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SHORT COMMUNICATION

# Molecular diversity and spore density of indigenous arbuscular mycorrhizal fungi in acid sulfate soil in Thailand

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**Abstract** Acid sulfate soil (ASS) has an extremely low pH (3.0) and a high capacity to fix phosphate; symptoms of phosphate deficiency are commonly observed in many crop plants. Arbuscular mycorrhizal (AM) fungi form mutualistic relationships with plant roots, and improve uptake of phosphate from soil. However, there is little information on the actual situation of AM fungi in ASS in Thailand. The purpose of the present study is to determine the indigenous AM fungal density and species in ASS in Thailand. AM fungal spores were retrieved and identified by molecular approaches from ASS field at the central plain of Thailand. This study showed that AM fungal spore density in ASS was 0.232 spores per g dry soil. Among the plant species growing in the natural ASS, there was no AM fungal colonization in the roots of four plant species, i.e. *Digitaria* sp., *Fimbristylis* sp., *Mimosa pudica* L., and *Sesbania* sp.; however, AM colonization was found in *Wedelia* roots. Using phylogenetic analysis, four operational taxonomic units (OTUs), i.e., one *Glomus*, one *Entrophospora*, one *Paraglomus* and one unknown species were identified from the AM fungal spores. Five OTUs, i.e., two *Glomus*, one *Acaulospora*, one *Entrophospora* and one unknown Glomeromycota were indentified from *Wedelia* roots. To our knowledge, this is the first report of the actual situation of AM fungi in ASS in Thailand determined by using molecular approaches.

**Keywords** Acid sulfate soil · Arbuscular mycorrhizal fungi · LSU rDNA · Spore density

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## Introduction

Acid sulfate soil (ASS) is distributed all over the world, and covers 12.5 million ha of arable land around the world, and about 1.5 million ha in Thailand. The proportion of ASS reaches 10% of the cultivated acreage worldwide (Land Development Department 2002 http://www.ldd.go.th./). Due to the accumulation of aluminum ($Al^{+3}$) and ferric ($Fe^{+3}$) ions in the ASS, the pH is extremely low (3.0) and most crop plants cannot be cultivated without improvement of the soil acidity. Furthermore, since ASS has a high capacity to fix phosphate, symptoms of phosphate deficiency are commonly observed in many crops (Jugsujinda et al. 1978; Sanyal et al. 1993). In fact, ASS is a major cause of low yield of crops, and hence improvement of ASS is an urgent issue (Kang et al. 2001). Application of lime is the most practical and useful means to improve the crop yield in acid soil conditions, but the increasing cost of lime is gradually making it difficult to cultivate the crops in ASS regions.

It is well known that arbuscular mycorrhizal (AM) fungi improve the uptake of immobile mineral nutrients such as phosphate, thereby improving plant growth (Smith and Read 1997). AM fungi are obligate biotrophs, which is a requirement to colonize host plants for new spore formation. Spore production is highly dependent on environmental conditions: in general, AM fungal spore formation is suppressed by extreme acidity and alkalinity. Therefore, even if AM fungi exist in ASS, the density and diversity of the AM fungi in ASS would be extremely low. However, there is little information on the actual situation of AM fungi in ASS in Thailand. The purpose of the present study is to determine the indigenous AM fungal density and species in ASS in the central plain of Thailand.

## Materials and methods

### Sampling and measurements of chemical properties of ASS

Fifteen independent soil samples (depth 10 cm, diameter 20 cm) were collected on 8 January 2005 from the ASS field of the Royal Acid Sulfate Soil Improvement Experiment Station (ASSIES) in Banna of Nakhon Nayok Province of Thailand (14°14′34″N, 100°59′01″E). After the individual soil samples were air-dried, they were combined and passed through a 2-mm mesh sieve for analysis. The soil pH was determined with a 1:2.5 ratio of soil (dry weight) and distilled water (volume) using a pH meter (HI-9811; Hanna, Tokyo, Japan), and the content of available phosphate was analyzed by the method of Bray and Kurtz (1945). The phosphate absorption coefficient was determined by the method of Nanjo (1986) and total phosphorus was analyzed for molybdenum yellow colorimeter methods (Sekiya 1970). The soil analysis was repeated 15 times.

### Spore collection and counting

The AM fungal spore density in the soil was counted according to the method of Brundrett et al. (1996). Samples of 20 g of fresh soil were passed through 500-μm and then 53-μm meshes. The residues on the 53-μm mesh were subjected to sucrose density gradient centrifugation to obtain the spores. The number of the spores in the soil was counted under a stereo microscope (SZX12; Olympus, Tokyo, Japan), and then under an optical fluorescence microscope (BX50; Olympus). The measurement of spore density was repeated 15 times.

### Root sampling

The indigenous plants inhabiting the surrounding environment in ASSIES were examined in an area of about 750 $m^2$ (50 m long×15 m wide) of the ASS field on 27 January 2007. Fine root samples from indigenous plants species belonging to the genera of *Digitaria*, *Fimbristylis*, *Mimosa*, *Sesbania* and *Wedelia* growing in the ASS field were collected. Each root sample was divided into two sub-samples. One sub-sample was used to determine mycorrhizal colonization, while the other one was dried with silicon gel prior to molecular analysis.

### Assessments of AM fungal colonization

Samples of fine roots were cleared in 10% w/v KOH and were stained with 0.5% trypan blue solution (Oba et al. 2006). After staining the roots with trypan blue, the mycorrhizal colonization was quantified using the grid line intersect method (Giovannetti and Mosse 1980). Counting was done using a stereo microscope at ×90 magnification (SZX12; Olympus). An average of 200 root pieces per plant and 7 plants per an indigenous plant species were measured for AM fungal colonization.

### DNA extraction from roots and spores

DNA extraction from root samples was done using the method of Wu et al. (2007). DNA extracted from five pieces of 1- to 2-cm-long root fragments per one plant were pulverized in a 2.0-ml tube containing five 2.0-mm zirconium balls using an MS-100 microhomogenizing system (Tomy Digital Biology, Tokyo, Japan) at 4,200 rpm for 1 min. After the addition of 500 μl of 2× cyltrimethylammonium bromide (CTAB) solution [2% CTAB, 0.1 M Tris-HCl (pH 8.0), 20 mM ethylenediaminetetraacetic acid (EDTA; pH 8.0), 1.4 M NaCl, and 0.5% β-mercaptoethanol], the sample was homogenized again at 4,200 rpm for 1 min with the system and incubated in a block heater at 65°C for 1 h. After mixing with 500 μl of chloroform:isoamyl alcohol mixture (24:1, v:v), it was centrifuged at 15,000 rpm for 7 min. The supernatant was transferred to a 1.5-ml tube, mixed with an equal volume of isopropyl alcohol and kept at −30°C

**Table 1** Soil chemical properties and AM fungal spore density in ASS

| pH ($H_2O$) | Total phosphorus (mg P $g^{-1}$) | Available phosophate (mg $P_2O_5$ $g^{-1}$) | Phosphate absorption coefficient | AM fungal spore density (spores $g^{-1}$ DW) |
|---|---|---|---|---|
| 3.24 | 0.31 | 0.04 | 2673 | 0.232 |

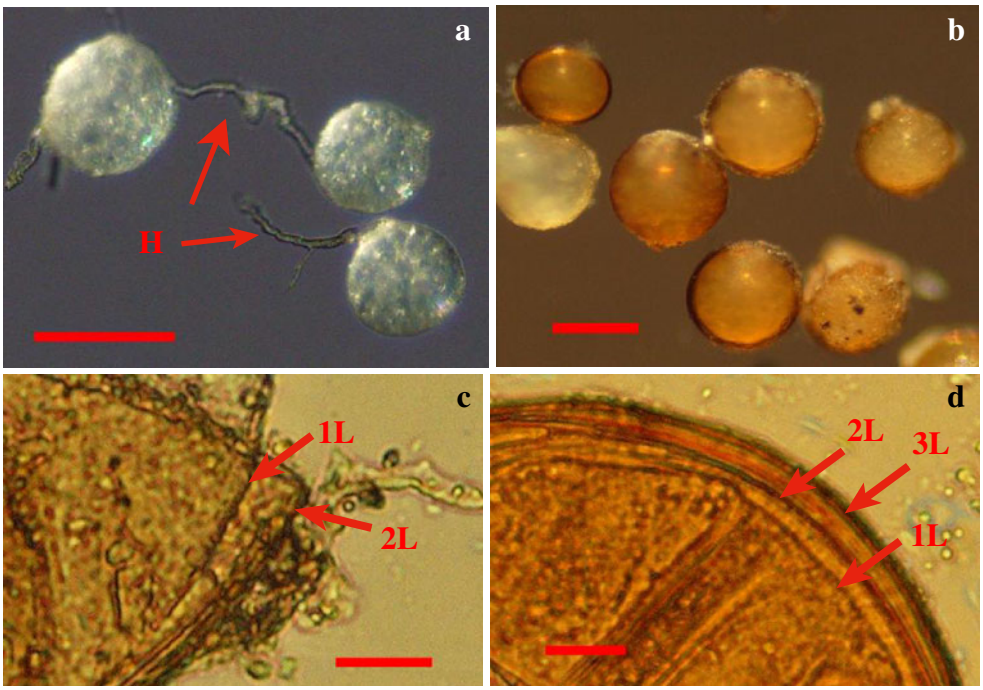


**Fig. 1** The indigenous spores of AM fungi collected from ASS. **a** Hyphal attachment spore, **b** no hyphal spore, **c**, **d** spore layer. : *Scale bars* (**a**, **b**) 100 μm, (**c**, **d**) 20 μm. *H* hyphae, *1L* first spore layer, *2L* second spore layer, *3L* third spore layer

for 10 min to precipitate the DNA. After centrifugation at 8,000 rpm at 4°C for 10 min, the DNA pellet was washed once with 80% ethanol, dried, resuspended in 120 μl of Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)], and stored at −30°C until use. In DNA extraction from AM fungal spores, 5–25 spores collected in ASS were washed five times with sterilized distilled water and transferred to a 0.5-ml tube containing 20 μl of sterilized distilled water and five 2.0-mm zirconium balls, and were then homogenized at 4,000 rpm for 1 min with the system. After adding a small amount of sterilized distilled water, the pellet in the tube was homogenized again at 4,000 rpm for 1 min and then centrifuged at 8,000 rpm for 2 min. The supernatant was transferred to a 1.5-ml tube and stored at −30°C until use for polymerase chain reaction (PCR).

## Nested PCR amplifications

The DNA samples extracted from the plant roots and spores were used as PCR templates after 20-fold dilution. The amplification of a region in the fungal large subunit ribosomal DNA (LSU rDNA) was conducted using a nested PCR method (Gollotte et al. 2004). The fungus-


**Fig. 2** Indigenous plants species inhabiting the ASS. **a** *Fimbristylis* sp., **b** *Mimosa pudica* L., **c** *Sesbania* sp., **d** *Digitaria* sp., **e** *Wedelia* sp.

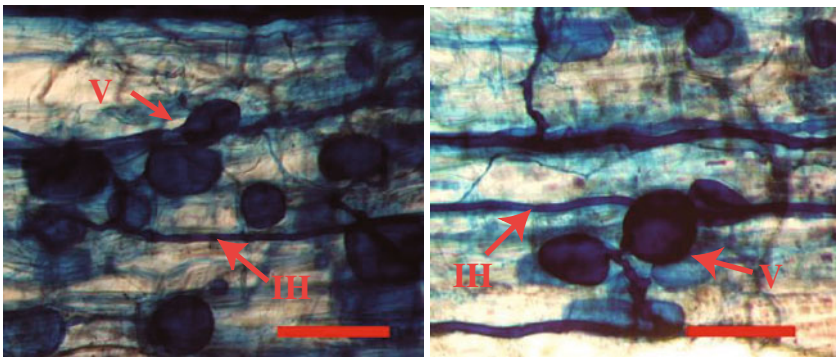


**Fig. 3** AM fungal colonization in *Wedelia* roots. *Scale bar* 50 μm. *V* vesicle. *IH* internal hyphae

specific primer LR1 (5′-GCA TAT CAA TAA GCG GAG GA-3′) (van Tuinen et al. 1998) and FLR2 (5′-GTC GTT TAA AGC CAT TAC GTC-3′) (Trouvelot et al. 1999) were used in the first PCR to amplify the 5′ end of the LSU rDNA region. The first PCR products were diluted 100-fold and used as templates for the second PCR using the nested primers FLR3 (5′-TTG AAA GGG AAA CGA TTG AAG T-3′) and FLR4 (5′-TAC GTC AAC ATC CTT AAC GAA-3′) (Gollotte et al. 2004) under the same PCR conditions. PCR was performed in a 10-μl reaction mixture containing 1 μl of template DNA, 1 μl of 10× PCR buffer, 0.2 mM of each deoxyribonucleotide triphosphate, 0.3 μM of each primer, and 0.25 U of TaKaRa *Taq* DNA polymerase (Takara Shuzo, Tokyo, Japan), using a thermal cycler (Mastercycler Ep gradient; Eppendorf, Hamburg, Germany). The PCR protocol was composed of an initial treatment at 94°C for 1 min, 30 cycles of treatments at 94°C for 30 s, 54°C for 30 s, and 72°C for 1 min, and a final treatment at 72°C for 10 min. The first PCR product, diluted 100-fold, served as a template for the second PCR reaction targeting AMF; amplification conditions were as described above. Amplification products were separated by gel electrophoresis on 1% agarose gel in TAE buffer (40 mM Tris, pH 8.0, 40 mM acetic acid and 1 mM EDTA) and DNA was visualized by staining with ethidium bromide.

## Cloning and sequencing

Subcloning was conducted by the method of Renker et al. (2006). Second PCR products yielded positive amplicons from the indigenous plant roots and AMF spores DNA extracts samples were subcloned into pT7 Blue vector using the Perfectly Blunt cloning kit (Novagen, Madison, WI, USA) according to the manufacturer's instructions. The vector was transformed into *Escherichia coli*. The transformants were plated on LB agar plates containing 50 μg $ml^{-1}$ ampicillin and X-Gal/IPTG. Screening for recombinant cells was carried out by blue/white selection. The presence of inserts in the plasmid was confirmed with colony PCR using M13 forward and reverse primers.

Nucleotide sequences were determined with an ABI BigDye terminator v3.1 cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan) according to the instruction supplied and with a PE Applied Biosystems Automated DNA sequencer (model 3130xl; Applied Biosystems).

## Reconstruction of phylogenetic trees, rarefaction curves

The AM fungal species were inferred from sequence homologies with sequences registered in the DNA Data Bank of Japan (DDBJ, http://www.ddbj.nig.ac.jp/). Multiple alignments were performed using CLUSTAL W algorithm. Neighbor-joining phylogenic trees were constructed using MEGA4 (Tamura et al. 2007), *Mortierella verticillata* (Accession no. AF157199) was used as an outgroup. Nucleotide sequences with greater than 97% identity were referred as a same operational taxonomic unit (OTU) group. Rarefaction curves were constructed with the freely available software Analytic Rarefaction Program version 1.3 (http://www.uga.edu/strata/software/Software.hyml) in order to determine whether the number of sequenced samples tested sufficiently represented AM fungal OTU in the plant roots and AM fungal spores. The sequences obtained in this study have been deposited in the DDBJ database and assigned accession numbers from AB494433 to AB494437 and AB494439 to AB494442.

**Table 2** AM fungal colonization in indigenous plant species inhabited in ASS

| Plant species | AM fungal colonization (%) |
|---|---|
| *Digitaria* sp. | 0.0 |
| *Fimbristylis* sp. | 0.0 |
| *Mimosa pudica* L. | 0.0 |
| *Sesbania* sp. | 0.0 |
| *Wedelia* sp. | 3.0 |

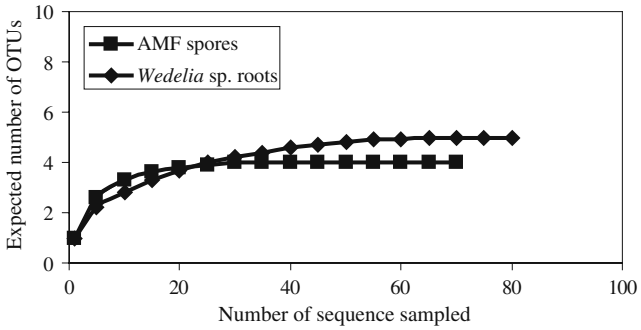


**Fig. 4** Rarefaction curves of the OTUs obtained from the indigenous spores (*rhombus*) and *Wedelia* roots (*square*). Nucleotide sequences with greater than 97% identity were referred as a same OTU group

## Results

### Soil chemical properties and spore density in ASS

The ASS had a very low soil pH of 3.24, and the phosphate absorption coefficient was high with a value was 2,673 (Table 1). Moreover, the content of available phosphate and total phosphorus was 0.04 mg and 0.31 mg per g dry soil, respectively. Furthermore, genera of *Glomus*-like (Fig. 1a) and *Acaulospora*-like (Fig. 1b) spores were observed even under pH 3 in the natural ASS field, and the spore density was 0.232 spores per g dry soil (Table 1). The spore color was clean white to yellow brown, diameter about 100 μm and they were formed singly in soil. Moreover, they had two or three layered walls (Fig. 1c, d).

### AM fungal colonization

The vegetation composition of the sampling plot was in patches, and the indigenous plant species inhabiting the sampling plot in ASSIES were identified as *Digitaria* sp., *Fimbristylis* sp., *Mimosa pudica* L., *Sesbania* sp. and *Wedelia* sp. (Fig. 2). Among the plant species growing in the natural ASS, no AM fungal colonization of four of them, i.e. *Digitaria* sp., *Fimbristylis* sp., *Mimosa pudica* L., and *Sesbania* sp., was observed. No arbuscule were visible, but internal hyphae and vesicle were observed only in *Wedelia* roots (Fig. 3), with AM fungal colonization of 3.0% (Table 2).

### Phylogenetic analysis

In this study, 83, 74-sequence samples were analyzed from the *Wedlia* roots and indigenous AM fungal spores, respectively. The rarefaction curves on the basis of analyzed sequence numbers of both the *Wedlia* roots and the indigenous AM fungal spores almost reached a plateau (Fig. 4). Overall, seven different AM fungal OTUs were detected in the present study (Fig. 5). Four OTUs were detected from

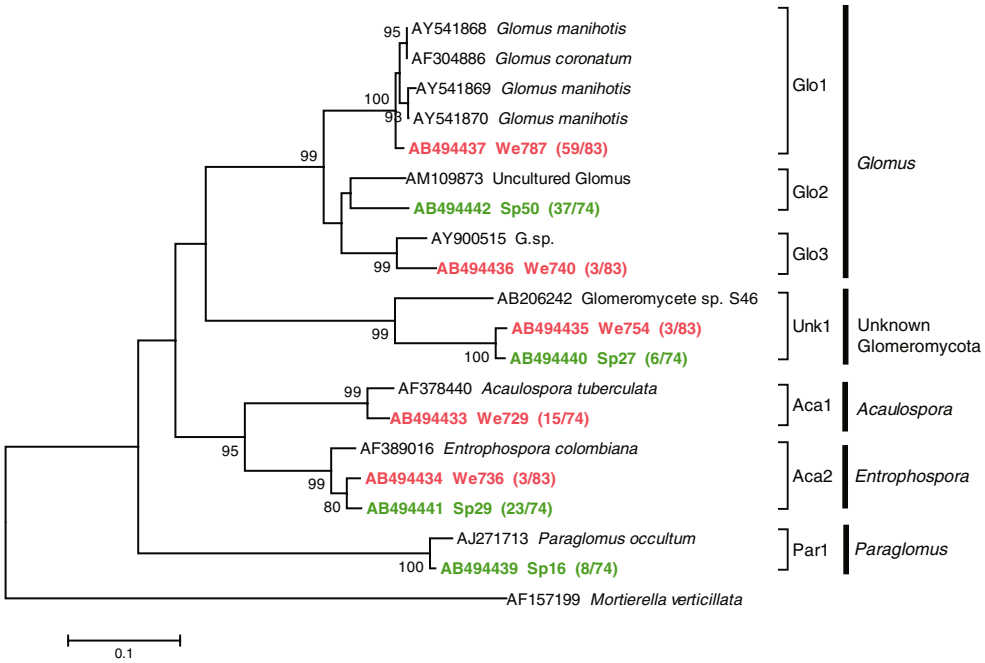


**Fig. 5** Neighbor-joining tree of partial LSU rDNA sequences from AM fungi, isolated from AM fungal spore (*green*) and *Wedelia* root samples (*red*) collected from ASS, including known AM fungal sequences from DDBJ database for comparison. Bootstrap values (only values >80 are shown) were estimated from 1,000 replicates. Representative sequences from roots and spores are incorporated. Each individual sequenced sample is labeled with a prefix (*We* and *Sp* denote the *Wedelia* roots and AM fungal spores, respectively) followed by the sample number. *Arabic numbers in parentheses* indicate the number of detected sequence clones (*above slash*) and the number of total sequenced clones (*below slash*)

the AM fungal spores, i.e., one *Glomus* (Glo2), one *Entrophospora* (Aca2), one *Paraglomus* (Par1) and one unknown Glomeromycota (Unk1). Glo2 showed no similarity to any known species but was related to uncultured *Glomus*. Aca2 and Par1 showed high-sequence similarity to *Entrophospora colombiana* and *Paraglomus occultum*, respectively. Unk1 was related to *Glomeromycete* sp. S46 (Fig. 5). Glo2 and Par1 were only detected in AM fungal spores. Five OTUs, i.e. two *Glomus* (Glo1, Glo3), one *Acaulospora* (Aca1), one *Entrophospora* (Aca2) and one unknown Glomeromycota (Unk1) were found in *Wedelia* roots. Glo1 showed high-sequence similarity to *Glomus manihotis*. Glo3 was related to *Glomus* sp. Aca1 also showed high-sequence similarity to *Acaulospora tuberculata*. Glo1, Glo3 and Aca1 were detected only in the *Wedelia* roots. AM fungal OTU of the spore did not correspond with that of colonized plant roots. Two OTUs, Aca2 and Unk1 were common to *Wedelia* roots and AM fungal spores.

## Discussion

This is the first report that indigenous AM fungal species were observed and detected by using molecular approaches in ASS in Thailand. From molecular analysis, the AM fungal OTU compositions of the indigenous spores did not correspond with that of colonized roots. Recently, increasing evidence from molecular approaches has shown a poor match between the species of AM fungal spores and AM fungi in roots of grassland plants (Clapp et al. 1995; Merryweather and Fitter 1998; Renker et al. 2005; Börstler et al. 2006; Hempel et al. 2007; Li et al. 2010). These results are similar to our results in ASS. Indeed, our results showed that *G. manihotis* was identified only from roots and no sporification was observed in ASS. Similarly, Li et al. (2010) also showed that AM fungal OTU within plant roots were not found from AM fungal spores in disturbed ecosystems using a molecular approach. As described above, since the AM fungal communities in plant roots cannot necessarily reflect that of AM fungal spores in soil. Hence, *G. manihotis* may also be found only from roots in this study.

Furthermore, Uhlmann et al. (2006) reported that *Glomus aggregatum* was frequently found in many spores in soil, although the AM colonization in plant roots was not high. Conversely, although *Glomus intraradices* frequently colonized plant roots, this species did not form many spores in soil (Uhlmann et al. 2006). Clapp et al. (1995) and Sanders et al. (1996) reported that *G. intraradices* frequently sporulated in the plant roots. As described above, the sporification ability may differ among species. In addition, co-occurring plant species in grassland ecosystems or woodlands can host very different AM fungal communities, and that host–plant preferences exist (Helgason et al. 2002; Vandenkoornhuyse et al. 2002). Thus, the AM fungal OTU composition of the spores in ASS did not correspond with that of colonized roots. Moreover, the spores of *G. manihotis* are often a dominant species in highly acidic soils (Bartolome-Esteban and Schenck 1994), but our results did not correspond with their published data. Some AM fungus may produce many spores under certain conditions or during certain seasons of the year, whereas under different conditions, they may not sporulate at all (Bever et al. 1996). In this study, conditions for sporification of *G. manihotis* may be unfavorable due to the environmental conditions, seasons or host preferences between growing plants in ASS, thereby *G. manihotis* may be detected only in roots using molecular approaches.

Some AM fungal species, such as *G. manihotis*, *E. colombiana*, and *Paraglomus occultum*, have been detected in other acidic soils (Bartolome-Esteban and Schenck 1994; Moreira and Cardoso 2003; Moreira et al. 2007; Appoloni et al. 2008), as is the case in our study. Howeler et al. (1987) reported that *G. manihotis* and *E. colombiana* can colonize cassava root even in low pH soil. Bartolome-Esteban and Schenck (1994) found that spore germination of *G. manihotis* was not inhibited in acid soil with low pH conditions. Ghosh et al. (2006, 2008) showed that *P. occultum* was more efficient for AM colonization of plants from inoculation experiments under acid soil condition compared with other *Glomus* species. As described above, AM fungal species such as *G. manihotis*, *E. colombiana*, and *P. occultum* may have been favored to survive under ASS conditions. Thus, we suggest that AM fungal species detected in ASS may have been more resistant to an extreme acid soil condition. In addition, we anticipate that the indigenous AM fungal species found in ASS will provide promising lineages for use in acid soil conditions.

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