Distribution of Vesicular-Arbuscular Mycorrhizal Hyphae in the Rhizospheres of Trifoliate Orange and Bahia Grass Seedlings under an Intercropping System

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Summary

An experiment was conducted under green house conditions to observe the distribution of vesicular-arbuscular mycorrhizal (VAM) hyphae between plants. Special acrylic root boxes with three compartments were constructed. The two outer compartments, into which the trifoliate orange (*Poncirus trifoliata* Raf.) seedlings and Bahia grass (*Paspalum notatum* Flügg.) were transplanted, were separated from the center compartment by a barrier made of a nylon screen of 37 μ m mesh. The inoculation with a VAM fungus, *Gigaspora margarita* Becker and Hall, was done in the center compartment. The density of hyphae, percentage of infection, and the number of spores were higher in the Bahia grass compartment than those in the compartment with trifoliate orange.

A petri dish experiment showed that although the Bahia grass and trifoliate orange root exudates stimulated the hyphal growth of *G. margarita*, the length of hyphae in the Bahia grass treatment was longer than that of the trifoliate orange.

In this experiment it was possible to observe in detail the distribution of hyphae in the rhizosphere of two plants under intercropping system conditions. These hyphae would be related to the interconnection between plants to promote a network system; the formation and development of this network system is influenced by some root exudates.

Key Words: hyphae, network, root exudates, sod culture, symbiosis, vesicular-arbuscular mycorrhizal fungi.

Introduction

The introduction of sod culture systems may promote greater symbiotic interactions between species. The utilization of sod culture as a cultural practice may be beneficial to the plants by improving soil texture, preventing soil erosion, promoting better water penetration, and supplying organic matter. It may propagate some symbiotic microorganisms such as VAM fungi, especially the indigenous VAM species.

Harinikumar et al. (1990) observed that the intercropping system between maize and soybean stimulated proliferation of VAM fungi as compared to a monocropping system. This intercropping system is also useful in orchards of fruit trees, such as satsuma mandarin, where a sod culture by using Bahia grass might improve the mycorrhizal formation in the root as compared to clean culture (Ishii et al., 1996). In Japan, sod culture of Naginatagaya (*Vulpia myuros* L.) has

been utilized in citrus orchards.

Associations between VAM fungi and plant roots are usually non-specific in fungus-plant pairing. The consequence of this low host specificity indicates that VAM hyphae distributed in soils may connect two or more different plants and then promote a network system among plants. This network made by VAM interconnections can bring some benefits to the soil plant system, such as the extension of root longevity (Tommerup and Abbott, 1981) and also provide channels to allow nutrient transfer between plants (Martins, 1993; Cruz and Martins, 1997; Martins and Cruz, 1997; Xiaolin and Zhang, 1997). The benefit of the nutrient transfer through VAM hyphae may be explained by an increased efficiency of nutrient utilization, so that nutrients are maintained in the plant system and not lost to the soil by leaching or adsorption.

In the sod culture system in citrus, this interconnection mediated by VAM hyphae is useful because the trees may benefit by the factors cited above. However, this interconnection involves not only a direct link between plants through hyphae, but also a complex of hyphae which are able to branch and link with other hyphae of the same species of VAM fungi distributed in the soil (Rutto, 2000).

Received; June 2, 1999. Accepted; November 29, 1999.

A part of this paper was presented at the 1999 Spring Meeting of the Japanese Society for Horticultural Science. This work was supported in part by a Grant-in-Aid for Scientific Research (No. 11460014) from the Ministry of Education, Science and Culture, Japan.

An establishment of the symbiosis between VAM fungi and their host plant roots involves a complex of interactions between the two symbionts (Gianinazzi-Pearson and Gianinazzi, 1988), especially under intercropping. The hyphal growth may be considerably stimulated in the presence of living host roots whose exudates and volatile chemical compounds act as stimulators (Gemma and Koske, 1988). Some of these compounds have been identified as flavonoids (Tsai and Phillips, 1991). In Bahia grass roots one of the stimulatory compounds has been identified as eupalitin, a flavonoid (Ishii et al., 1997). According to Gianinazzi-Pearson et al. (1989), some root exudates of a VAM host plant increased in vitro spore germination and hyphal growth of *Gigaspora margarita* as compared to those of a non-VAM plant.

However, there are a few reports concerning a direct interaction of hyphae in vivo. When these hyphae are distributed between two rhizospheres under an intercropping system, they are able to interconnect two plants. In this study, we observed the distribution of VAM hyphae that could form a network system between trifoliate orange and Bahia grass seedlings. Moreover, we investigated whether the growth of VAM hyphae in vitro could be stimulated by exudates from the host roots.

Materials and Methods

Experiment 1. Observation of a hyphal distribution in the rhizosphere of trifoliate orange and Bahia grass

This experiment was carried out in a greenhouse without temperature controlling system. Three special acrylic boxes (2 cm wide, 30 cm long, and 15 cm deep) were constructed; each box was divided into three compartments (Fig. 1). The central compartment (2 cm wide, 4 cm long and 15 cm deep) was separated from

the two outer ones by a barrier made of a nylon screen of 37 μ m mesh that allows the VAM hyphae to penetrate but not the plant roots. The pots were covered with aluminum paper to block the light and prevent the formation of algae.

Each pot was filled with sand previously sterilized with 70% methanol for about 10 min, rinsed with tap water, and fertilized with 140 ppm of N, 10 ppm of P, 140 ppm of K, and 50 ppm of Mg.

In early March the trifoliate orange and Bahia grass seeds were sown in trays containing vermiculite. Three months later a trifoliate orange seedling was transplanted into an outer compartment, and Bahia grass was transplanted into the other. The center compartment was inoculated with approximately 100 spores of the VAM fungi, *G. margarita*.

Three months after the transplantation, the aluminum cover was removed and hyphal density was measured by observing an area of 12.5 x 7.5 mm in each compartment using a camera equipped with a 0.5 inch, 900,000 pixel Charge Coupled Device (CCD) image sensor (Keyence VH-6300, Osaka, Japan). On a computer display, this area was divided into 192 squares and the density of hyphae was calculated according to the following equation: Density of hyphae (%) = (Squares with hyphae / Total squares (192)) x 100 (Fig. 1).

Soil samples were taken to count the number of spores in 25 g of soil, according to a procedure of Ishii et al. (1996). Roots samples, 10 mm long, were rinsed with distilled water, fixed in Karnovsky's solution (Karnovsky, 1965), and dehydrated through a series of graded solutions of ethanol and the t-butanol freeze-drying method (Inoue and Osatake, 1988). These roots were mounted on aluminum stubs with silver-conducting paint, coated with a thin layer of gold using an ion sputter (JEOL JET-1100, Tokyo, Japan), and photographed under a scanning electron microscope (SEM) (JEOL JSM-T200, Tokyo, Japan).

Fig. 1. Photograph of a root box to observe the interconnection of Bahia grass and trifoliate orange roots through VAM hyphae.

Left, Root box general design; Right, CCD image (x 25) of VAM hyphae.

From the same plants other roots samples were taken, 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. Effect of Bahia grass and trifoliate orange root exudates on the growth of VAM hyphae in vitro

Seeds of trifoliate orange and Bahia grass which were de-husked, sterilized with 5% sodium hypochlorite solution, and rinsed with sterile water, were grown in vitro. They germinated within 3 days in the dark at 25 °C in a petri dish with 1.5% agar (5 seeds per petri dish) as a medium. The petri dishes were then transferred to a growth chamber with lights. Seedlings were grown for 12 days, and then they were removed from the agar. Control dishes, which contained only 1.5% agar without any seedlings, were treated in the same way. Two petri dishes were prepared for each of the 3 treatments. The surface of *G. margarita* spores were sterilized for 15 min in 5% chloramine T plus 0.04% streptomycin, then four spores were transferred to each petri dish. The spores 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 density of hyphae was higher in the Bahia grass compartment than in the trifoliate orange compartment. In both compartments the density was higher in the area 3 cm away from the central compartment as compared to that area 6cm away (Table 1). The number of spores in the soil was higher in the Bahia grass compartment than in the trifoliate orange compartment. A similar result was observed in the percentage of VAM infection; more infections occurred on Bahia grass roots than on

Table 1. Density of VAM hyphae in the compartments of Bahia grass and trifoliate orange in a root box system.

Root section ^z	Density of hyphae (%)
Bahia grass 6cm	63.2 ± 1.8 ^y
Bahia grass 3cm	73.3 ± 1.1
Center	77.8 ± 1.8
Trifoliate orange 3cm	50.3 ± 2.6
Trifoliate orange 6cm	52.1 ± 1.9

^z Distance in either plant compartment away from the central compartment where VAM spores were inoculated.

^y Mean ± standard error (SE) (n=18). It was taken 6 images from each one of 3 boxes.

Table 2. The percentage of VAM infection in Bahia grass (BG) and trifoliate orange (TO) roots and the number of spores in the sections of root boxes

Side of the pot	VAM infection(%)	No. of spores/25 g soil
BG side	87.0 ± 2.5 ^z	877 ± 32
TO side	66.3 ± 4.4	567 ± 26

^z Mean ± SE (n=6). It was taken 2 samples from each one of 3 boxes.

trifoliate orange roots (Table 2). In both species, the VAM infection exceeded 25%.

The CCD images and SEM photomicrographs showed a high presence of VAM fungi infection sites, such as hyphal penetration into trifoliate orange roots and formation of new spores in/on Bahia grass roots. The CCD images disclosed new spores whose color and size were similar to those used as the inoculum (*G. margarita*) (Fig. 2).

Experiment 2

In the petri dishes containing root exudates of trifoliate orange or Bahia grass, a significant increase in hyphal growth of *G. margarita* occurred compared to the control with 1.5% agar. The growth of VAM hyphae in the petri dishes with Bahia grass root exudates surpassed that containing trifoliate orange exudates (Table 3).

Discussion

The presence of infected sites, confirmed by the CCD camera and SEM, in the compartments of the trifoliate orange and Bahia grass indicates that the VAM fungi had grown from the center compartments to the outer ones. In the CCD camera images, the spores in Bahia grass and trifoliate orange compartments have the same surface characteristics as do those of *G. margarita*, and no other contaminating spores were found in the soils of the outer compartments. Thus, the colonization in the plants could be attributable to the germination of the spores and the subsequent growth of mycelia from the center compartment. The network system made by VAM hyphae from the center to outer compartments might promote links between the two plant species. Newman et al. (1994) has shown similar results in which many forest plants and grasses were inter-linked by a common mycelium. Read and Birch (1988) found in a field experiment that VAM infection into the root of one plant might arise from a contact of a mycelium which spreads from a previously VAM infected plant. As a consequence of this pattern of infection, many plants may be inter-linked by the mycorrhizal hyphae.

The direct interconnection between both plants mediated by VAM hyphae is not clear. However, experiments with divided pots have shown that even

Fig. 2. Photomicrograph of a VAM hyphal invasion into trifoliate orange roots (A and a) and new VAM spores formed in/on Bahia grass roots (B and b).

A; CCD image, x 175; a; SEM photomicrograph, x 1000 (See arrows);

B; CCD image, x 175; b; SEM photomicrograph, x 750 (See arrows).

Table 3. Hyphal length of *Gigaspora margarita* cultured in a media which contained water and root exudates of Bahia grass or trifoliate orange seedling.

Treatment	Hyphal length(mm)
Control(water)	33.3 $\pm$ 1.9 ^z
Root exudates of Bahia grass	68.2 $\pm$ 3.5
Root exudates of trifoliate orange	42.8 $\pm$ 3.3

^z Mean $\pm$ SE (n=8).

when both plants were separated by nylon screen of 40 μ m mesh, which allow only the hyphae to cross, a nutrient transfer occurred. Some isotope elements, such as carbon (¹⁴C) (Martins, 1993), nitrogen (¹⁵N) (Cruz and Martins, 1997; Martins and Cruz, 1997), and phosphorus (³²P) (Xiaolin and Zhang, 1997) which were applied to the donor plants, were detected in the shoot of the receiver plant. Rutto (2000) found that in an in vitro experiment the hyphae were able to form branches by themselves and repair broken hyphae by forming by-pass units to link hyphal strands in the presence or absence of spores. Therefore, an establishment of a network system made by interconnections between the two plants through VAM hyphae occurred.

The results on hyphae density, VAM infection, and

number of spores indicated that exudates from Bahia grass roots were more effective for promoting VAM infection than were those from trifoliate orange roots. An increase in VAM infection sites in the Bahia grass compartment is attributed to the greater root density per unit volume of soil which expose these obligate symbiotic fungi to Bahia grass roots more than trifoliate orange roots. Hence, susceptibility to infection seemingly depends on the fungal species, the plant root characteristics, and the interactions between them, even though the VAM spores can colonize both plants. Also, the chemical compounds exuded from roots could work as stimulators or inhibitors according to the rhizospheric conditions. Hungria et al. (1997) found that bean plants intercropped with maize plants exuded more phenolic compounds which increased the number of VAM spores in the soil. However, under natural environmental conditions many species of microorganisms are affected by many other factors not shown in this experiment. The presence of root exudates which stimulates the VAM infection is just one factor. Even though the other aspects were not evaluated, root exudates are likely to contribute to the propagation and establishment of soil microorganisms, especially the symbionts.

The data of the petri dish experiment (Table 3) indicate an exudation of some chemical compounds from the roots that stimulate the growth of VAM fungi;

the effects of these compounds depend on the plant which releases them. In this study, those from Bahia grass were more promotive than were those from trifoliate orange. These results may explain the data shown in the root box experiment, where the VAM hyphae germinated and grew into the host roots, even if they lived a bit away from them in a separate compartment. Several authors have already documented the promotive effects of plant root exudates on spore germination and hyphal growth of VAM fungi under in vitro conditions (Bécard and Piché, 1989). They believe that root exudates can work as a transcriptional signal which induces the root colonization by VAM fungi.

In the roots of Bahia grass, three VAM stimulatory substances were isolated by flash chromatography, and one was identified as eupalitin (Ishii et al., 1997). These substances would have other effects on microbial population in the rhizosphere (Hartwig et al., 1991), so that enhancement of VAM spore germination and hyphal growth might bring some advantages to the plants in question.

We observed an in vivo distribution of VAM hyphae in the rhizospheres of the two kinds of plants grown side by side under an intercropping system condition in a greenhouse. If these hyphae could interconnect two neighboring plants to establish a network system, we speculate that a network system on these plants might be established under similar environmental conditions in sod culture. Further studies are required to evaluate the behavior of VAM hyphae in the presence of non-VAM plant roots and to identify any other chemical compounds from the roots of Bahia grass and trifoliate orange roots that would stimulate VAM establishment in soils.

Acknowledgments

The authors are very grateful to Mr. K. Kuwada and Yamaki Co. Ltd. for the use of the CCD camera.

Literature Cited

Bécard, G. and Y. Piché. 1989. New aspects on the acquisition of biotrophic status by a vesicular-arbuscular mycorrhizal fungus, *Gigaspora margarita*. *New Phytol.* 112: 77-83.

Cruz, A. F. and M. A. Martins. 1997. Transfer of nitrogen between plants interconnected by arbuscular mycorrhizal fungi (AMF). *R. Bras. Ci. Solo* 21: 559-565 (In Portuguese).

Gemma, J. N. and R. E. Koske. 1988. Pre-infection interactions between roots and the mycorrhizal fungus *Gigaspora gigantea*: chemotropism of germ-tubes and root growth response. *Trans. Brit. Mycol. Soc.* 91: 123-132.

Gianinazzi-Pearson, V., B. Branzanti and S. Gianinazzi. 1989. *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant

flavonoids. *Symbiosis* 7: 243-255.

Gianinazzi-Pearson, V. and S. Gianinazzi. 1988. Morphological integration and functional compatibility between symbionts in vesicular-arbuscular mycorrhizal associations. *NATO ASI Series H* 17: 73-84.

Harinikumar, K. M., D. J. Bagyaraj, B. C. Mallesha. 1990. Effect of intercropping and organic soil amendments on native mycorrhizal fungi in an oxisol. *Arid Soil Res. and Rehab.* 4: 193-197.

Hartwig, U. A., C. M. Joseph and D. A. Phillips. 1991. Flavonoids released naturally from alfalfa seeds enhance growth rates of *Rhizobium meliloti*. *Plant Physiol.* 95: 797-803.

Hungria, M.; D. S. Andrade, A. Colozzi-Filho and E. L. Balota. 1997. Interactions among soil microorganisms and bean and maize grown in monoculture or intercropped. *Pesq. Agropec. Bras.* 32: 807-818 (In Portuguese).

Inoue, T. and H. Osatake. 1988. A new drying method of biological specimens for scanning electron microscopy: The t-butyl alcohol freeze-drying method. *Arch. Histol.* 51: 53-59.

Ishii, T. and K. Kadoya. 1994. Effects of charcoal as a soil conditioner on citrus growth and vesicular-arbuscular mycorrhizal development. *J. Japan. Soc. Hort. Sci.* 63: 529-535.

Ishii, T., A. Narutaki, K. Sawada, J. Aikawa, I. Matsumoto and K. Kadoya. 1997. Growth stimulatory substances for vesicular-arbuscular mycorrhizal fungi in Bahia grass (*Paspalum notatum* Flügg.) roots. *Plant and Soil* 196: 301-304.

Ishii, T., Y. H. Shrestha and K. Kadoya. 1996. Effect of sod culture system of Bahia grass (*Paspalum notatum* Flügg.) on vesicular-arbuscular mycorrhizal formation of satsuma mandarin trees. *Proc. Int. Soc. Citriculture* 2: 822-824.

Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* 27: 137A-138A.

Martins, M. 1993. The role of the external mycelial network of arbuscular mycorrhizal fungi in the carbon transfer process between plants. *Mycol. Res.* 97: 807-810.

Martins, M. and A. F. Cruz. 1997. The role of the external mycelial network of arbuscular mycorrhizal fungi: III. A study of nitrogen transfer between plants interconnected by a common mycelium. *Rev. Microbiologia* 29: 228-233.

Newman, E. I., C. L. N. Devoy, N. J. Easen, K. J. Fowles. 1994. Plant species that can be linked by VA mycorrhizal fungi. *New Phytol.* 126: 691-693.

Phillips, J. M. and D. S. Hayman. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. Br. Mycol. Soc.* 55: 158-161.

Read, D. J. and C. P. D. Birch. 1988. The effects and implications of disturbance of mycorrhizal mycelial

- systems. Proc. Royal Soc. Edinb. 94: 13-24.
- Rutto, K. L. 2000. Studies on the axenic culture of vesicular-arbuscular mycorrhizal fungi as a contribution to sustainable agriculture. M.S. Thesis. Ehime Univ., Matsuyama, Ehime.
- Tommerup, I. C. and L. K. Abbott. 1981. Prolonged survival and viability of VA mycorrhizal hyphae after root death. Soil Biol. Biochem. 13: 431-433.
- Tsai, S. M. and D. A. Phillips. 1991. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spp. spores *in vitro*. Appl. Environ. Microbiol. 57: 1485-1488.
- Xiaolin, L. and J. Zhang. 1997. Phosphorus transfer via vesicular mycorrhizal hyphal link between roots of red clover. p. 749-750. In: T. Ando, K. Fujita, T. Mae, H. Matsumoto, S. Mori and J. Sekiya (eds.). Plant Nutrition - for Sust. Food Prod. and Envir. Kluwer Academ. Publ., Dordrecht, Boston, London.

カラタチ-バヒアグラス間作の根圏における VA 菌根菌糸の分布

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摘 要

植物間の VA 菌根 (VAM) 菌糸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、温室内で実施した。このルートボックスは、中央部に枠 (37 μ メッシュのナイロン製フィルムを張り付けたものであり、根の侵入は防げるが、VAM 菌の菌糸は通り抜ける) を 2 面 (枠間の長さ 2cm) 設けて、3 区分したものである。この外側の区分には、それぞれカラタチ、バヒアグラスを移植し、中央部には *Gigaspora margarita* 胞子を接種した。接種 3 カ月後、両植物の根圏における菌糸分布を CCD カメラや走査型電子顕微鏡で観察するとともに、両栽植部における菌糸密度、菌根感染率並びに新しく形成された胞子数を比較調査した。その結果、いずれの植物の根圏においても菌糸、胞子などを鮮明に

観察することができた。しかし、バヒアグラス栽植部の菌糸密度、菌根感染率および胞子数はカラタチ栽植部と比較して高かった。

バヒアグラスおよびカラタチ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、いずれの根浸出物も菌糸生長を促進した。しかし、この傾向はカラタチ根浸出物区よりもバヒアグラスの方が大であった。

以上、本実験ではカラタチ-バヒアグラス間作の根圏における VA 菌根菌糸の分布状態を詳細に観察することができた。菌糸は植物間の相互連結を伴うネットワークシステム形成に関与しており、根からの浸出物はそのシステムの形成や発達に影響を及ぼしていると考えられる。