Network Establishment of Vesicular–Arbuscular Mycorrhizal Hyphae in the Rhizospheres between Trifoliate Orange and Some Plants

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Summary

The distributions of vesicular–arbuscular mycorrhizal (VAM) hyphae in the rhizospheres of trifoliate orange (Poncirus trifoliata Raf.) and some plants grown under greenhouse conditions were explored. Furthermore, the effect of the 25% MeOH eluates of bahiagrass (Paspalum notatum Flügg.) and millet (Pennisetum glaucum L. R. Br.) roots fractionated by flash chromatography on the network establishment of VAM hyphae was examined. Special acrylic root boxes with three compartments separated by nylon screens were used for the experiment. Seedlings of trifoliate orange were transplanted into one outer compartment in all boxes, and seedlings of bahiagrass, millet, cockscob (Celosia cristata L. cv. Parade), radish (Raphanus sativus L. cv. Natsumino), and tomato (Lycopersicon esculentum Mill. cv. Fukuju) were transplanted into the other outer compartment. A VAM fungus, Gigaspora margarita Becker and Hall, was inoculated in the center compartment. In plots with only trifoliate orange seedlings, 25% MeOH eluates of bahiagrass root extract (BRE) and millet root extract (MRE) were applied into the other outer compartment once a week. A plot with only trifoliate orange seedlings and without BRE and MRE applications was also prepared as a control. The density of hyphae, percentage of infection, and number of spores in the bahiagrass and millet compartments were higher than those in the compartments with trifoliate orange. These parameters in the cockscob, radish and tomato compartments, however, were markedly low as compared with the trifoliate orange compartments. Although few VAM hyphae and spores were observed in the control (no plant compartment), the density of hyphae in the compartments treated with BRE and MRE increased to around 18%, and a few spores were found in these compartments.

Root exudates of trifoliate orange, bahiagrass, and millet stimulated the hyphal growth of G. margarita in vitro. Particularly, the stimulation by bahiagrass and millet was significantly greater than that of the control (agar only). But the hyphal length of cockscob, radish, and tomato treatments was shorter than that of the control.

These results suggest that under field conditions, root exudates will affect the behaviour of VAM fungi in the soil. Moreover, some compounds in BRE and MRE may act as chemotropic signals for attracting VAM hyphae.

Key Words: hyphae, network, root extracts, sod culture, vesicular–arbuscular mycorrhizal fungi.

Introduction

The favorable effect of sod culture in orchards is attributed to many factors including the propagation of symbiotic microorganisms, such as VAM fungi. Naginatagaya [Vulpia myuros (L.) C. C. Gmel.] and bahiagrass have been used as sods in some orchards of Japan. We observed that the introduction of sod culture

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with bahiagrass (Ishii et al., 1996) and V. myuros (Kirino et al., 1998) improves VAM formation in satsuma mandarin (Citrus unshiu Marc.) on trifoliate orange rootstocks. Galvez et al. (1995) reported that some grasses used as overwintering cover crops are potentially very beneficial for VAM fungi. Most soils are not conducive to keeping them viable without host plants, because VAM fungi are obligate symbionts (Douds and Schenck, 1991).

Under natural environmental conditions, the symbiotic association between plants and VAM fungi is not specific, so that a network of hyphae forms in the soil among several kinds of plants. This network system which links plants results from the ability of the hyphae to branch (Rutto, 2000).
The network establishment of VAM fungi in sod culture system is influenced by many factors from the soil and plants. The germination of spores and growth of hyphae may be stimulated by root exudates (Cruz et al., 2000), such as flavonoids (Tsai and Phillips, 1991; Ishii et al., 1997). Asparagus (Asparagus officinalis L.) plants, however, produce derivatives of cinnamic acid known to be allelochemicals, which can mediate changes in the soil microbial community (Hartung and Stephens, 1983). Waker et al. (1990) found that one allelochemical compound in asparagus roots depressed the VAM infection and the growth of mycorrhizae. These authors suggested that the accumulation of phenolic allelochemicals produced by asparagus could be a reason why asparagus monocropping alters the species composition of the VAM fungal community.

In this study, we examined the establishment of VAM hyphal network by plants that are usually non-mycorrhizal as well as by plants used in sod culture. Moreover, the effects of BRE and MRE on the growth of VAM hyphae in soils which would greatly contribute to the proliferation of VAM spores in the rhizosphere in the absence of hosts were evaluated.

**Materials and Methods**

*Experiment 1. Distribution of VAM hyphae in the rhizospheres of VAM and non-VAM plants*

This experiment was done in a greenhouse condition without a temperature-controlling system. Special acrylic root boxes were constructed, and each root box (3-cm wide, 45-cm long and 15-cm deep) was divided into three compartments. The center compartment (3-cm wide, 5-cm long and 15-cm deep) was separated from the outer zones by a barrier made of a nylon screen of 37 μm mesh that allows the VAM hyphae but not the roots to penetrate. The pots were covered with an aluminum film to block the light and prevent the formation of algae. Each box was filled with sterilized clay substrate. The sterilization was done by adding chloropicrin into the substrate, and covered with a plastic film. One week later the plastic film was removed to release chloropicrin for one week prior to transplanting.

Seeds were pregerminated in trays containing vermiculite. A trifoliate orange seedling was transplanted into one outer compartment in each box, and each of the cockscob, bahiagrass, millet, radish, and tomato were separately transplanted into the outer compartments in the center compartment. A plot with only trifoliate orange seedlings was prepared as the control. The inoculation was done in the center compartments with a VAM fungus, G. margarita (Central Glass Co., Ltd.). One week after the inoculation, each box was amended with 140 ppm of N, 10 ppm of P, 140 ppm of K, and 50 ppm of Mg.

Two months later, the aluminum cover was removed and the density of hyphae in an area of 12.8 × 9.3 mm of each compartment was observed using a Charge Coupled Device (CCD) camera (Keyence VH-7000). On a computer screen, this area was divided into 200 squares and the density of hyphae was calculated by using the following equation:

\[ \text{Density of hyphae} (\%) = \left( \frac{\text{squares with hyphae}}{\text{total squares (200)}} \right) \times 100. \]

Soil samples (25g) were taken to evaluate the number of spores according to the procedure of Ishii et al. (1996). Roots were sampled, washed, and stained by the technique of Phillips and Hayman (1970). The percentage of VAM infection in the roots was determined according to Ishii and Kadoya (1994).

*Experiment 2. Effects of bahiagrass and millet root extracts as signals for VAM fungi*

In this experiment, the root boxes, substrates, planting, and inoculation were made the same as those in Experiment 1. The outer compartments without seedlings were treated once a week until harvest with 25% MeOH eluates of BRE and MRE equivalent to 1 gFW, which were obtained according to the procedures of Ishii et al. (1997). A plot with only trifoliate orange seedlings was prepared as the control. The density of hyphae, number of spores and percentage of infection were evaluated by using the same methods as described in the experiment 1.

*Experiment 3. Effects of several kinds of root exudates on growth of VAM hyphae in vitro*

Seeds of trifoliate orange, bahiagrass, millet, cockscomb, radish, and tomato were de-husked, sterilized with 5% sodium hypochlorite solution, rinsed with sterile water, and sown in a petri dish with 1.5% agar (5 seeds per petri dish). After they germinated within 3 days in the dark at 25°C, the petri dishes were transferred to a growth chamber with fluorescent illumination. Seedlings were grown for 12 days, and then were removed from the agar. Control dishes, which contained only 1.5% agar without any seedlings, were treated in the same way. Each treatment and the control were duplicated. The surface of G. margarita spores was sterilized for 15 min by using a disinfectant (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, each petri dish was inoculated with 4 spores. The petri dishes 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).

**Results**

*Experiment 1*

The compartments of trifoliate orange, bahiagrass, and millet had high densities of hyphae in the plots of
trifoliate orange × bahiagrass and trifoliate orange × millet (Fig. 1). In particular, the hyphae in the compartments of bahiagrass and millet were denser than those in the trifoliate orange compartments. Results of the trifoliate orange × cockscomb, trifoliate orange × radish and trifoliate orange × tomato plots revealed that the densities of hyphae in the trifoliate orange compartments were higher than those in the compartments of the other seedlings. In the control plots (trifoliate orange × no plant), the density of hyphae in the trifoliate orange compartments was 51.3%; few hyphae were observed in the adjacent no plant compartments.

In plots of trifoliate orange × bahiagrass and trifoliate orange × millet, the percentage of VAM infection in bahiagrass and millet was higher than that in trifoliate orange, whereas in plots of trifoliate orange × cockscomb, trifoliate orange × radish and trifoliate orange × tomato, the rate of infection in trifoliate orange was higher than that in cockscomb, radish and tomato (Fig. 2). Similarly, the number of spores was greater in bahiagrass and millet compartments than in trifoliate orange compartments in plots of trifoliate orange × bahiagrass and trifoliate orange × millet. In plots of trifoliate orange × cockscomb, trifoliate orange × radish and trifoliate orange × tomato, the number of spores in 25 g soil varied between 100 and 200 in the trifoliate orange compartments, whereas no spore was found in

Fig. 1. Density of VAM hyphae in both compartments of trifoliate orange and other plants in a root box system. TO: Trifoliate orange roots, NP: No plants, CR: Cockscomb roots, TR: Tomato roots, RR: Radish roots, BR: Bahiagrass roots, MR: Millet roots. Horizontal bars indicate standard error (SE)(n=18). It was taken 6 images from each one of 3 boxes.

Fig. 2. Percentage of VAM infection in the roots of trifoliate orange and other plants in a root box system. TO: Trifoliate orange roots, NP: No plants, CR: Cockscomb roots, TR: Tomato roots, RR: Radish roots, BR: Bahiagrass roots, MR: Millet roots. Horizontal bars indicate SE (n=3).

Fig. 3. Number of VAM spores in the sections of trifoliate orange and other plants separated by a nylon mesh in a root box system. TO: Trifoliate orange roots, NP: No plants, CR: Cockscomb roots, TR: Tomato roots, RR: Radish roots, BR: Bahiagrass roots, MR: Millet roots. Horizontal bars indicate SE (n=3).

Fig. 4. VAM hyphal invasion into trifoliate orange roots (A) and new VAM spores formed in soil with millet plants (B). (CCD image, × 175)(See arrows).
Fig. 5. Density of VAM hyphae in both the compartment transplanted with trifoliate orange and the compartment with the 25% MeOH eluates of bahiagrass and millet root extracts in a root box system. TO: Trifoliate orange roots, NP: No plants, BRE: 25% MeOH eluates of bahiagrass root extracts (1 gFW equivalent), MRE: 25% MeOH eluates of millet root extracts (1 gFW equivalent). Horizontal bars indicate SE (n=18). It was taken 6 images from each one of 3 boxes.

Fig. 6. Percentage of VAM infection in trifoliate orange roots in the plots treated with 25% MeOH eluates of bahiagrass and millet root extracts in a root box system. TO: Trifoliate orange roots, NP: No plants, BRE: 25% MeOH eluates of bahiagrass root extracts (1 gFW equivalent), MRE: 25% MeOH eluates of millet root extracts (1 gFW equivalent). Horizontal bars indicate SE (n=3).

The compartments of cockscomb, radish, and tomato (Fig. 3). VAM hyphal invasion into roots and VAM spore formation in soils occurred frequently in plots of trifoliate orange × bahiagrass and trifoliate orange × millet (Fig. 4).

Experiment 2

Few hyphae were observed in the no plant compartments in the plot of trifoliate orange × no plant. However, when BRE and MRE were applied to the no plant compartments (trifoliate orange × BRE and trifoliate orange × MRE), the density of hyphae in the no plant compartments of these plots approached 18% (Fig. 5). In the same treatments, the percentage of VAM infection in trifoliate orange roots was higher than that in the plot of trifoliate orange × no plant (Fig. 6). In the same plots the number of spores in the trifoliate orange compartments

Fig. 7. Number of VAM spores in both the compartment transplanted with trifoliate orange and the compartment with the 25% MeOH eluates of bahiagrass and millet root extracts in a root box system. TO: Trifoliate orange roots, NP: No plants, BRE: 25% MeOH eluates of bahiagrass root extracts (1 gFW equivalent), MRE: 25% MeOH eluates of millet root extracts (1 gFW equivalent). Horizontal bars indicate SE (n=3).

Fig. 8. Hyphal length of G. margarita cultured in agar media that contain root exudates of some seedlings. Horizontal bars indicate SE (n=4).

was around 200, and 10 and 12 spores per 25 g soil were found in the compartments of BRE and MRE, respectively. In the no plant compartments, however, very few spores were observed (Fig. 7).

Experiment 3

Root exudates from bahiagrass, millet and trifoliate orange stimulated the VAM hyphal growth compared to the 1.5% agar control (Fig. 8). In particular, the bahiagrass and millet root exudates were the most promotive, whereas the hyphal growth in the agars containing the root exudates of cockscomb, radish, and tomato was significantly less than that in the control.

Discussion

Cruz et al. (2000) previously reported the presence of new hyphae and spores in the compartments of trifoliate orange and other plants, which indicated that the VAM fungi had grown from the center compartments to the outer ones. The CCD camera images also showed that the spores formed newly in the outer compartments had the same surface characteristics as those of G. margarita.
Thus, the colonization in the plants of the outer compartments resulted from the germination of the spores in the center compartments and subsequent growth of mycelia to the outer ones. The interconnection formed between two or more different plants by VAM hyphae promotes a network in the soil-plant system. Under natural environmental conditions this network may benefit the plants, especially when a nutrient exchange between plants maintains an adequate level in the plant/VAM system.

Cruz et al. (2000) demonstrated that the network system by VAM hyphae was easily discernible in the rhizosphere between trifoliate orange and bahiagrass. By using 25% MeOH eluates of bahiagrass root extracts, Ishii et al. (1995) first succeeded in the axenic culture of Gigaspora ramisporohora. They also reported that several VAM stimulatory compounds containing flavonoids, such as eupatin, which significantly stimulate hyphal growth in vitro existed in the 25% MeOH eluates (Ishii et al., 1997).

The data of hyphal density, VAM infection percentage, and spore number demonstrate that the root exudates either promote infection or not, depending on the host plant, which would indicate that under natural environmental conditions, some root exudates stimulate, while others inhibit VAM infection. Cruz et al. (2000) found that exudates from bahiagrass and trifoliate orange roots both stimulated the growth of VAM hyphae; the former being more promotive than the latter. The few hyphae and spores found in the outer compartments without plants are attributed to the release of root exudates from the trifoliate orange growing in the opposite outer compartments.

VAM hyphae in the center compartments were attracted to one outer compartment without plants and produced a few spores when treated with BRE and MRE. Some compounds in BRE and MRE seemingly act as chemotropic signals for attracting the hyphae. Root extracts of bahiagrass are functional in vitro (Ishii et al., 1995; Ishii et al., 1997). VAM stimulatory substances in BRE, which are water soluble and diffusible in soils, seemingly induce the proliferation of VAM fungi even in the absence of the host plants.

Pedersen et al. (1991) who reported that asparagus soil extracts inhibited VAM colonization, suggested that this inhibition might be due to the accumulation of phenolic allelochemicals produced by asparagus, which affects the VAM community. The effects of plant-produced allelochemicals in a long-term monocropping system have been suggested to alter the composition of soil microflora including mycorrhizal fungi (Waker and Stephenson, 1990; Waker et al., 1990), while utilization of non-mycorrhizal plants in a crop rotation may induce the decrease of VAM fungal populations (Harinikumar and Bagyaraj, 1988). Our results indicated that root exudates from cockscomb, radish, and 'Fukuju' tomato significantly inhibited in vitro hyphal growth. Cockscomb and radish are usually non-mycorrhizal plants but sometimes incur low percentages of VAM infection (Fig. 1). Although almost all of the tomato plants formed VA mycorrhizae, 'Fukuju' cultivar showed a very low percentage of VAM infection even when inoculated with other species of VAM fungi (unpublished data). Thus, these allelochemical-containing plants may inhibit the infection by VAM fungi. The degree of the inhibition, however, depends on the plant species. Although bahiagrass and millet roots contain compounds that inhibit the growth of VAM fungi (Ishii et al., 1997 and unpublished data), their root exudates stimulated it. Our results indicate that the root exudates from the plants that are rarely infected by VAM fungi are inhibitive, whereas those from highly susceptible plants are stimulative.

In this study, we present in vitro the distribution of VAM hyphae in the rhizospheres between trifoliate orange rootstock and several kinds of plants. These hyphae might promote an interconnection between plants constructing a network system. This observation may be good evidence that a hyphal network may exist between citrus trees and other plants under an intercropping culture. Although cockscomb, radish, and tomato are not used as plants for intercropping in citrus orchards, our results indicate that when the plants that are not or less infected by VAM fungi are grown in the orchards, their use may lead to a poor network by VAM hyphae. Our results (Figs. 1 and 2) reveal that the development of network by VAM hyphae in bare soils may be inferior to soils in which plants that are rarely infected are grown. It would be related to the kinds of substances released from the roots. Substances released generally contain both VAM inhibitors and stimulators, so that the network establishment may be affected by the quality and quantity of these compounds. A large amount of organic carbon, which is a major substance for microbial activity, also exists in the root exudates. Therefore, we suppose that even in soils cultivated with plants slightly susceptible to VAM fungi, the possibility of formation and development of network by VAM hyphae will be greater than in bare soils, whereas root exudates from plants such as bahiagrass and millet would significantly enhance the development of network by VAM hyphae.

**Literature Cited**


カラタチと二、三の植物間の根圏における VA 菌根菌系によるネットワーク形成

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摘 要

カラタチと二、三の植物間の VA 菌根 (VAM) 菌系の分布状態を観察するために、特別なアクリル製のルートボックスを用い、温室内で実験を実施した。ルートボックス（45 × 15 × 3 cm）は、中央区分に径（37 μm メッシュのナイロン製フィルムを張り付け、根の侵入を防げるが、VAM 菌の菌糸は通り抜ける）を 2 面（桝間の長さ 5 cm）設けて、3 区分した。この中央区分に Gigaspora margarita 菌系を接種するとともに、外側の区分の一方にカラタチを、他方に菌糸が大きいバヒアグラスおよびキビが、菌糸形成が見られないか、あるいはきわめて小であるケイトウ、ダイコンおよびトマトをそれぞれ移植した。また、カラタチだけを移植したルートボックスも用意し、無栽植区分に、フラッシュクロマトグラフィーで得られたバヒアグラス (BRE) およびキビ (MRE) 根 1 gFW 相当量の 25% メタノール溶出物 (VAM 菌系生長促進物質を含む) を 1 週間ごとに収穫時まで土壌に施した。実験開始 2カ月後、各植物の根圏や、BRE および MRE 溶出区分における菌糸分解を CCD カメラで観察するとともに、各植物の菌糸感染率を新しい形成された胞子数を比較調査した。その結果、カラタチ×バヒアグラス区およびカラタチ×キビ区では、カラタチ、バヒアグラス、キビ栽培区分とともに、菌糸密度、菌の菌糸感染状態および土壌中の VAM 菌系胞子数は大であった。特に、カラタチ栽培区分よりもバヒアグラスあるいはキビ栽培区分の方が顕著であった。しかしながら、カラタチに、VAM 菌が感染しにくいケイトウ、ダイコンあるいはトマトを栽植した区（カラタチ×ケイトウ、カラタチ×ダイコンおよびカラタチ×トマト）では、カラタチ栽培区分の菌糸密度は 50% 以上で、菌糸感染率および胞子数も大であったが、ケイトウ、ダイコンおよびトマト栽培区分の菌糸密度は 20% 程度と低く、また菌糸形成も悪く、新しい胞子がほとんど見られなかった。対照（カラタチ×無栽植）区ではカラタチ栽培区分において数多くの菌糸が観察されたが、無栽植区分では菌糸や胞子がほとんど見られなかった。しかし、無栽植区分に、BRE および MRE を処理したところ（カラタチ×BRE およびカラタチ×MRE）、この区の菌糸密度はいずれの処理区においても 18% 程度に増加し、新しい胞子が形成されていった。

根から浸出される物質が G. margarita の菌糸生長に及ぼす影響を調査したところ、カラタチ、バヒアグラスおよびキビの根浸出物は菌糸生長を促進したが、この傾向はバヒアグラスおよびキビにおいて大であった。しかしながら、ケイトウ、ダイコンおよびトマトの根浸出物区では菌糸生長が阻害される傾向であった。

これらの結果は、根からの浸出物が圃場においても土壌に生息する VAM 菌の行動に著しく影響を及ぼすことを示唆している。また、BRE および MRE に含まれる物質は VAM 菌糸を引きつけるシグナルとして作用しているものと推察される。