhibiting the growth of various pathogenic termites, weeds, insects, nematodes, and fungi by disrupting the respiration process in the cytochromes of pathogen cells (Sehrawat et al*.,* 2022). Other studies have reported several diazotrophic bacteria that have a multiple plant-growth promoting activities. For instance, *Paenibacillus* sp. RFNB5 produced indole acetic acid (IAA) and ACC deaminase, *Burkholderia* sp. RFNB13 produced IAA and siderophores (Islam et al*.,* 2012), *Paenibacillus* sp. JS-4 excreted IAA and antibacterial compound (Liu et al*.,* 2019). *Pseudomonas* sp. prduced ACC deaminase and phosphate solubilization (Ahemad and Kibret, 2014), as well as *Exiguobacterium roggenkampii* and *Klebsiella oxytoca* exhibited phosphate solubilization, IAA production, *nifH* gene, and siderophore production (Flores et al., 2025).

BLAST analysis using the 16S rRNA gene sequences of BK1043, BK1042, and AT1043 showed homology with *Paenibacillus alginolyticus* DSM 5050 (98.13 %), *Burkholderia cepacia* NBRC 14074 (99.03 %), and *Arthrobacter enclensis* NIO-1008 (99.09 %), respectively (Table 5). The phylogenetic analysis based on 16S rRNA gene sequences exhibited 3 clusters (Fig 3). A single cluster was formed by isolate BK1043, BK1042, and AT1043 with reference strains in genus *Paenibacillus*, *Burkholderia,* and *Arthrobacter,* respectively.

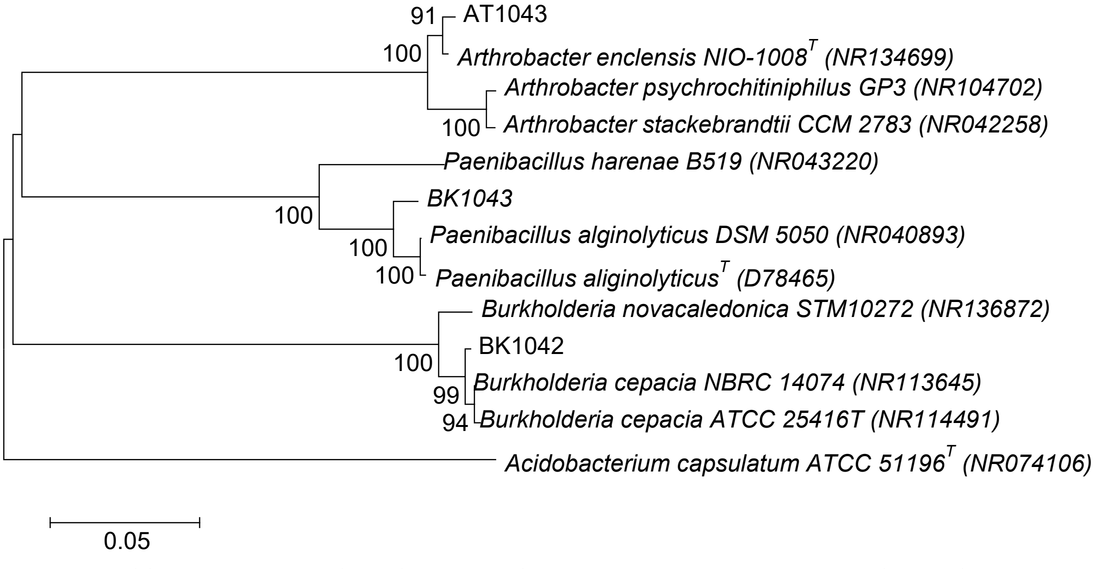

Fig 3. Neighbor-Joining tree based on 1078 bp 16S rRNA gene sequences shows the positions of BK1043, BK1042, and AT1043 in the genera *Paenibacillus*, *Burkholderia*, and *Arthrobacter*, respectively (Tamura-Nei model with bootstrap 1,000 replicates). A number on branch nodes represents bootstrap value (>70%) expressed as a percentage (n=1000). Scale bar: 0.05 substitution per nucleotide position. The 16S rRNA coding sequence of *Acidobacterium capsulatum* ATCC 51196**T** was observed as an outgroup.

To date, more than 20 *Paenibacillus* species have been recognized to fix N. They harbor a highly conserved *nif* gene cluster containing 9 genes within 10.5-12 kb region (Grady et al., 2016). In addition, some of its diazotrophic members (inhabited various plant rhizosphere) were able to perform multiple beneficial activities towards plant growth such as IAA and antibacterial production by *Paenibacillus* WF-6, *Paenibacillus* YB-3, *Paenibacillus* SZ-1b (Liu et al*.,* 2019) as well as IAA and siderophore production by *P. polymyxa* (Ahemad and Kibret, 2014). However, there is still limited information about *P. alginolyticus* related to their potential as diazotrophic bacteria and PGPR. *Arthrobacter* as members of *Actinobacteria* has been widely reported to perform BNF activities in the rhizosphere (Biabani et al., 2012; Boukhatem et al., 2022). Some N-fixing *Arthrobacter* can perform other plant growth promoting traits.

Table 5. Sequence similarities of selected isolates based on 16S rRNA gene.

Isolates	Description	Identity (%)	*E*-value	Query Cover (%)	Accession (NCBI Reference Sequence)
BK1043	*Paenibacillus alginolyticus* DSM 5050	98.13	0.0	100	NR040893
BK1042	*Burkholderia cepacia* NBRC 14074	99.03	0.0	100	NR113645
AT1043	Arthrobacter enclensis NIO-1008	99.09	0.0	100	NR134699

For instance, siderophore and phosphate solubilization by *Arthrobacter* sp. V54 and *Arthrobacter* sp. V64 are obtained from the maize rhizosphere (Vanissa et al*.,* 2018). Nevertheless, there is no report about *A. enclensis* as a diazotrophic bacteria and PGPR. Several members of *Burkholderia* have been known as N-fixing bacteria including *B. nodosa* (Tang et al*.,* 2020), *B. mimosarum, B. vietnamiensis* (Santos et al*.,* 2001), and *B. cepacia* (Miladiarsi et al*.,* 2017). Some others also exhibit multiple beneficial traits, for instance by *Burkholderia* sp. J62 which carries out phosphate solubilization and antibiotic, IAA and ACC deaminase production (Jiang et al., 2008) as well as *Burkholderia vietnamiensis* with siderophore production, phosphate solubilization, and indole-3-acetic acid synthesis activities (Han et al., 2025). The ability of *B. cepacia* solubilize inorganic phosphate and IAA production are widely reported (Maxton et al*.,* 2017). However, the abilities of *B. cepacia* to produce NH3 and HCN are not widely studied.

Deep insight into the previous bacterial population data, the more abundance of *B. cepacia* (BK1042) than *P. alginolyticus* (BK1043) and *A. enclensis* (AT1043) may suggest its crucial roles in black potato rhizosphere on site B by perform ammonia, IAA, HCN production, or other beneficial features. Nevertheless, in this study, bacterial population was counted only based on morphological characters and culture approach which can mislead understanding of the actual abundance and functional roles of each strain as N-fixing bacteria or even inaccessible microbes. Notably, further analysis through metagenomic and metatranscriptomic analysis can be carried out to resolve these limitations.

Materials and methods

Site description and sample collection

Soil samples and plant specimens were obtained from two sites of agricultural land in Bangka Islands, Indonesia. Site A and B were >4 years and <1 year-aged farmed-land and established on 2°04'51.0"S 105°57'44.9"E and 2°04'16.4"S 105°57'54.5"E, respectively. The distance between two sites was about 1.3 km. The black potatoes and groundnuts were planted between rows of oil palm trees as intercropping plant and cover crops with the distance between three plants was about 2-5 m.

Rhizosphere soil of black potatoes and groundnuts (5 g), bulk soils (5 g), as well as nodulated-roots (5 g) and 5 tubers (size in 1-3 cm) were collected randomly from 3 collection points for each site in triplicates, mixed in sterile ziplock plastic bags up to a total of about 10 g, then immediately stored on ice. All samples were kept at 4ºC for 72 hours until soil and bacteriological analysis for culture-based methods were done (Luan et al*.,* 2015). Both plants that used in present study were 2-3 months old after planting in the oil palm field.

Soil analysis

A portion of rhizosphere soils and bulk soils were mixed into one sterile ziplock plastic bag up to a total 10 g for each site. The two samples then delivered to The Laboratory of Department of Land Science and Resources, Faculty of Agriculture, IPB University as outsourcing service provider of soil analysis. The services were included total organic compound (TOC) by Walkley and Black method, total nitrogen (TN) by Kjeldahl method, available P concentration by Bray-1 technique, available K by NNH_4OAc method, water content by gravimetric method, pH measurement, cation exchange capacity (CEC) calculation, and soil texture analysis (Motsara and Roy, 2008).

Isolation and screening of diazotrophs

The composite soils as well as nodules and tubers in triplicates were used for diazotroph isolation. First, about 1 g of soil samples were transferred to 9 mL sterile 0.85 % NaCl solution as 10^{-1} and homogenized. 1 mL of soil suspension was transferred to other 9 mL of sterile distilled water to make 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} dilution and 0.1 mL of each diluted soil suspension was spread onto N-free (pH 6.9) agar medium (Park et al*.,* 2005). Yeast mannitol agar (YMA) medium was used to distinguish rhizobial group among the purified isolate (Situmorang et al*.,* 2009). The tuber and nodule samples were washed using distilled water, sterilized by ethanol 70 % and $HgCl_2$ 0.1 % for 2 min, rinsed by sterile distilled water, crushed, and sample suspension was streaked onto YMA medium (Idris et al., 2015). All plates were incubated for 3 days at 30ºC. Total plate count and morphological characterization were performed to estimate bacterial population. Both isolation processes were performed in duplicates.

Ammonia production assay

Phenate method was carried out to determine the potential of bacterial isolates for ammonia production in N-free medium (Eaton et al., 2005). All isolates were grown on 10 mL of N-free medium supplemented by tryptone (1 % w/v), incubated in shaker incubator 125 rpm for 4 days at 30ºC, harvested for cell biomasses, and resuspended with 10 mL sterilized N-free medium (repeated three times). The bacterial suspension was adjusted to (optical density/OD_{600}=0.6) and incubated in a shaker incubator 125 rpm for 1 week at 30ºC. Thereafter, 0.08 mL of phenol solution (≥89% v/v), 0.08 mL of natrium nitroprusida (0.5 % w/v), and 0.2 mL of oxidizing solution was added to 2 mL of supernatant. NH_3-N concentration was measured using Vis Spectrophotometer at (λ) 640 nm. Non-inoculated medium and 0-1 ppm NH_3 solution were used as negative control and standard curve construction.

Hemolytic test

Blood agar plate was used to examine mammal-pathogenicity traits of selected isolates. A loop ose of 48 h culture colony was streaked onto the blood agar lawn and grown for 72 h at 30ºC (Turista and Puspitasari, 2019). Hemolytic assay was performed in triplicates.

Hypersensitive test

Phytopathogenic traits of selected isolates were examined through hypersensitive reaction tests on tobacco plants (*Nicotiana tabacum* L.) with infiltration methods. The selected isolates were inoculated on 20 mL of nutrient broth (NB) medium and incubated in a shaker incubator 125 rpm for 24 h at 30ºC. The bacterial suspension was adjusted to (OD_{600}=0.8) to obtain 10^8 cfu/mL and about 1 mL of aliquot was infiltrated onto abaxial surface of tobacco leaves with sterile syringe 1 mL (Jayaraman et al., 2017). The plant was maintained for one week in a glass house. *Xanthomonas oryzae* pv. oryzae STG21, non-inoculated medium, and sterile distilled water were used for positive and negative control, respectively. This experiment was carried out in triplicates and repeated two times.

Antagonism trait test

Antagonism assay among selected bacteria was performed following Long et al. (2005) method. On this test, one selected isolate was cultured with another to examine their antagonistic interactions when applied as a consortium. Bacterial cultures were grown on 20 mL of NB medium, incubated in a shaker incubator at 125 rpm for 24 h at 30ºC, and diluted to (OD_{600}=0.8). 200 µL of aliquot was transferred into soft nutrient agar (NA) medium and overlaid onto a sterile petridish (bacterial lawn). Furthermore, one loop ose of other 48 h culture was spotted (applied bacteria) on the prepared agar lawn and incubated for 48 h at 30ºC. This assay was performed in triplicates and sterilized distilled water was used as negative control.

nifH gene detection

All selected bacteria were subjected to *nifH* gene amplification. Genomic DNA extraction from bacterial cells was carried out using Presto™ Mini gDNA bacteria Kit (Geneaid) according to the manufacturer's instructions. Amplification of *nifH* gene was performed using the PolF (5'-TGCGAYCCSAARGCBGACTC-3') and PolR (5′-ATSGCCATCATYTCRCCGGA-3′) primer set (Poly et al., 2001). The reaction mixture (10 µL) contained 5 µL of GoTaq Green Master Mix (Promega), 0.5 µL of each primer, 3 µL of nuclease water (Promega), and 1 µL of DNA template (100 ng/µL). Thereafter, PCR was carried out with thermal cycler machine under the following conditions: initial denaturation at 95ºC for 2 min, followed by 30 cycles of denaturation at 95ºC for 30 s, annealing at 55ºC for 1 min, and extension at 72ºC for 1 min. After final extension at 72ºC for 5 min, the PCR mixtures were kept at 4ºC (Hadianta et al., 2014). The PCR amplicons (360 bp) were subjected to 1.5% agarose gel electrophoresis.

Acetylene reduction assay

The biological nitrogen fixation (BNF) activity of selected isolates was examined using acetylene reduction assay (ARA) (Kifle and Laing, 2016). Bacterial isolates were inoculated on 20 mL of NB medium, incubated in shaker incubator with 125 rpm for 24 h at 30ºC, and adjusted to (optical density/OD_{600}=0.8). Thereafter, 500 µL of aliquot was transferred into 10 mL vials containing 5 mL of N-free semi-liquid medium, covered by rubber cap, and incubated for 1 week at 30ºC. The headspace air was replaced with C_2H_2 (10 % v/v) using sterile 5 mL syringe, and after incubation at 30ºC for 2 h. The concentration of the formed ethylene (C_2H_4) was analyzed by gas chromatography. The specific activity of nitrogenase was determined by ratio C_2H_4/total cell protein of the culture, which assessed by Lowry method (Rodrigues et al., 2008). Acetylene reduction assay was performed in duplicates and the C_2H_4 measurement was performed by Laboratorium of Biology, Soil Research Centre, Bogor.

Phosphate solubilization

Pikovskaya (PKV) agar medium was used to determine qualitatively phosphate solubilization activity among the isolates (Song et al., 2008). A loop of 24 h bacterial culture was spotted on the agar lawn and incubated for 5 days at 30ºC. Halo zone that formed around the colony indicated the positive result as phosphate solubilizer. Phosphate solubilization index (SI) then calculated by dividing the sum of colony and halo zone diameter towards colony diameter.

Indole acetic acid (IAA) production

Bacterial isolates were subjected to indole acetic acid (IAA) production assay using Salkowski reagent. Bacterial cultures were inoculated on 20 mL of NB medium, incubated in shaker incubator with 125 rpm for 24 h at 30ºC, and adjusted to (optical density/OD_{600}=0.8). About 200 µL of aliquot was transferred into 20 mL fresh NB medium supplemented by 0.1 g/L of L-tryptophan and incubated in shaker incubator with 125 rpm for 5 days at 30ºC in dark. After centrifugation, 2 mL of Salkowski reagent and 3 drops of o-phosphoric acid was added to 1 mL of liquid phase, homogenized, incubated for 30 min at 30ºC in dark condition, and measured at (λ) 530 nm using Vis Spectrophotometer (Astriani et al*.,* 2016). A standard curve was established using a serial IAA concentration (0-200 ppm).

Hydrogen cyanide production

The ability of isolates to produce hydrogen cyanide (HCN) was examined following method of Zadeh et al*.* (2010). A loop ose of 24 h bacterial culture was streaked on the Nutrient Agar (NA) lawn supplemented with 4.4 g/L glycine and incubated for 24 h at 30ºC. A piece of sterile filter paper (d=90 mm) was immersed in a solution of 5% picric acid and 2 % Na_2CO_3, placed at top of culture (as cover), and incubated for 5 days at 30ºC. The HCN production activity of cultures was indicated by the discoloration of filter paper range from orange to brown. All plant growth promoting assays were carried out in triplicates.

Identification of 16S rRNA gene

Genomic DNA extraction was carried out using Presto™ Mini gDNA bacteria Kit (Geneaid). The 16S rRNA gene amplification was performed using the 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGWGTGTACAAGGC-3') primer set (Marchesi et al., 1998). The reaction mixture (10 µL) contained 5 µL of GoTaq Green Master Mix (Promega), 0.5 µL of each primer, 3 µL of nuclease water (Promega), and 1 µL of DNA template (100 ng/µL). PCR was carried out using thermal cycler machine under the following condition: initial denaturation at 94ºC for 4 min, followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 45 s, and extension at 72ºC for 90 s. After final extension at 72ºC for 7 min, the PCR mixtures were kept at 4ºC (Peng et al*.,* 2018). The PCR products (1,300 bp) were observed on 1.5% agarose gels. The amplicon of 16S rRNA gene (10 µL) was delivered to 1st BASE DNA Sequencing Service, Malaysia to identify the gene sequence. All sequences were deposited in DNA Data Bank of Japan (DDBJ) and GeneBank database as LC515210, LC515209, and SUB6743268, respectively.

Bioinformatic and data analysis

The 16S rRNA gene outputted sequences were filtered and trimmed using seqtrace v.0.9.0 to produce a high-quality sequencing chromatogram with spaced peaks, each a single color, and minimal baseline noise. Afterward, the sequences were blasted using online basic local alignment search tool-nucleotide (BLAST-N) against GeneBank database. Thereafter, isolate sequence and 3 reference sequences with the highest similarity, *e*-value, and query cover percentage were aligned to construct a phylogenetic tree based on Neighbor-Joining method and bootstrap 1000 replicates using MEGA7 software (Kumar et al*.,* 2016). The bacterial diversity on site A and B including Shannon's index (H'), Simpson's index (d'), richness (S), and evenness (J), was estimated using vegan package (https://cran.r-project.org/web/packages/vegan/index.html) on R software v.3.5.

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Statement of contributions

All authors contributed to the experimental conception and design. Material preparation and data collection were carried out by AA, while data analysis was performed by AT and NRM. The first draft of the manuscript was written by AA, and all authors provided comments on previous versions. All authors read and approved the final manuscript.

Conclusion

The best nitrogen fixing bacterial candidates with growth-promoting activities including BK1043, BK1042, and AT1043 were obtained in this study. All 3 isolates were non-human and plant pathogens, showing synergistic activities, able to produce ammonia, and referred to as nitrogen-fixing bacteria in corresponded to *nifH* and nitrogenase features. However, isolate BK1042 showing the negative results in *nifH* detection and ethylene assay. In addition, all 3 isolates were able to produce indole acetic acid and hydrogen cyanide, except BK1043. Based on 16S rRNA gene identification, BK1043, BK1042, and AT1043 were belonged to *Paenibacillus alginolyticus*, *Burkholderia cepacia*, and *Arthrobacter enclensis,* respectively. This study is the first report describing the isolation and characterization of diazotrophic bacteria associated with black potato rhizosphere as well as their assessment as plant-growth promoting bacteria.

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