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Isolation and identification of nitrogen-fixing bacteria associated with *Dioscorea alata* L. and *Dioscorea esculenta* L.

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Yams are important tuber crops in the tropics. Nitrogen is one of the limiting factors in yam production. Yam accessions that can rely on biological nitrogen fixation must be identified if we are to achieve efficient and sustainable yam production. The aim of this study was to isolate and identify the endophytic diazotrophic bacteria of 19 accessions of two yam species. Forty-one endophytic bacteria were isolated from surface-sterilized roots, stems and leaves of yam accessions grown in nutrient-poor subsoil for 160 days. The 16S rRNA gene sequencing showed that the 41 isolates were grouped into 18 genera including *Bacillus*, *Bradyrhizobium*, *Devosia*, *Ensifer*, *Mycobacterium*, *Neorhizobium*, *Paenibacillus*, *Pseudoxanthomonas*, *Rhizobium* and *Xanthomonas*. Most endophytes showed a positive response to nitrogenase activity and were found in the stems (21) and the roots (14), while only six were found in the leaves. In *Dioscorea esculenta*, the acetylene reduction assay (ARA) values ranged from 4.1 to 57.4 nmol tube/24 h and from 4.1 to 164 nmol tube/24 h in *Dioscorea alata*. In addition, the endophyte species were different among yam species and among yam accessions and were affected by fertilization. These results indicate that the endophytic diazotrophic bacteria of yams are diverse.

Key words: Diazotroph, Endophytes, Nitrogenase, Yam

INTRODUCTION

Nitrogen (N) is of great importance to agriculture as it is required in larger amounts than other nutrients and is involved in vital biological processes. Inappropriate use of N fertilizers results in environmental problems (e.g., the eutrophication of soils and waters, climate change). The production of N fertilizers is unsustainable, and so the need to provide plants with biologically fixed N is being increasingly investigated. Plant endophytic bacteria are defined as bacteria that live inside plant tissues without causing visible disease symptoms. Diazotrophic bacteria are endophytic bacteria capable of biological nitrogen fixation (BNF) in both legumes and non-leguminous plants (Iniguez *et al*., 2004). BNF has been documented for several important crops, including rice (Mbai *et al*., 2013; Ferrando & Scavino, 2015), sugarcane (Magnani *et al*., 2013; Muangthong *et al*., 2015) and sweet potato (Asis & Adachi, 2005). However, there is a lack of information on endophytic diazotrophs in tropical plants (Suhandono *et al*., 2016).

Yams (*Dioscorea* spp.) are important tuber crops grown throughout all the tropical zones, with the highest fresh tuber production in Africa. In West Africa more than 60 million people depend on this crop for their food (Asiedu & Sartie, 2010). In its traditional growing systems in Africa, yams are cultivated without or with very little application of mineral fertilizers (e.g., N). In Asian countries such as Japan and Myanmar where yams are also cultivated, little attention has been given to this crop. Although cultivated as the dominant staple food crop in Papua New Guinea, *Dioscorea esculenta*, for example, is usually grown in poor soils with low fertilizer input (O'Sullivan & Ernest, 2007). Trials on the mineral fertilization of yams showed that several genotypes do not respond to mineral N application (Diby *et al*., 2009; Ettien *et al*., 2014). Recently, nitro-

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gen-fixing bacteria associated with yams were reported for the accession E-2 of *D. esculenta* by Rezaei *et al.* (2017). Additionally, Takada *et al.* (2017) also reported that the accession A-19 of the water yam (*D. alata*) grows in non-fertile soil and absorbs nitrogen from the air. These reports indicate that nitrogen-fixing bacteria may contribute considerably to yam growth. However, in several studies conducted with different crops, it has been reported that communities of endophytic bacteria vary depending on plant genotype (Elbeltagy *et al.*, 2001), suggesting an endophytic bacteria-host plant specificity. This study was designed to assess the nitrogen-fixing bacterial population associated with different accessions belonging to *Dioscorea alata* and *Dioscorea esculenta*.

MATERIALS AND METHODS

A pot experiment was conducted from May to November 2016 on 19 accessions belonging to two species of yam (*Dioscorea alata* and *Dioscorea esculenta*) on Miyako Island, Tokyo University of Agriculture (TUA) Miyako Farm, Okinawa, Japan (N 24° 70′, E 125° 28′). The samples were planted in 5 *l* pots in a greenhouse on May 30th, 2016. Each pot was filled with 3 kg of nutrient-poor subsoil collected on Miyako Island. The treatments consisted of nitrogen application and control without nitrogen application. Urea was used as a mineral source of nitrogen and was applied 60 days after planting (DAP). The characteristics of this soil are given in Takada *et al.* (2017). Nineteen yam accessions were tested in this study including the accessions A-19 (Takada *et al.*, 2017) and E-2 (Rezaei *et al.*, 2017) as shown in Table 1. These accessions, with different origins, are all maintained by TUA. The experimental design was a completely randomized bloc with one repetition for the accessions.

Preparation of plant samples

Endophytic nitrogen-fixing bacteria in yams were extracted at 160 DAP using the culture-dependent method. Leaves, stems and roots were tested to determine whether they were harboring nitrogen-fixing bacteria. Three to five widely open and healthy leaves without petioles were collected from the base, middle and top of the plant and thoroughly washed with tap water to remove dust and soil particles. The leaves were cut into pieces of about 2 × 2 cm^2. The same procedure was followed when selecting stems. The stems were cut into segments of about 2–3 cm long. The roots were washed with tap water to remove all attached soil particles and then placed on paper tissues. About 2 g of each sample were used in the surface sterilization process.

Surface sterilization

Samples were washed with two liters of distilled water containing 2 drops of TWEEN® 20 (about 1 m*l*)

Table 1 List of accessions used in the study on nitrogen-fixing bacteria associated with yam

Accessions code No.	Yam species	Origin	Accessions code No.	Yam species	Origin
A-4	*D. alata*	Indonesia	A-68	*D. alata*	Kagoshima University, unknown
A-16	*D. alata*	Nepal	A-73	*D. alata*	Myitkyina, Kachin state, Myanmar
A-17	*D. alata*	Kochi, Japan	A-74	*D. alata*	Pyin Oo Lwin, Mandalay division, Myanmar
A-18	*D. alata*	Okinawa, Japan	A-86	*D. alata*	Kutkhaing, Shan state, Myanmar
A-19	*D. alata*	Palau	A-112	*D. alata*	Okinawa, Japan
A-23	*D. alata*	Papua New Guinea	A-116	*D. alata*	Okinawa, Japan
A-44	*D. alata*	Keelung, Taïwan	E-1	*D. esculenta*	Lashio, Myanmar
A-58	*D. alata*	Chiayi, Taïwan	E-2	*D. esculenta*	Okinawa
A-61	*D. alata*	Kagoshima, Japan	E-3	*D. esculenta*	Hateruma island, Okinawa, Japan
A-62	*D. alata*	Pyin Oo Lwin, Mandalay, Myanmar			

to remove any epiphytes. During surface sterilization, the plant samples were immersed successively in a solution of 70% ethanol for 5 min, and 3% sodium hypochlorite (NaClO) for 10 min. The samples were then transferred to a clean bench, rinsed 4 times with sterile distilled water, and macerated with a sterilized pestle and mortar with 10 m*l* of autoclaved normal saline (0.9% NaCl). One milliliter of the macerate was mixed with 1 m*l* of 30% glycerol in Eppendorf tubes and stored at −15℃ for bacteria isolation and identification.

Isolation of bacterial colonies

Nitrogen-free modified Rennie (MR) medium (Elbeltagy *et al*., 2001) was used as the growing medium. The MR medium was prepared from solutions A and B. Solution A (300 m*l*) consisted of 0.2 g $MgSO_4 \cdot 7H_2O$, 3 m*l* $CaCl_2$ (0.2 g in 10 m*l* water), and 5 m*l* of 0.5% bromothymol blue (0.25 g in 50 m*l* 0.2 M KOH). The pH was adjusted to 6.8 with 0.1 M HCl. Solution B (700 m*l*) was made with 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.1 g NaCl, 5.0 g sucrose, 3.0 g mannitol, 2.0 g DL-malic acid, 100 mg Yeast extract (Difco), 1.25 m*l* $Na_2MoO_4 \cdot 2H_2O$ (0.2 g in 10 m*l* water) and 1 m*l* NaFe(III)-EDTA (0.28 g in 10 m*l* water). The pH was adjusted to 6.8 with 5 M KOH. A solid MR growing medium was prepared with 20 g gellan gum. As with as solution A, solution B was added to the solidifying powder and autoclaved at 121℃ for 20 min. Under a clean bench, the two solutions were mixed and 1 m*l* each of filter-sterilized biotin and *para*-aminobenzoic acid were added. Plant macerates were injected into 40-m*l* glass tubes containing 4 m*l* of semi-solid N-free MR growth medium and closed with a foam stopper. The tubes were then incubated at 30℃ for 7–10 days. In further studies, tubes with formations of bacterial pellicles were considered to contain at least one nitrogen-fixing bacterium. These bacterial pellicles were streaked on a solid N-free MR medium and incubated under the same conditions as mentioned above. On each plate, bacterial colonies of different shapes and colors were considered to be different strains and were separately cultured on a fresh solid N-free MR medium until pure colonies were obtained. Bacteria with weak growth were excluded from the analysis.

Acetylene reduction assay (ARA)

To confirm the nitrogenase activity of the isolated bacteria, an ARA was conducted on randomly selected pure bacterial colonies. A semi-solid N-free MR medium was prepared, and 4 m*l* was introduced into 35-m*l* glass tubes that were closed with foam stoppers. One loop of the bacterial isolates was injected into the tubes and kept at 30℃ for 9 days. The foam stoppers were replaced with rubber stoppers, and an atmosphere containing 10% acetylene was created in the tubes by removing 3 m*l* air and replacing it with an equal volume of acetylene gas. The ethylene concentration was measured 24 h later by injecting 1 m*l* of the atmosphere into a gas chromatograph equipped with a flame ionization detector (FID) and N Porapak column.

Bacterial genomic analysis

Pure bacterial colonies were subjected to an analysis of their 16S rRNA genes. A bacterial cell suspension (20 μl) was treated with proteinase K (5 μl) at 60℃ for 20 min and 95℃ for 5 min to disrupt cell membranes. The 16S rRNA genes were amplified from the extracted DNA template with the universal primers 9F (5'-GAGTTTGATCCTGGCT-3') and 1541R (5'-AAGGAGGTGATCCAGCCGCA-3'). The PCR reaction mixture consisted of 10x ExTaq buffer 5 μl, dNTP Mix 4 μl, 1 μl of each primer, distilled water (nuclease-free) 37.75 μl and enzyme ExTaq 0.25 μl. PCR was performed as follows: 35 cycles of denaturation at 94℃ for 30 sec, annealing at 55℃ for 30 sec, extension at 72℃ for 90 sec and final extension at 72 ℃ for 2 min. The PCR products were purified and the bacterial DNA fragments sequenced by Macrogen Japan Corp. The obtained sequences of 400 to 800 bp were used for a similarity search of the 16S rRNA fragments in the 16S database of EzBioCloud (Yoon *et al*., 2017). The accession numbers of the DNA sequences were submitted to the DNA Data Bank of Japan (DDBJ).

RESULTS

Endophytic diazotrophic bacteria isolated from yams

Endophytic bacteria were isolated from roots, stems and leaves. Urea-treated plants were compared with untreated plants (control). The number of isolates was higher in urea-treated plants (21). For both the control and the urea treatment, the bacteria were isolated from all the tested organs. Seven isolates in the urea-treated plants belonged to the class *Bacilli*, nine to the class *Actinobacteria*. The

classes *Alphaproteobacteria* and *Betaproteobacteria* were represented by two isolates each, and one strain belonged to the class *Gammaproteobacteria*. In the control plants, the class *Bacilli* was dominant with 4 isolates, but the *Actinobacteria* and *Betaproteobacteria* strains were not detected. Overall, 21, 14 and six endophytic nitrogen-fixing bacteria were isolated from the stems, roots and leaves of yams, respectively. The phylum *Proteobacteria* accounted for 56.1% followed by the phylum *Firmicutes* at 34.15% and the phylum *Actinobacteria* at 9.76%. The genus *Bacillus* accounted for 31.71% of total isolates, followed by the genus *Rhizobium* (24.39%). Forty-one endophytes were isolated on a nitrogen-free growing medium from yam plants (Tables 2 and 3).

In *Dioscorea esculenta* (Table 2), all tested accessions could harbor fast growing endophytic bacteria on an N-free MR medium. In total 11 isolates belonging to seven genera were found in this species. Seven isolates belonged to the phylum *Proteobacteria*, all being in the class *Alphaproteobacteria*. Two isolates belonged to the class *Bacilli* and two to the class *Actinobacteria*. Three bacteria were isolated from accession E-1 and three from accession E-2, while six were found in E-3. The bacteria of the phylum *Actinobacteria* were not isolated from accession E-1. No bacterial isolates were found in the leaves, while only two isolates were found in the roots of accessions E-1 and E-2. In this study, a greater number of isolates were recovered from the stems (83%).

In *Dioscorea alata* (Table 3), fast growing endophytic diazotrophic bacteria were not found in some of the tested accessions. No bacteria were identified from accessions A-16, A-18, A-19, A-62 and A-74. In accessions from which bacteria were isolated and identified, most of the isolates in all of the tested organs were from urea-treated plants. Roots and stems harbored the same number of bacteria (12). Five strains each were isolated from A-17 and A-112, and four strains each were found in A-23 and A-86. In A-86 and A-112, all the isolates were from urea-treated plants, while in A-17, all the bacteria were from the control plants.

Acetylene reduction assay

All the randomly selected endophytic bacteria exhibited positive ARA (Tables 2 and 3). In *D. esculenta*, the amount of ethylene produced after 24 hours of incubation ranged from 4.1 to 57.4 nmol C_2H_4/tube/24 h. The highest nitrogenase activity was 57.4 nmol C_2H_4 by *Bradyrhizobium* sp., isolate E1T0R_Y6, found in the root of E-1 under unfertilized conditions. In *D. alata*, the ARA values ranged from 4.1 to 164 nmol C_2H_4, the highest value being in isolate A23T1R_Y40. In *D. alata*, isolates showing a high ARA value were found in urea-treated plants.

Table 2 Population of diazotrophic bacteria isolated from accessions of *Dioscorea esculenta*

Isolate[a]	Origin			Close to: (%)	ARA[b]
	Organ	Accession	Treatment		
E1T0R_Y6 (LC373043)	Root	E-1	T0	*Bradyrhizobium lupini* (100)	57.4
E2T0R_Y14 (LC373045)		E-2	T0	*Bacillus altitudinis* (99.4)	NT
E3T1S_Y82 (LC373077)	Stem	E-3	T1	*Curtobacterium citreum* (99.7)	4.1
E3T1S_Y17 (LC373047)		E-3	T1	*Sphingomonas* sp. (98.6)	8.2
E3T1S_Y83 (LC373078)		E-3	T1	*Neorhizobium huautlense* (99.8)	8.2
E3T1S_Y84 (LC373079)		E-3	T1	*Bacillus aryabhattai* (100)	8.2
E2T1S_Y5 (LC373042)		E-2	T1	*Kocuria* sp. (95.1)	NT
E2T0S_Y16 (LC373046)		E-2	T0	*Rhizobium multihospitium* (99.0)	8.2
E3T0S_Y23 (LC373048)		E-3	T0	*Rhizobium* sp. (98.3)	8.2
E1T1S_Y73 (LC373072)		E-1	T1	*Rhizobium multihospitium* (100)	4.1
E3T1S_Y80 (LC373076)		E-3	T1	*Neorhizobium huautlense* (99.9)	8.2

[a]Bacterial isolates are followed by DNA sequences accession number submitted to the DNA Data Bank of Japan (DDBJ). Numbers in parenthesis in related species represent the sequence similarity percentage.

[b]ARA denotes the acetylene reduction assay value, expressed in nmole C_2H_4/tube/24 h. Plants were treated with urea (T1) as source of mineral nitrogen or without urea (T0) as a control. NT: not tested.

Table 3 Related bacterial genus of diazotrophic bacteria isolated from accessions of *Dioscorea alata*

Isolate[a]	Origin			Close to: (%)	ARA[b]
	Organ	Accession	Treatment		
A86T1R_Y60 (LC373061)	Root	A-86	T1	*Bacillus altitudinis* (100)	65.6
A68T1R_Y54 (LC373056)		A-68	T1	*Achromobacter xylosoxidans* (100)	NT
A58T1R_Y51 (LC373054)		A-58	T1	*Devosia yakushimensis* (99.4)	123
A23T1R_Y55 (LC373057)		A-23	T1	*Rhizobium phenanthrenilyticum* (99.1)	NT
A112T1R_Y65 (LC373065)		A-112	T1	*Sphingomonas* sp. (98.2)	12.3
A23T1R_Y40 (LC373052)		A-23	T1	*Ensifer* sp. (98.3)	164
A17T0R_Y78 (LC373075)		A-17	T0	*Mesorhizobium* sp. (98.3)	NT
A23T0R_Y39 (LC373051)		A-23	T0	*Paenibacillus panacisoli* (98.9)	12.3
A17T0R_Y27 (LC373049)		A-17	T0	*Nitratireductor* sp. (98.5)	8.2
A68T1R_Y52 (LC373055)		A-68	T1	*Pseudoxanthomonas indica* (99.4)	NT
A112T1R_67 (LC373067)		A-112	T1	*Rhizobium massiliae* (100)	12.3
A112T1R_Y66 (LC373066)		A-112	T1	*Rhizobium multihospitium* (100)	4.1
A86T1S_Y61 (LC373062)	Stem	A-86	T1	*Bacillus aryabhattai* (100)	8.2
A61T1S_Y71 (LC373070)		A-61	T1	*Bacillus aryabhattai* (100)	8.2
A4T1S_Y87 (LC373080)		A-4	T1	*Bacillus aryabhattai* (100)	8.2
A17T0S_Y77 (LC373074)		A-17	T0	*Enterobacter cloacae* (100)	NT
A44T0S_Y94 (LC373082)		A-44	T0	*Bacillus aryabhattai* (100)	8.2
A112T1S_Y63 (LC373064)		A-112	T1	*Burkholderia contaminans* (100)	NT
A17T0S_Y77 (LC373074)		A-17	T1	*Enterobacter cloacae* (100)	NT
A86T1S_Y72 (LC373071)		A-86	T1	*Mycobacterium cosmeticum* (100)	NT
A23T1S_Y37 (LC373050)		A-23	T1	*Rhizobium* sp. (98.5)	NT
A116T1S_Y46 (LC373053)		A-116	T1	*Rhizobium* sp. (97.8)	41
A61T0S_Y69 (LC373069)		A-61	T0	*Rhizobium multihospitium* (100)	12.3
A17T0S_Y76 (LC373073)		A-17	T0	*Xanthomonas sacchari* (99.2)	NT
A73T1L_Y58 (LC373059)	Leaf	A-73	T1	*Bacillus aryabhattai* (99.8)	8.2
A86T1L_Y59 (LC373060)		A-86	T1	*Bacillus aryabhattai* (100)	8.2
A61T0L_Y68 (LC373068)		A-61	T0	*Bacillus aryabhattai* (100)	8.2
A4T1L_Y90 (LC373081)		A-4	T1	*Bacillus arryabhattai* (100)	8.2
A73T0L_Y57 (LC373058)		A-73	T0	*Bacillus subtilis* (100)	8.2
A112T1L_Y62 (LC373063)		A-112	T1	*Curtobacterium oceanosedimentum* (100)	16.4

[a]Bacterial isolates are followed by DNA sequences accession number submitted to the DNA Data Bank of Japan (DDBJ). Numbers in parenthesis in related species represent the sequence similarity percentage.
[b]ARA denotes the acetylene reduction assay value, expressed in nmole C_2H_4/tube/24 h. Plants were treated with urea (T1) as source of mineral nitrogen or without urea (T0) as a control. NT: not tested.

DISCUSSIONS

The objective of this study was to assess the nitrogen-fixing bacteria associated with several yam accessions unlike in previous studies. In our study, endophytic diazotrophs could not be isolated from five yam accessions; all in *D. alata*, including A-19. The reaction time during the surface-sterilization step was longer than in other studies (Muangthong *et al*., 2015), resulting in possible damage to plant tissue and endophytic bacterial cells. This could also explain why only the accessions of *D. alata*, which have softer tissues than *D. esculenta*, were affected by the strong surface-sterilization treatment.

Several strains belonging to different genera were isolated from yam accessions. The class *Alphaproteobacteria* was the dominant class and included the genera *Bradyrhizobium* (1), *Devosia* (1), *Ensifer* (1), *Mesorhizobium* (1), *Neorhizobium* (2), *Nitratireductor* (1), *Rhizobium* (9), and *Sphingomonas* (2). Our finding was in line with previous studies showing that strains of the class *Alphaproteobacteria* are found in a large number as nitrogen-fixing bacteria in soils and tissues of legumes and non-legumes (Tsoy *et al*., 2016). We isolated *Ensifer* sp. A23T1R_Y40 with the highest acetylene reduction. Some strains of this genus such as *Ensifer medicae* are capable of fixing biological nitrogen (Reeve *et al*., 2010; Li *et al*., 2016). There have been few reports

on the presence of this genus in non-legumes. Recently, one strain was isolated from the roots of rice (Zhang *et al*., 2010). To our knowledge, this is the first study reporting the genus *Ensifer* as endophytic nitrogen-fixing bacteria in a tuber crop.

The bacterial classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* have been reported as nitrogen-fixing bacteria in plants and soils (Taule *et al*., 2012). In our study, we identified strains of the genera *Achromobacter* and *Burkholderia*, and *Enterobacter*, *Pseudoxanthomonas* and *Xanthomonas*, in the classes *Betaproteobacteria* and *Gammaproteobacteria*, respectively. A strain, A68T1R_Y52 showing 99.38% similarity with *Pseudoxanthomonas indica* was isolated in this study. Grapevine roots have been reported to harbor strains of the genus *Pseudoxanthomonas*, which possesses plant growth-promoting characteristics (Marasco *et al*., 2013). However, evidence of nitrogen fixation by this genus has yet to be reported. In this study, the isolate A68T1R_Y52 grew quickly on an N-free MR medium.

In the phylum *Firmicutes*, thirteen strains were isolated and identified as being closely related to *Bacillus* spp. (12) and *Peanibacillus panacisoli* (1). Several strains belonging to these two genera were shown to be capable of biological nitrogen-fixation in non-legumes (Ding *et al*., 2005). Grady *et al*. (2016) reported that strains of *Paenibacillus* species play important roles, including hormone production and phosphate solubilization. Several strains of this genus were identified as nitrogen-fixing bacteria (Xie *et al*., 2014). This is the first report on the ability of *P. panacisoli* to fix atmospheric nitrogen as plant endophytic bacteria. Different strains of *Bacillus* spp. were isolated from tissues of several crops such as rice (Mano *et al*., 2006, 2007; Mbai *et al*., 2013) and sugarcane (Magnani *et al*., 2010) with plant growth-promoting characteristics.

Numerous strains belonged to the phylum *Actinobacteria* have been involved in plant growth-promoting activity, including nitrogen fixation, phosphate solubilization and phytohormones production (Sathya *et al*., 2017), with the genus *Mycobacterium* as one of the most common isolates., *Kocuria* sp. E2T1S_Y5 was isolated in the stem of *D. esculenta* E-2. Important crops such as rice have been shown to harbor strains belonging to the genus *Kocuria* in their seeds (Kaga *et al*., 2009). These bacteria were also identified in the Jatropha (*Jathropha curcas*) plant as endophytic bacteria (Madhaiyan *et al*., 2015). To our knowledge, this study is the first report on the potential nitrogen fixation of *Kocuria* sp.

The application of mineral N in the form of urea did not reduce the number of endophytic nitrogen-fixing bacteria as previously reported (Lin *et al*., 2012). The amount of urea applied in this study was 10 times less than the amount recommended for yams in Okinawa prefecture (Japan). Although the effect of nitrogen application on the dynamics of endophytic bacteria of yams is not yet known, the amount of urea applied in this study (using N-deficient soil) could provide the bacteria with an adequate amount of nutrient N to realize a better growth and colonization ability. All the isolates tested in this study could grow satisfactorily on an N-free medium, supporting their ability to fix nitrogen from air. Representative isolates were selected and tested for acetylene reduction, and they could all reduce acetylene. Further studies are needed to evaluate the potential of these isolates as plant growth-promoting bacteria through biological nitrogen fixation in yams. This study revealed the strong effect of plant genotype on the number of endophytic diazotrophs found in yams. Several studies have reported that plant roots harbor more endophytic bacteria than stems and few bacteria are found in leaves (Gyaneshwar *et al.*, 2001; Koomnok *et al*., 2007) due to the fact that most endophytic bacteria in plants are recruited from the surrounding soil. However, our study showed that in yams, the plant genotype has a strong effect. Significant differences were observed among accessions of *D. esculenta*. Thus, no fast-growing endophytic diazotrophs were found in the roots of accession E-3, in which all isolated bacteria were in the stems. An investigation of the physiological and molecular factors involved in bacteria movements within yam plant organs is needed. Overall, accessions of *D. esculenta* harbored most endophytic bacteria in the upper parts (stems and leaves). In *D. alata*, there was no difference between the number of diazotrophs in the roots and the stems. This finding was in line with that of Koomnok *et al*. (2007) who reported that depending on rice genotype, a similar number of nitrogen-fixing bacteria was found in the leaves, stems and roots as also reported in sugarcane by Muangthong *et al*. (2015).

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ダイジョ系統とトゲイモ系統に関連する窒素固定細菌の分離と同定

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ヤムイモ（*Dioscorea* spp.）は熱帯地域などでは重要な作物である．近年，ヤムイモの生長には内生する窒素固定細菌が寄与している可能性が指摘され始めている．しかしヤムイモには多様な系統が存在するため，本研究ではダイジョ（*Dioscorea alata* L.）の16系統とトゲイモ（*Dioscorea esculenta* L.）の3系統，併せて2種19系統のヤムイモを窒素施用と無施肥の条件で160日栽培し，茎，根，葉の部位に内生する窒素固定細菌の分離と16S rRNA遺伝子配列に基づく同定を行った．その結果，全部位から窒素固定細菌が併せて41株が分離された．分離株は *Bacillus* 属，*Bradyrhizobium* 属，*Devosia* 属，*Ensifer* 属，*Mycobacterium* 属，*Neorhizobium* 属，*Paenibacillus* 属，*Pseudoxanthomonas* 属，*Rhizobium* 属，*Xanthomonas* 属など18属と同定された．分離株の窒素固定能はアセチレン還元活性で評価し，トゲイモからの分離株は4.1から57.4 nmol C_2H_4 tube/day/24 h，ダイジョからの分離株は4.1から164 nmol C_2H_4 tube/day/24 hであり，試験した株はすべて固定能を有していた．そして，部位や系統の違い，施肥の有無による内生窒素固定細菌種が異なり，ヤムイモに内生する窒素固定細菌は多様であることが明らかとなった．