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PLANT AND BACTERIAL EXTRACTS INVOLVED ON ARBUSCULAR MYCORRHIZAL FUNGUS STIMULATION AND PATHOGENS SUPPRESSION

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ABSTRACT

'Amanatsu' orange (*Citrus* × *natsudaidai* Hayata) seedlings were cultivated under aluminum [$Al_2(SO4)_3$)], salinity (NaCl) and water stress conditions in green house. 'Amanatsu' root extracts (ARE) were obtained from these plants by using a flash chromatograph, and an experiment *in vitro* to measure the effect of the extracts on hyphal growth of arbuscular mycorrrhizal fungus (AMF) was carried out. The hyphal growth was stimulated by ARE from aluminum and water stressed plants, whereas it was inhibited by ARE from the plants with NaCl. In particular, the hyphal growth in Petri dishes with ARE from aluminum and water stressed plants were around 70 % higher than that with agar only. In a second experiment, the 25 % MeOH eluates of Bahiagrass (*Paspalum notatum*) and millet (*Pennisetum glaucum*) root extracts stimulated, *in vivo*, AMF infection on trifoliate orange (*Poncirus trifoliata*) rootstocks. A third experiment was carried out to evaluate bacterial extracts on AMF (*Gigaspora margarita* and *Glomus clarum*) hyphal growth and soil-borne plant pathogens suppression. The results indicated that the 25 % MeOH fraction could stimulate the hyphal growth whereas mainly the 75 and 100 % MeOH fraction inhibited the pathogen growth *in vitro*.

Additional key words: Aluminum stress, hyphae, methanol fractions, salinity, water stress

RESUMEN

Extractos de plantas y bacterias implicados en la estimulación de hongos micorrízicos arbusculares y la inhibición de patógenos

Plántulas de naranjo (*Citrus* × *natsudaidai* Hayata) 'Amanatsu' fueron cultivadas en presencia de salinidad (NaCl), aluminio [$Al_2(SO4)_3$)] y estrés hídrico bajo invernadero. Extractos de las raíces de las plantas (ERP) fueron obtenidos mediante el uso de un cromatógrafo de columna. Luego se realizó un experimento *in vitro* utilizando estos extractos para medir el crecimiento de las hifas de hongos micorrízicos arbusculares (HMA). El crecimiento de las hifas en placas de Petri fue estimulado por los ERP de los tratamientos con estrés hídrico y aluminio, mientras que fue inhibido por los ERP del tratamiento con NaCl. El crecimiento de las hifas con los ERP de estrés hídrico y aluminio fue alrededor de 100 % mayor que las cultivadas en sólo agar (sin ERP). En un segundo experimento, una fracción de 25 % de extractos metanólicos (MeOH) de pasto bahía (*Paspalum notatum*) y mijo (*Panicum miliaceum*) estimularon, *in vivo*, la infección del HMA sobre naranjo trifoliado (*Poncirus trifoliata*). Un tercer experimento se llevó a cabo para evaluar extractos bacterianos sobre el crecimiento de las hifas de HMA (*Gigaspora margarita* y *Glomus clarum*) y la supresión de patógenos del suelo. Los resultados indicaron que la fracción MeOH 25 % pudo estimular el crecimiento de las hifas mientras que, principalmente, las fracciones MeOH 75 y 100 % inhibieron el crecimiento *in vitro* de patógenos.

Palabras clave adicionales: Estrés hídrico, estrés por aluminio, estrés salino, fracciones de metanol, hifas

INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) establish a compatible interaction with plants. This mutualistic interaction has important implications for plant nutrition and growth with special concerning on biocontrol of diseases (Hernández-Montiel et al. 2013). AMF are an ecologically important component of the soil microbial community that play a critical role in establishment and maintenance of the plant communities. During the formation of the AMF symbiosis the root exudates seem to have an important signaling function. They exhibit an

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attractive effect on AMF hyphal growth in soils (Sun et al., 2012). Chemical composition of soil and composition of rhizosphere microflora are very complex and variable, and the interaction between plant roots and AMF are also complex. The major substrate for the microbial activity in the rhizosphere is organic carbon (rhizodeposition) released by plant roots, which contains mainly sugars, amino acids, organic acids and phenolics. Leakage of organic compounds and other chemicals to the rhizosphere is a phenomenon that can represent a significant loss of photosynthates from the plant, which serve as signals to initiate symbiosis with rhizobia and AMF (Badri and Vivanco, 2009).

The germination *in vitro* of AMF spores has been documented (Ellouze et al., 2012, Gulbis et al., 2013), and the possibility to maintain these fungi in axenic culture has motivated investigation on stimulatory substances for the growth of AMF. In fact, CO_2 enrichment and AMF could influence on cutting growth and some physiological traits during rooting (Nowak and Nowak, 2013). The fungal growth depends on some substances present in root exudates and volatiles, and recently, flavonoid compounds have been reported to stimulate the plant-fungus interactions (Ishii et al., 1997; Steinkellner et al., 2007).

Some experiments have shown that the hyphal growth of *Gigaspora margarita* is strongly influenced by 25% MeOH eluates of bahiagrass (Horii et al., 2009) and millet root extracts (unpublished data). However, no attempts were made in roots under some environmental stress such as, aluminum, salinity and water stress. It is assumed that the roots under environmental stress exude more compounds as a signal for AMF infection, since these roots need mycorrhizal activity. Also, it is necessary to study *in vivo* conditions the effects of the root extracts which have promoted the hyphal growth.

The AMF are well known to improve the growth of plants in soils under stress conditions, where the low fertility is a common situation. However, the necessity to obtain large quantities of prime inoculums has motivated more researches about stimulatory substances of AMF development.

During the process of plant colonization, AMF interact with bacteria where spores and hyphae provide specific niches for certain populations of bacteria also found in the cytoplasm of AMF spores (Cruz and Ishii, 2012). The beneficial effects of mycorrhizae in the rhizosphere are the result of synergistic interactions among all rhizosphere microbes, which are crucial for plant growth, and many positive results within this system have been obtained for ornamental plants (Matysiak and Falkowski, 2010). Bacteria living in or around AMF spores have been well reported (Cruz et al., 2008; Horii and Ishii, 2006), which might contribute to hyphal growth and biocontrol of soil-borne plant pathogens (SBPP) (Cruz and Ishii, 2012). However, the specific compounds released from these bacteria, which could have these properties, are not yet clarified.

The purpose of this study is to investigate AMF stimulatory substances in roots of 'Amanatsu' orange plants under stress conditions (salinity, aluminum or water stress), and to compare the influence of different fractions from the bahiagrass and millet root extracts on the AMF colonization of trifoliate orange seedlings. Moreover, the research evaluated the effects of these active compounds from bacteria on stimulation of AMF and inhibition of SBPP.

MATERIALS AND METHODS

This research was carried out at Ehime University (Experiment 1 and 2) and Kyoto Prefectural University (Experiment 3), Japan.

Experiment 1: Effect of root exudates from stressed roots on hyphal growth. Sand substrate was sterilized with chloropicrin, and covered with a plastic film. One week later the plastic was removed and the substrate was kept on air during one week to release the chloropicrin. The substrate was amended with 140 ppm N, 10 ppm P, 140 ppm K, 50 ppm Ca and 50 ppm Mg; and each pot was filled with 3.5 kg of this substrate. Two groups of 16 pots were conformed: the first one was inoculated with about 70 spores of *Gigaspora margarita* per pot, leaving the other group without inoculation. Later, one-year-old 'Amanatsu' seedlings were transplanted to the pots. Four weeks after transplanting, solutions containing either 600 mM of NaCl or 5 mM of $Al_2(SO4)_3$ were applied once a week to four plants of each

group. Three months after transplanting a water stress treatment was applied by leaving four plants of each group without irrigation for a period of 18 days. At the end of this period, plants were notoriously water stressed, well beyond the maximum reading of tensiometers that had been installed in the pots. Four well irrigated control plants were maintained in each group during this period.

The experiment was conducted as a completely randomized design with factorial arrangement of the treatments (2 x 4) which examined two inoculation groups (inoculation or no inoculation) and four kinds of stress (salinity, aluminum and water stress, plus a control), with four replicates (total of 32 pots). After the period of water stress the experiment was harvested. The roots were washed and kept in a freezer, then the 'Amanatsu' root extracts (ARE) were taken by flash chromatograph according to the Figure 1 (Ishii et al., 1997) with some modifications. The 25 % methyl alcohol (MeOH) eluates of ARE were used in the experiment *in vitro*.

In order to examine the response of AMF to the 25 % MeOH eluates of the ARE spores of *G. margarita* were used. The surface of the spores was sterilized for 15 min in the solution (0.7 g chloramine T + 5.6 mg streptomycin + 2 mg chloramphenicol/ 100 mL distilled water) containing a few drops of Tween 80. After the spores were rinsed in sterile water, they were transferred to each Petri dish containing 10 mL of 1.5 % sterilized agar media and ARE equivalent to 0.4 g of 'Amanatsu' root fresh weight. Two Petri dishes per treatment were prepared with four spores in each one, which were incubated at 25 °C in the dark. Two weeks later, hyphal growth was observed by using an image-processing system equipped with a light microscope and a personal computer (Ishii and Kadoya, 1994).

Experiment 2: Effect of BRE and MRE on AMF infection. Bahiagrass and millet seeds were sown in trays containing zeolite in early spring. In early October the roots were collected. Isolation and purification of the stimulatory substances (BRE and MRE) from the roots were carried out as shown in Figure 1. The stimulatory substances in the roots were extracted with 80 % MeOH solution, and then fractionated by flash chromatograph (Fuji Silysia Chemical Ltd.) equipped with a Chromatorex ODS DM 1020T column (20 mm in diameter and 25 cm length).

To investigate the effects of each fraction of BRE and MRE, trifoliate orange (*Poncirus trifoliata* Raf.) were used in an *in vivo* experiment. One-year-old trifoliate orange seedlings were transplanted in plastic pots (0.8 L) with 2:1:1 mixture of vermiculite, zeolite and perlite. Each pot was amended with 140 ppm N, 10 ppm P, 140 ppm K, and 50 ppm Mg. Two weeks later, the pots were inoculated with 5 g of a commercial inoculum containing 50-70 spores of *Gigaspora margarita*. Two weeks after the inoculation the pots were treated, five times a week, with 25 µL and 50 µL of BRE and MRE, respectively (1 g of root-fresh weight equivalent), each with 0, 10, 25, 50 and 100 % MeOH fractions. Another treatment with only AMF inoculation was prepared as a control. Two months after transplanting the seedlings were harvested and the plant biomass represented by total fresh weight (TFW) and root fresh weight (RFW) was measured. The roots were washed, stained according to the methods of Phillips and Hayman (1970), and observed by using a light microscope to rate the degree of root infection by AMF as described by Ishii and Kadoya (1994).

Experiment 3: Effects of MeOH bacterial extracts on AMF hyphal growth and soil-borne plant pathogens suppression. Three kinds of bacteria isolated from AMF spores were used in this experiment. They were previously molecularly identified as *Janthinobacterium lividum* (KCIGM01) and *Paenibacilus polymyxa* (KCIGM04) (Cruz et al., 2008); and *Pseudomonas* sp. (KCIGC01) (Cruz et al., 2010). The KCIGM01 and KCIGM04 were taken from *G. margarita* spores, whereas the KCIGC01 from *Glomus clarum*. These bacteria were grown in 8 L of liquid media composed of polypepton and Bacto yeast extract at 5 and 1 $g \cdot L^{-1}$, respectively, for 3 days at 25 °C. MeOH was added into the liquid media to reach 70 % of concentration. Then the extraction and fractionation of bacterial compounds followed the procedures of the flowchart (Figure 1) described in the experiment 1. However, in this case, the 0, 10 and 20 % MeOH fractions were discarded to avoid the influence of the bacterial growth media.

The bacterial fractions from KCIGM01 and

KCIGM04 were used to evaluate the hyphal growth of *G. margarita* and those from KCIGC01 to evaluate hyphal growth of *Gl. clarum* using the same procedures of the experiment 1. To verify the pathogens suppression three kinds of SBPP (*Fusarium oxysporum*, *Rhizoctonia solani* and *Rosellinia necatrix*) were incubated separately in Petri dishes containing potato dextrose agar. Two sterilized paper disks containing 50 µL of each bacterial extraction were placed on the periphery of these dishes. After 5 days of incubation at 25 °C, the mycelium growth was visually evaluated to estimate the degree of antagonism based on the criteria of the following inhibition number scale: 0-no; 1-slightly; 2-moderate; 3-strong; 4-extremely strong.

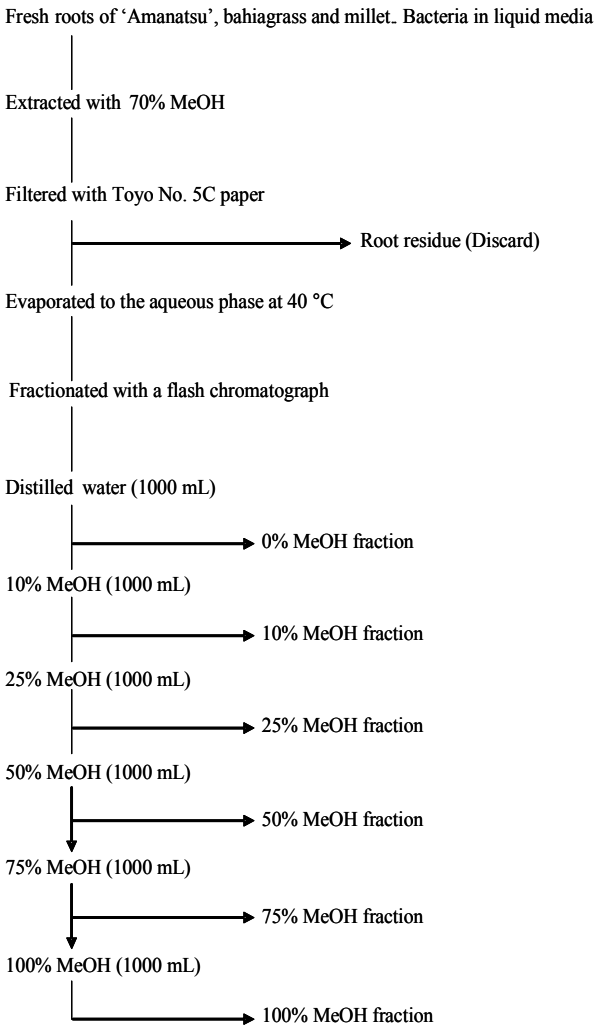

Figure 1. Flow sheet for separation of root extracts (Ishii et al., 1997)

RESULTS

Experiment 1: The hyphal growth in the Petri dishes was stimulated by the ARE treatments except for that from the plots treated with NaCl. In particular, the hyphal growth in the aluminum and water stress treatments was about 70 % greater than that from the non stressed plants (control), and more than twice larger than the hyphal growth in agar only (without ARE). Although, there was no statistical difference between the NaCl and the agar only treatment ($P>0.05$), the hyphal growth in NaCl Petri dishes tended to be slightly longer than that from the agar without ARE (Table 1).

Table 1. Effect of 25 % MeOH fraction of 'Amanatsu' root extracts (ARE) from plants grown under several stress conditions on *in vitro* hyphal growth of HMF (*Gigaspora margarita*)

Treatment	Hyphal length (mm)
Agar only (no ARE applied)	36.5 a
Control plants (non stressed)	50.3 b
Plants under salinity (600 mM NaCl)	39.8 a
Plants under aluminum [5 mM $Al_2(SO_4)_3$]	82.4 c
Plants under water stress (18 days without irrigation)	84.3 c

Experiment 2: The 25 % MeOH fraction of BRE and MRE significantly stimulated the AMF infection percentage of trifoliate orange seedlings, as compared with the other fractions and the control treatment with AMF only (Table 2). There was no significant difference among the fractions for the TFW and RFW in each group of BRE and MRE. However, the plant growth (TFW and RFW) in treatments with MRE were higher ($P\leq0.05$) than those with in BRE, and AMF only (Table 3).

Experiment 3: In general view the 25 and 50 % MeOH fractions of all bacteria could stimulate the hyphal growth, in particular, the 25 % fraction from KCIGC01 was the strongest one (Table 4). Other fractions did not show consistent stimulation of hyphal growth as compared to the

control treatment.

The growth of SBPP was inhibited mainly by the 75 and 100 % MeOH fractions. In the case of KCIGC01, the inhibition was also important in the 50 % fraction, meaning that most concentrations of these extracts were effective to suppress de growth of all SBPP (Table 4). Extracts from KCIGM01 and KCIGM04 at high concentrations were effective to suppress de growth of *R. necatrix*, while they could suppress *F. oxysporum* and *Rh. Solana* only at specific concentrations (either 75 or 100 % MeOH). Among all the treatments, the highest suppression of SBPP was found in the 100 % MeOH from KCIGM04 against *F. oxysporum* (3.88 in the number scale).

Table 2. Effect of different fractions of MeOH extracts from roots of bahiagrass (BRE) and millet (MRE) on the AMF (*Gigaspora margarita*) colonization of trifoliate orange seedlings

Fraction of	AMF colonization
AMF only	65.3 c
BRE 0 %	54.5 c
BRE 10 %	38.5 b
BRE 25 %	82.5 d
BRE 50 %	41.5 b
BRE 100 %	18.5 a
MRE 0 %	46.2 b
MRE 10 %	41.2 b
MRE 25 %	81.2 d
MRE 50 %	38.5 b
MRE 100 %	16.9 a

Means followed by the same letters in columns are not significant different according to Scott-Knott test ($P \leq 0.05$)

Table 3. Effect of MeOH extracts from roots of bahiagrass (BRE) and millet (MRE) on mean total fresh weight (TFW) and root fresh weight (RFW) of trifoliate orange seedlings

MeOH extracts	TFW	RFW
	(g per plant)	
AMF only	1.77 a	0.66 a
BRE	1.51 a	0.67 a
MRE	2.39 b	0.91 b

No interaction between the two MeOH extracts and their different fractions. Means followed by the same letters in columns are not significant different according to Scott-Knott test ($P \leq 0.05$)

DISCUSSION

The results show that the root extracts from plants under aluminum and water stress conditions can stimulate the hyphal growth, compared to the root extracts from plants under normal conditions. However, the root extracts from salinity conditions inhibited the hyphal growth. Perhaps, the plants under salinity stress tend to exude more stimulators than those required for AMF colonization, thus inducing inhibition of growth rather than AMF stimulation. In this case, the mycorrhizal activity would continue in other pathways, but with inhibited hyphal growth. The stimulatory effect of root exudates of AMF host plants on fungal growth has been elucidated in other studies (Hage-Ahmed et al., 2014).

Furthermore, we investigated the effect of fractions of BRE and MRE on AMF formation of trifoliate seedlings in experiment 2. The results showed that the AMF infection was stimulated by 25 % fraction of both extracts, suggesting that higher or lower concentration of the extracts may be partially inhibitory as previously demonstrated by Ishii et al. (1997) and Horii et al. (2009). It has been shown that specific compounds in the root exudates can stimulate hyphal growth and AMF infection (Steinkellner et al., 2007). Ishii et al. (1997) isolated three compounds in the 25 % MeOH fraction of BRE and one of them was identified as eupalitin, an O-methyled flavonol. Whereas the fresh weights were not influenced by the fractions in the extracts, the plants with MRE consistently showed higher values than BRE (Table 2).

The pre-symbiotic stage of fungus-plant is stimulated by some diffusible root exudates (Horii et al., 2009) and/or volatile compounds (Dong et al., 2009), and the growth response is generally characterized by a stimulation of hyphal elongation and hyphal branching (Giovannetti et al., 1993). Recently, some reports have demonstrated that a physical environmental factor, the light irradiation, can also stimulate hyphal elongation and branching (Nagahashi and Douds, 2000). Hyphal branching of *Gigaspora gigantea* has also been induced by near-UV light (390 nm)

and blue light (430 nm) irradiation (Nagahashi et al., 2000).

AMF partner bacteria functioned very well on SBPP suppression, promotion of hyphal growth and stimulation of nutrient biodynamics represented by P solubilization and nitrogenase activity (Cruz and Ishii, 2012). The mechanisms of this phenomenon could be chemically explained through bacterial exudates or physically by their aggregation with biofilm. As per definition, the biofilm is a jointing of several bacteria that compose a kind of cover in order to protect themselves and sometimes the host, thus it plays an important role in the ecosystem and bacterial adaptation to any environment. In other experiments (unpublished data), 50 and 100 % MeOH fraction from BRE and 50 % fraction from chickpea (*Cicer arietinum*) could inhibit *Fusarium oxysporum* f. sp. *lycopersici* growth. These results suggest that in several plants, including gramineae and leguminous, the biocontrol compounds might be located in those fractions.

Table 4. Effects of MeOH bacterial extracts on AMF *(Gigaspora margarita* and *Glomus clarum*) hyphal growth and soil-borne plant pathogen suppression

Bacteria	MeOH fraction (%)	AMF hyphal length (mm)	Pathogen suppression*		
			F. oxysporum	*Rh. solani*	*R. necatrix*
	Control	49 c	0.00 a	0.00 a	0.00 a
KCIGM01 (*J. lividum*)	25	80 e	0.24 a	0.22 a	0.33 a
	50	108 f	0.41 a	0.51 a	0.12 a
	75	2 a	2.24 d	1.13 b	2.38 c
	100	3 a	0.65 a	0.13 a	3.63 d
KCIGM04 (*P. polymyxa*)	25	110 f	0.00 a	0.33 a	0.17 a
	50	67 d	0.21 a	0.41 a	0.71 a
	75	15 b	1.13 b	0.22 a	0.51 a
	100	2 a	3.88 e	0.88 b	2.88 c
KCIGC01 (*Pseudomonas* sp.)	25	252 h	1.51 b	2.25 d	1.25 b
	50	163 g	1.96 c	3.21 e	3.23 d
	75	88 e	2.23 c	3.17 e	1.83 c
	100	95 e	2.83 d	1.28 c	2.54 c

*Suppression scale: 0-no; 1-slightly; 2-moderate; 3-strong; 4-extremely strong.
Means followed by the same letters in columns are not significant different according to Scott-Knott test (P≤0.05)

The author hypothesizes that other substances in these plant roots and bacterial extracts might stimulate the growth of AMF and suppress the SBPP. Further researches are necessary in order to chemically identify the active compounds from the fractions mentioned in these results.

CONCLUSIONS

The 'Amanatsu' orange roots under aluminum and water stress could stimulate AMF hyphal growth, but not those with NaCl.

Bahiagrass and millet root extracts could promote the AMF colonization

Bacterial extracts were able to function as AMF stimulator, and soil-borne plant pathogen inhibitor.

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