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Identification and function of *Gigaspora margarita* growth-promoting microorganisms

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Abstract

In an *in vitro* study, 5 kinds of helper-microorganisms which promote the growth of *Gigaspora margarita* Becker and Hall were isolated. Three fungal strains (KHIMF1, KHIMF2 and KHIMF3) and two bacterial strains (KHIMB1 and KHIMB2) were characterized using sequence similarities of the rDNA-internal transcribed spacer (ITS), slide culture methods and gram staining. The fungal strains were similar to *Woollsia* root-associated fungus XV (Accession Number #AY230785) (KHIMF1), *Candida parapsilosis* (KHIMF2), or *Cladosporium cladosporioides* (KHIMF3), respectively. The bacterium KHIMB1 could not be identified, but KHIMB2 had a 95% sequence homology with *Methylobacterium* sp. The effects of volatile compounds and exudates released from these microorganisms on hyphal growth of *G. margarita* were investigated. The volatile compounds of all of the fungal strains and KHIMB1 stimulated the hyphal growth of *G. margarita*. Ethylene, a volatile stimulator of mycorrhizal fungul growth, was produced by these microorganisms. The exudates of KHIMB2 increased the hyphal length of *G. margarita*. Scanning electron microscopy showed that the surface of *G. margarita* could be colonized by the isolated helper-microorganisms. Our results suggest that although different types of helper-microorganisms are associated with *G. margarita*, the mechanisms which stimulate its growth relate to ethylene or non-volatile compounds released from the helper-microorganisms. We hypothesize that ethylene or non-volatile compounds released from the helper-microorganisms will stimulate the growth of *G. margarita in situ.*

Keywords: Arbuscular mycorrhizal fungi, helper-microorganism, ethylene, *Gigaspora margarita*

1. Introduction

Arbuscular mycorrhizal (AM) fungi are known to influence and to be influenced by other microorganisms in the soil (Andrade et al., 1997). The interaction between soil microorganisms and AM fungi is important for plant growth and several positive interaction between AM fungi and associated microorganisms have been described (Linderman, 1992). Mosse (1962) reported that the infection with *Endogone* stimulated the establishment of AM infections. Mayo et al. (1986) found that spore germination of *Glomus versiforme* was stimulated by *Pseudomonas* and *Corynebacterium* spp. isolated from non-sterilized spores. Furthermore, the presence of yeasts or their soluble and volatile exudates could stimulate spore germination and hyphal growth of *Glomus mosseae* (Sampedro et al., 2004). Bacterium-like organisms (BLO) were found in the vacuoles of *G. margarita* (Bonfante et al., 1994; Cruz, 2004), but it is not clear how such organisms influence AM fungi.

Ishii et al. (1995) succeeded in the axenic culture of the AM fungus, *Gigaspora ramisporophora*, by using the 25% methanol eluates of bahiagrass roots. However, the successful re-growth of sub-cultured hyphae under axenic conditions was slow. We hypothesized that better growth could be obtained by growing AM fungi in the presence of microorganisms isolated from the spores of AM fungi.

The present study aimed at characterizing such microorganisms, and investigating the effect of chemical compounds produced by them on spore germination and hyphal growth of AM fungi. Those microorganisms surrounding the AM spores were observed using a scanning electron microscope (SEM).

2. Materials and Methods

Sampling helper-microorganism strains

To sterilize the surface of *G. margarita* spores, a solution of 0.7 g chloramines T + 5.6 mg streptomycin + 2.1 mg chloramphenicol in 100 ml distilled water containing a few

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drops of Tween 80 was used. Sterilization for 15 min by the solution killed almost all microorganisms but had no effects on germination of *G. margarita* spores. A 20 min-treatment, however, was enough to kill *G. margarita* spores. On the basis of these results, the following method was used.

The surface of *G. margarita* spores (Central Grass Co., Ltd., Tokyo) was treated for 15 min using the sterilizing solution mentioned above. The aim of the 15 min-treatment was to reduce the number of microorganisms. After sterilization, the spores were rinsed in sterilized water, and individually transferred to a Petri dish (7 cm in diameter) containing 10 ml of 1.5% agar only. After 1 week, 32 microorganisms were isolated from the agar surrounding the *G. margarita* spores using a dilution plate method, Potato dextrose agar (PDA) (Nichiei Co., Ltd., Tokyo) medium was used.

The 22 fungi and 10 bacteria were isolated and cultivated on potato dextrose agar (PDA) (Nichiei Co., Ltd., Tokyo) medium and peptone and yeast (Nissui Co. Ltd., Tokyo) medium, respectively. A small plug of agar was cut out with a cork borer and microbial plug 5 mm diameter was prepared. This was transferred to a Petri dish containing 10 ml of 1.5% agar. Spores of *G. margarita* previously sterilized for 15 min by the aforesaid solution were individually transferred to the agar media and kept in contact with the plug of cultural microorganism. The spores were incubated at 27°C in the dark. After 1 week, hyphal growth of *G. margarita* was measured using an image-processing system equipped with a light microscope and a personal computer (Ishii and Kadoya, 1994).

Identification of helper-microorganisms

Fungi were cultured in potato dextrose (PD) media (200 g potato soup and 20 g sucrose in 1 L distilled water) on a shaker at 27°C for 3 days. Fungal hyphae were filtered and then crushed in liquid N_2 and 5 ml Lysis buffer added (50 mM Tris HCl, pH 8, 150 mM EDTA, 0.1% lauroyl sarkosine, and 500 μg/ml pronave E). The samples were incubated at 60°C for 20 min, and centrifuged at 3,000 rpm for 10 min. The supernatant was extracted once with phenol-chloroform-isoamyl alcohol (25:1:1, v/v/v), and then centrifuged at 3,000 rpm for 10 min. DNA in the supernatant was precipitated with equal volume of isopropanol, then centrifuged at 14,000 rpm for 15 min at 4°C. The DNA was washed with 70% ethanol, dried, and suspended in 50 μl of sterilized water. One μl of this solution was used as the template of polymerase chain reaction (PCR).

The oligonucleotide primers for ITS rDNA specific PCR (ITS5: 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ITS4: TCCTCCGCTTATTGATATGC-3', and 18SF1: 5'-AGGTTTCCGTAGGTGAACCT-3' and 58SR1: 5'-TTCGCTGCGTTCTTCATCGA-3') (Makimura et al., 2001) were made by Prorigo Japan Co, Ltd. (Tokyo). Each PCR contained 5 μl of 10× reaction buffer, 0.2 mM each dATP, dCTP, dGTP, and dTTP, and 2.5 U of Taq polymerase (all from TOYOBO Co. Ltd., Tokyo), as well as 5 pmol of each primer and DNA template solution. Sterilized water was added to increase the volume to 50 μl.

Bacteria were cultured in peptone-yeast media (5 g peptone and 5 g yeast in 1 L distilled water) on a shaker at 27°C for 24 h. DNA was extracted by using ISO PLANT Kit (Nippon Gene Co. Ltd., Tokyo). The oligonucleotide primers for 16S rDNA partial sequence specific PCR (27f: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1492r: 5'-GGCTACCTTGTTACGACTT-3' and 907f: AAACTCAAATGAATTGACGGG-3' and 1492r) were made by Prorigo Japan Co, Ltd., Tokyo. Each PCR contained the same solution as mentioned above.

DNA amplification was carried out using the Takara PCR Thermal cycler PERSONAL (Takara Co. Ltd., Tokyo). There was an initial denaturation at 94°C for 3 min, This was followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 42°C for 2 seconds, extension at 74°C for 30 seconds, and a final extension at 20°C for 3 min. The PCR products were extracted from agarose gel using GENECLEAN KIT (BIO 101 Systems Co. Ltd., Tokyo). Purified DNA was sequenced using a BigDye DNA Sequencing Kit (Applied Biosystems Co. Ltd., Tokyo) and a Genetic Analyzer 310 (Applied Biosystems). The nucleotide sequence data were compared with available sequences using the BLAST program (http://www.ebi.ac.uk/blastall/nucleotide.html).

The colony characteristics of the sampled strains were observed from cultures grown 27°C for 14 days at on PDA (Nichiei Co. Ltd., Tokyo) plates for fungi, or peptone and yeast (Nissui Co. Ltd., Tokyo) plates for bacteria. The morphological features of helper-microorganisms were observed with light microscopy using gram staining (B&M method) and slide culture. For the latter, a thin layer of medium is prepared on slide glass, and the microbe is cultured on the medium under sterile condition (Bartholomew and Mitter, 1952).

AM growth stimulants released from helper-microorganisms

The fungi and bacteria were grown on PDA medium and peptone and yeast medium, respectively. Plugs of the medium were cut out with a cork borer of 5 mm in diameter. The microbial plug was transferred to a Petri dish containing 10 ml of 1.5% agar. To investigate the effects of substances (exudates and volatile compounds; EVC), the spores of *G. margarita* were individually transferred to the agar media and placed in contact with the plug.

To investigate the effects of volatile compounds (VC), a well (1 cm in diameter) was punched out on the agar medium with a cork borer at the center of Petri dish. A microbial plug was put on the center of the well. Sterilized spores of *G. margarita* were individually transferred to the agar media at distance of about 2 cm from wells. The spores were incubated at 27°C in the dark. After 1 week, the spore germination rate and hyphal growth of *G. margarita* were measured.

To analyze the VC released by helper-microorganisms, a 5 mm diameter microbial plug was prepared as mentioned above. A control plug was placed in a 30 ml sample tube containing 5 ml of 1.5 % agar medium, sealed with silicon cap, and incubated at 27°C for 1 week. The atmosphere in the tube was sampled with a syringe. The sample gas was injected into a Hitachi gas chromatograph equipped with a flame ionization detector and an activated alumina (60–80 mesh) glass column (2 mm × 2 m) at 130°C. The concentration of ethylene evolved from the agar medium in the absence of helper-microorganisms was measured and found to be very low. This concentration was subtracted from the measured concentrations of ethylene released in the presence of helper-microorganisms.

Observation of helper-microorganisms by SEM

The spores of *G. margarita* sterilized by the solution as mentioned above were individually transferred to agar media and kept in contact with a microbial plug prepared as described above. After 1 week, germinated spores of *G. margarita* were freeze-dried (VA-250f, TAITEC Co. Ltd., Tokyo). The sample was observed with a SEM (JXA-840, JEOL Co., Ltd., Tokyo).

3. Results

Helper-microorganisms which promote the growth of G. margarita

Three fungal strains and two bacterial strains were isolated and tagged at KHIMF1, KHIMF2, KHIMF3, KHIMB1 and KHIMB2, respectively.

In the ITS rDNA analysis, the closest matches to strain KHIMF1 were with *Woollsia* root-associated fungus XV (97% identity to AY230785). Strain KHIMF2 showed the greatest sequence similarity to *Candida parapsilosis* (98% identity to CP10987). Strain KHIMF3 had as its closest relative *Cladosporium cladosporioides* (96% identity to AY361965). In the 16S rDNA analysis, strain KHIMB1 showed low sequence similarity to *Bacillus asahii* (89% identity to AB09209). Strain KHIMB2 had as its closest relative *Methylobacterium* sp. (95% identity to D3223) (Table 1).

Table 1. Sequence similarity of helper-microorganisms that promote the growth of *G. margarita* using molecular biology.

Strain	Identical species on database (Accession no.)	Identity
KHIMF1	*Woollsia* root-associated fungus XV (AY230785)	97%
KHIMF2	*Candida parapsilosis* (CP10987)	98%
KHIMF3	*Cladosporium cladosporioides* (AY361965)	96%
KHIMB1	*Bacillus asahii* (AB109209)	89%
KHIMB2	*Methylobacterium* sp. (D32233)	95%

The morphological characteristics of these helper-microorganisms were as follows: The color of the KHIMF1 colony was gray to white. The pointed ends of the hyphae were curled. The hyphae were about 1.3 μm wide (Fig. 1F-a and F-d).

The color of the KHIMF2 colony was yellow. The surface is sticky. The hyphae were about 1.0 μm wide. The pseudohyphae were surrounded by conidia (Fig. 1F-b and F-e).

The color of the KHIMF3 colony was black. The hyphae of this fungus grew quickly (1.2 cm/day). The hyphae were about 0.6 μm wide. The spores were in chains (Fig. 1F-c and F-f).

The color of KHIMB1 colony was white, and had a smooth surface. The cells were gram-positive and ellipsoid in shape. The size was about 4×2 μm (Fig. 1B-a).

The color of KHIMB2 colony was yellow. Cells were gram-negative. The size was about 6×5 μm (Fig. 1B-b).

From both morphological and molecular biological analyses, strains of the microorganisms were finally identified as follows: KHIMF1; *Woollsia* root-associated fungus XV (AY230785), KHIMF2; *Candida parapsilosis*, KHIMF3; *Cladosporium cladosporioides* and KHIMB2; *Methylobacterium* species. The molecular similarities and morphological features of KHIMB1 were not sufficient to identify this bacterium.

Effects of EVC and VC released from helper-microorganisms

Volatile compounds (VC) released from the fungi (KHIMF1, KHIMF2 and KHIMF3) greatly stimulated the hyphal growth of *G. margarita* (Fig. 2). Exudates and volatile compounds (EVC) from KHIMF1, however, had less effect on stimulation than VC. On the other hand, only EVC from KHIMB2 significantly stimulated the hyphal growth of *G. margarita*. For KHIMB2, EVC stimulated hyphal growth whereas volatile compounds did not suggesting that, in this case, the main effect was due to exudates. Finally, in KHIMB1 both VC and EVC of stimulated hyphal growth.

Ethylene was detected in the VC released from all of the helper-microorganisms except for KHIMB2. Ethylene concentration produced by KHIMF2 was 0.04±0.002 μl L^{-1}, and its concentration was the highest among the helper-microorganisms. Ethylene concentration produced by KHIMF1, KHIMF3, and KHIMB1 was 0.008±0.002 μl L^{-1}, 0.013±0.001 μl L^{-1}, and 0.009±0.001 μl L^{-1}, respectively (Fig. 3).

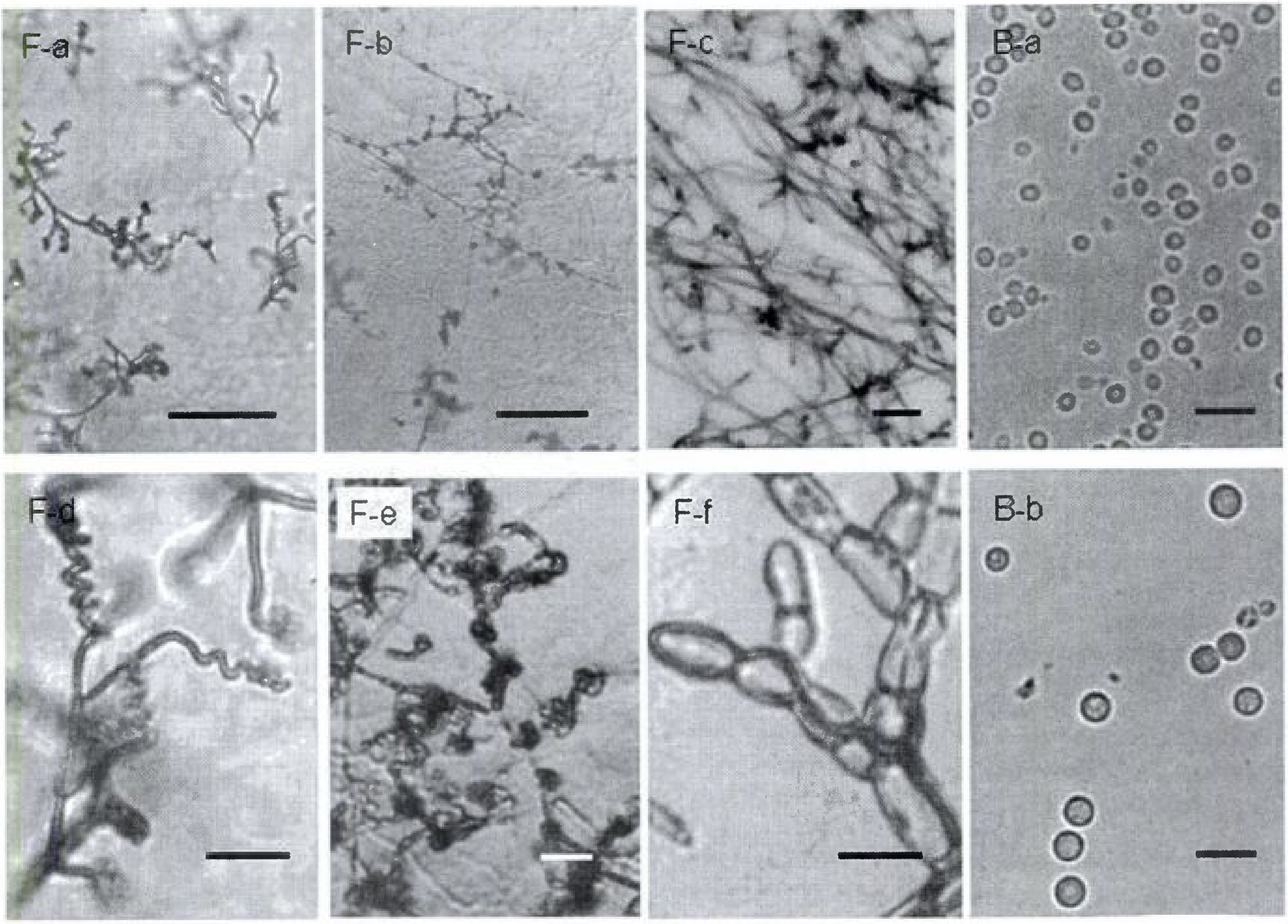

Figure 1. Photographs of helper-microorganisms which promote the growth of *G. margarita*. F-a: KHIMF1 (Bar 50 μm), F-b: KHIMF2 (Bar 50 μm), F-c: KHIMF3 (Bar 50 μm), F-d: KHIMF1 (Bar 10 μm), F-e: KHIMF2 (Bar 10 μm), F-f: KHIMF3 (Bar 10 μm), B-a: KHIMB1 (Bar 10 μm), B-b: KHIMB2 (Bar 10 μm).

Observation of helper-microorganisms by SEM

The hyphae of helper-fungi spread and flattened on the spore surface or on the hyphae of *G. margarita* (Fig. 4F-a, b and c). Furthermore, KHIMF1, KHIMF2 and KHIMF3 (Fig. 4F-d) showed the hyphal penetration into very small wounds on the *G. margarita* spore surface. The mycelia of KHIMB1 and KHIMB2 adhered to the surface of spores and hyphae of *G. margarita* (Fig. 4B-a and c).

4. Discussion

Three fungal strains, KHIMF1, KHIMF2 and KHIMF3, were identified as *Woollsia* root-associated fungus XV, *Candida parapsilosis* and *Cladosporium cladosporioides*. *Woollsia* root-associated fungus XV is a fungus that colonizes the root area of Epacridaceae called *Woollsia* which inhabit the forests and heaths. *Woollsia* forms ericoid mycorrhizae, and it is thought that *Woollsia* root-associated fungus interacts with ericoid mycorrhizal fungi (Midgley et al., 2004). It is very interesting that KHIMF1 could be a helper-fungus that promotes the growth of AM fungi as well as an ericoid mycorrhizal fungi. In BLAST analysis, the identification of KHIMB1 was not conclusive as the sequence of 16S-rDNA extracted from the bacterium compared poorly with that from *Bacillus asahii*. The sequence similarity of KHIMB2 was 95% when compared to the genus *Methylobacterium*. Although it has been shown that some kinds of bacteria or bacteria-like organisms (BLO) are intimately associated with arbuscular mycorrhizal spores, and have the capacity to inhibit or stimulate the germination of the spores (Mayo et al., 1986; Budi et al., 1999; Xavier and Germida, 2003), the identification of these microorganisms has not been reported. In this study, we have identified 4 kinds of helper-microorganisms that stimulate the growth of *G. margarita*, and found another that could not be identified. Observations using SEM showed that hyphae of KHIMF3 could penetrate *G. margarita* spores. While the contacts by the three associated fungi may affect the arbuscular mycorrhizal spores physically, they do not appear to stop germination or hyphal growth of *G. margarita*.

Most previous studies have dealt with interactions

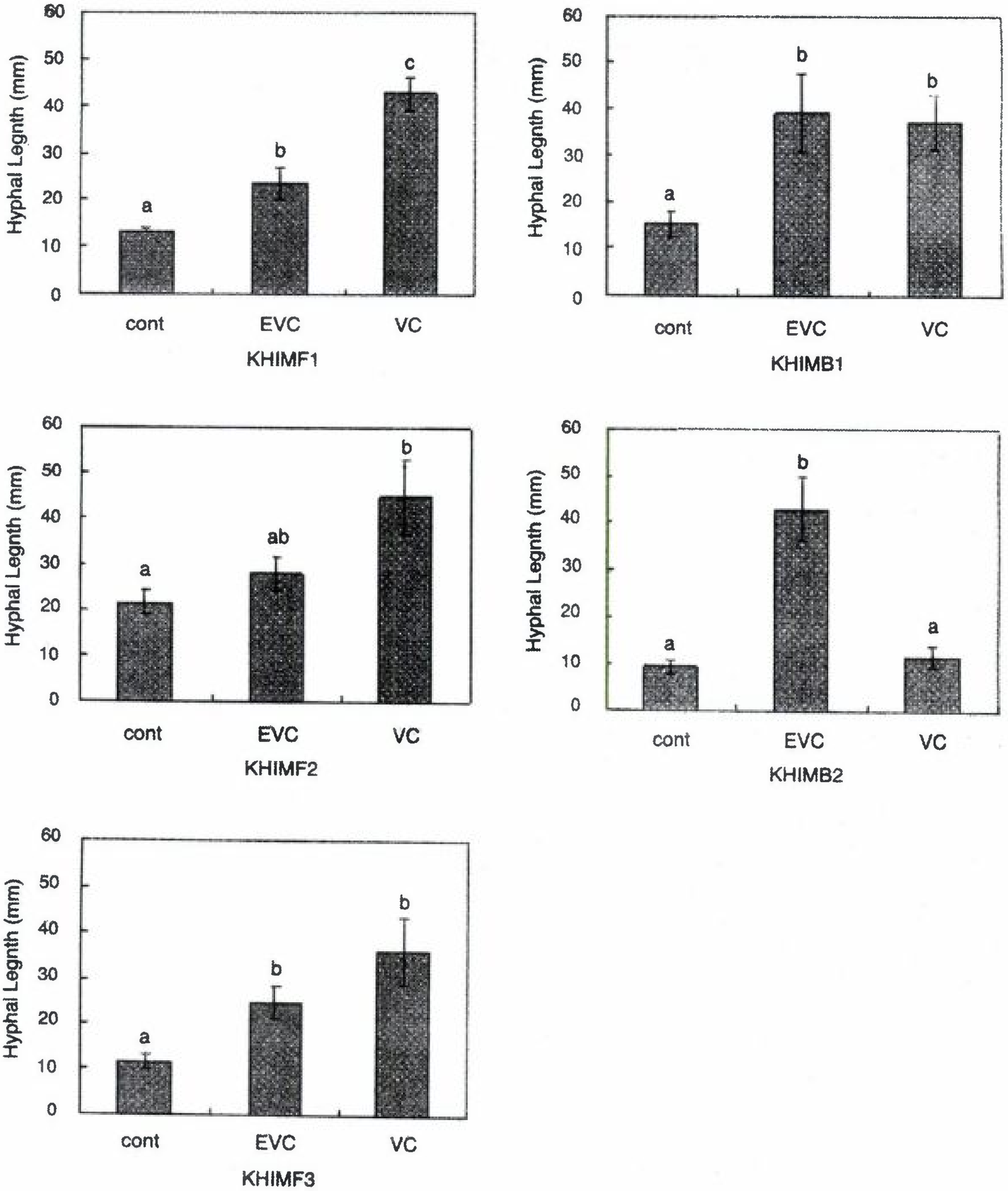

Figure 2. Effects of isolated helper-microorganisms on hyphal growth of *G. margarita*. The helper-microorganisms were co-cultured for 1 week. EVC: exudates + volatile compounds, VC: volatile compounds. Vertical bars represent the mean ±standard error (SE) (n=11). Different letters in each bar indicate significant differences at P<0.05 according to Duncan's multiple range test. Cont: control.

between selected bacteria or saprophytic fungi in relation to enhancement of AM root colonization (Fitter and Garbaye, 1994; Fracchia et al., 2000). Sampedro et al. (2004) found that the percentage of AM root colonization increased only when the soil yeasts were inoculated before *G. mosseae* was introduced. Our results indicate that different types of helper-microorganisms may produce stimulants that promote the growth of AM fungi. In the present study, volatile compounds from KHIMF1, KHIMF2, KHIMF3 and KHIMB1 significantly stimulated the growth of *G. margarita*. Ethylene released from these helper-microorganisms could play a vital role in growth stimulation of AM fungi. Some of the helper-microorganisms produce it at concentrations high enough to stimulate the growth of *G. margarita*. Ishii et al. (1996) indicated that spore germination and hyphal growth of AM

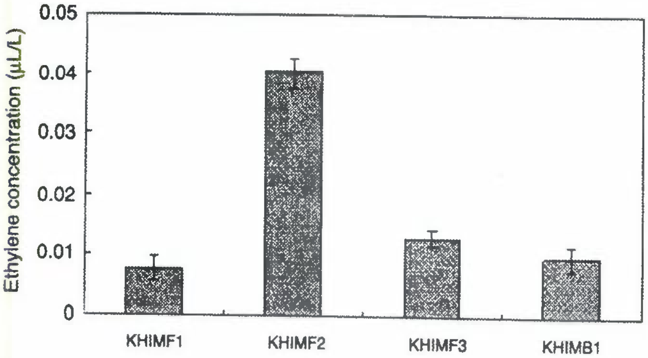

Figure 3. The concentration of ethylene produced by helper-microorganisms which promote the growth of *G. margarita*. Vertical bars represent the mean ± SE (n=3). Ethylene was detected after 1 week.

fungi was stimulated at concentrations ranging from 0.01 to 0.1 μl L^{-1} ethylene, but 0.2 μl L^{-1} and above were inhibitory. Guinel and Geil (2002) also suggested that ethylene could play important roles in the formation of AM symbioses. Although non-volatile compounds produced by KHIMB2 in a liquid culture system promoted the hyphal growth of *G. margarita*, the compounds are still unclear.

Our results suggest that different types of microorganisms are associated with AM fungi, and that they release ethylene or non-volatile compounds that significantly promote hyphal growth of *G. margarita*. Additional studies assessing the co-inoculation response of host plants to AM fungi and these growth-promoting microorganisms as an additional strategy for enhanced plant productivity may prove to be worthwhile.

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