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SHORT COMMUNICATION

Keitaro Tawaraya · Shigeki Watanabe · Horst Vierheilig
Tadao Wagatsuma

Formation of appressoria by the arbuscular mycorrhizal fungus *Gigaspora margarita* on roots of *Allium cepa* is linked with root age

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Abstract Spores of the arbuscular mycorrhizal fungus, *Gigaspora margarita*, were placed near the root tip, the middle of the root (equal distance from root base and root tip), or the root base (close to the shoot) of the first primary root of 9-day-old onion. Two weeks later, the number and position of appressoria and the appressoria with penetrating hyphae were determined in the first and the newly formed second primary roots. The total number of appressoria was not significantly different among the treatments. Inoculation near the root tip of the first primary root resulted in the formation of a large number of appressoria on the first primary root and the formation of about three times fewer appressoria on the second primary root. Inoculation near the base of the first primary root resulted in the formation of no appressoria on the first primary root, whereas many appressoria were formed on the second primary root. Our results suggest that the root age is a determinant of the appressorium formation.

Key words Appressorium · Arbuscular mycorrhiza · Glomeromycota · Root age · Root exudate

Roots of more than 80% of all land plant species are colonized by arbuscular mycorrhizal (AM) fungi belonging to the Glomeromycota (Schüßler et al. 2001). AM root colonization improves the plant growth via an enhanced uptake of nutrients, especially P (Smith and Read 1997). AM fungi can be found in the soil as spores and hyphae and in colonized roots. Usually, under optimal soil conditions, e.g., soil temperature, CO_2 levels, and moisture, AM fungal spores germinate irrespective of the presence of a host root in close proximity and extend germ tubes (Vierheilig and Bago 2005). After the first contact of a hypha with a host root, an appressorium is formed on the epidermal cell wall (Garriock 1989), and internal hyphae are developed from the appressorium. Thus, the appressorium can be described as the first fungal structure that is formed in close contact with the host plant.

Although appressoria formation is an essential step for the establishment of the AM association, relatively few data are available on the factors determining the appressoria formation. The first appressoria were formed by *Glomus mosseae* within 36 h after the beginning of interaction with *Ocimum basilicum* or *Helianthus annuus* (Giovannetti and Citernesi 1993). Nagahashi and Douds (1997) showed that appressoria only formed on epidermal cell walls isolated from carrot roots but did not form on epidermal cell walls isolated from non-host roots. Interestingly, on roots of so-called Myc^- plant mutants, which are resistant to AM colonization, more appressoria are formed compared to AM susceptible Myc^+ wild-type plants (Bradbury et al. 1993; Vierheilig and Piché 1996). The reason for this enhanced appressoria formation in the Myc^- plant mutants still remains unknown.

High P levels in the soil (Thomson 1986) or in plants (Tawaraya et al. 1994) decrease not only root colonization but also appressorium formation, which indicates that the degree of root colonization is linked with appressoria formation. Positive correlation between the number of appressoria and root colonization was also shown in symbiosis between soybean and six AM fungal species (Van Nuffelen and Schenck 1984). These results suggested that the formation of appressoria can be used as an indicator for the degree of further root colonization.

There are several reports that the susceptibility of a root to colonization by AM fungi changes depending on its age. Older parts of a root of leek and clover become colonized less easily than younger parts (Amijee et al. 1993). Colonization of roots increased significantly with increasing root age of *Andropogon gerardii* only in the low-P treatment (Henry and Kosola 1999). However, no data are yet available whether this age-dependent susceptibility of roots to

K. Tawaraya (✉) · S. Watanabe · T. Wagatsuma
Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan
Tel. +81-235-282870; Fax +81-235-258578
e-mail: tawaraya@tds1.tr.yamagata-u.ac.jp

H. Vierheilig
Institut für Pflanzenschutz, Universität für Bodenkultur Wien, Wien, Austria

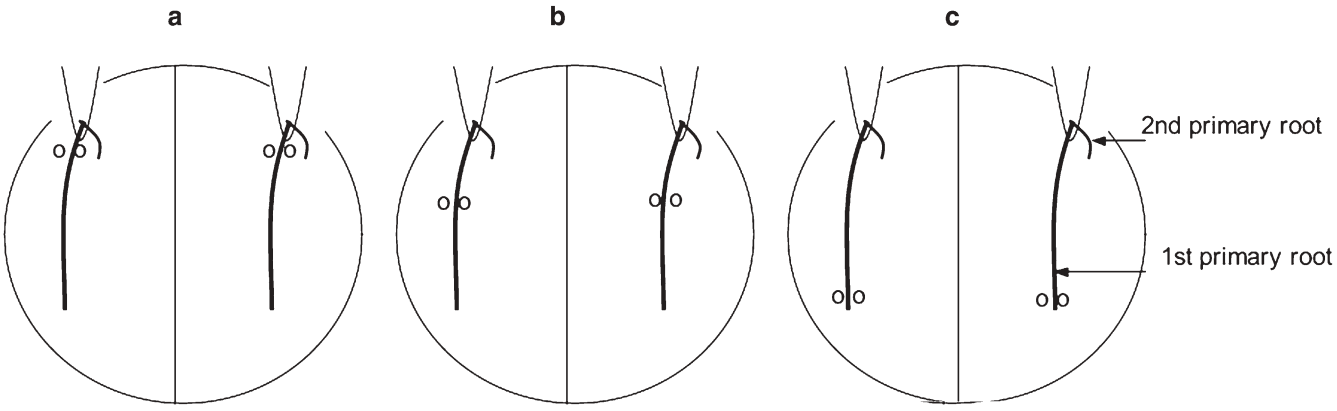

Fig. 1. Inoculation at the root base (a), the middle of the root (b), and the root tip (c) with spores (*white circles*) of arbuscular mycorrhizal fungus *Gigaspora margarita*

Table 1. Total number of appressoria, number and length of roots, shoot dry weight, and shoot P concentration 1 and 2 weeks after the placement of spores to the root base, the middle of root, and the root tip

Treatment	Total number of appressoria (no./plant)	First primary root	Second primary root		Total root length (mm/plant)	Shoot dry weight (mg/plant)	Shoot P concentration (mg/g)
		Length (mm/plant)	Number (no./plant)	Length (mm/root)			
One week							
Tip	0 a	76.3 a	2.0	16.9 a	110.1 a	0.47 a	ND
Middle	0 a	78.0 a	2.0	20.2 a	118.4 a	0.47 a	ND
Base	0 a	75.5 a	1.4	21.4 a	105.5 a	0.48 a	ND
Two weeks							
Tip	6.7 a	102.6 a	2.9	55.3 a	263.0 a	1.22 a	1.44 a
Middle	6.6 a	82.9 a	2.6	44.1 a	197.6 a	1.03 a	1.28 a
Base	7.0 a	91.9 a	2.1	54.6 a	206.6 a	1.20 a	1.14 a

a, not significantly different in three treatments ($P < 0.05$); ND, not determined

AM fungi can be linked with a certain stage of colonization. We hypothesized that different susceptibility depending on the root age may occur at the first stage of colonization, the appressorium formation, because it can be used as an indicator for the degree of further root colonization.

To reveal the preferable colonization site, we inoculated spores of AM fungi *Gigaspora margarita* to the first primary roots of onion at three different root positions – root base, middle of the root, and root tip – to examine the appressoria formation as follows.

Sea sand was desalted with 0.5 M HCl and autoclaved. Bicompartmentalized plastic Petri dishes (diameter, 90 mm) were modified to grow plants (Fig. 1). Holes for shoot growth were made on the one side (top) and drain holes were made on the other side (bottom). Autoclaved sand was placed into the dishes. One seedling of 9-day-old onion (*Allium cepa* L. cv. Senshuchukoki) was transplanted into the sand of each compartment of the Petri dish. At this point of time, the first primary root was 64.9 ± 0.6 (SE) mm long, and the second primary root was 2.1 ± 0.9 mm long. Each treatment was repeated four times.

Two spores of the AM fungus *Gigaspora margarita* Becker & Hall were placed at different sites of the first primary root: (1) 5 mm apart from the root tip, (2) 5 mm apart from the middle of the root (equal distance between root base and root tip), or (3) 5 mm apart from the base point of the root. Roots and spores were covered with sand. Thereafter, the dishes were covered and sealed with vinyl tape. In a growth chamber (27°C, 150 µM $m^{-2} s^{-1}$ photo synthetic photon flux density (PPFD), 16 h/8 h day/night photoperiod) dishes were placed vertically. Every week, 8 ml nutrient solution (Wagatsuma et al. 1988) was applied into the holes at the top of the dishes.

Shoots and roots were harvested 1 and 2 weeks after the inoculation. Each root was carefully separated from the sand and the total root length, and the number and length of first and second primary roots were determined. Roots were cleared and stained with a 500 mg l^{-1} aniline blue solution (Tawaraya et al. 1998). Stained roots were mounted on slides and were observed under a compound microscope with a magnification of 100–200. The number of appressoria and those with penetrating hyphae were quantified. The shoot dry weight was determined after drying the plant material at 70°C for 72 h. For P analysis, grounded shoots were digested in a HNO_3-$HClO_4$-H_2SO_4 solution. The P content in the digested solution was determined colorimetricallly with the vanadomolybdate-yellow assay (Olsen and Sommers 1982). Data were subjected to an analysis of vari-

ance, and means were compared by the least significant difference method (LSD, $P < 0.05$) using the statistical software StatView 4.5 (Abacus Concepts).

One week and 2 weeks after the inoculation (placement of the spores), neither the length of the first and the second primary roots nor the shoot dry weight and the shoot P concentration among treatments were significantly different (Table 1).

One week after the inoculation, no appressoria was formed in any treatments (Table 1), whereas 2 weeks after inoculation, appressoria were formed. The total number of appressoria per plant was not significantly different among the treatments (see Table 1).

When the number of appressoria was determined separately on the first and the second primary roots in each of the different spore placement, some differences could be observed. When spores were inoculated near the root tip, more appressoria were formed on the first primary root than those on the second primary root (Fig. 2A), and more appressoria were formed in the neighborhood of the root tip of the first primary root than the other part of the first primary root (data not shown). However, when roots were inoculated at the middle or the base of the root, more appressoria were formed on the second primary root than those on the first primary root.

When roots were inoculated at the root tip, more appressoria with penetrating hyphae were found on the first primary roots than on the second primary roots (Fig. 2B); however, when roots were inoculated at the middle of the root or the root base, more appressoria with penetrating hyphae were found on the second primary root than on the first primary root.

In a recent review by Vierheilig (2004), the effect of root exudation depending on P status of a host plant on AM fungal hyphal growth and root colonization has been extensively discussed. Root exudates of P-deficient onion increased AM fungal growth and root colonization (Tawaraya et al. 1996, 1998). In the present study, the P concentration in the shoots of the different treatments was not affected by the placement site of spore; thus, any P status effect on appressoria formation can be excluded.

The root growth pattern was similar in all treatments, indicating that the site of spore placement does not affect to the root growth. At the end of the experiment, the number of appressoria per plant was not significantly different among the treatments.

However, looking in more detail at the pattern of appressoria formation on roots (differentiating between first and second primary roots), a different pattern could be observed. In the first primary roots, a clear age-dependent pattern could be observed in appressoria formation. The younger the root where spores were placed, the more appressoria were formed. When spores were placed at the root base, no appressoria were formed at the oldest part at all.

This age-dependent root susceptibility to form appressoria was confirmed when we looked at the pattern of appressoria formation in the second primary roots. When spores where placed to the base of the first primary root (the oldest part of the root), the spores were in the vicinity

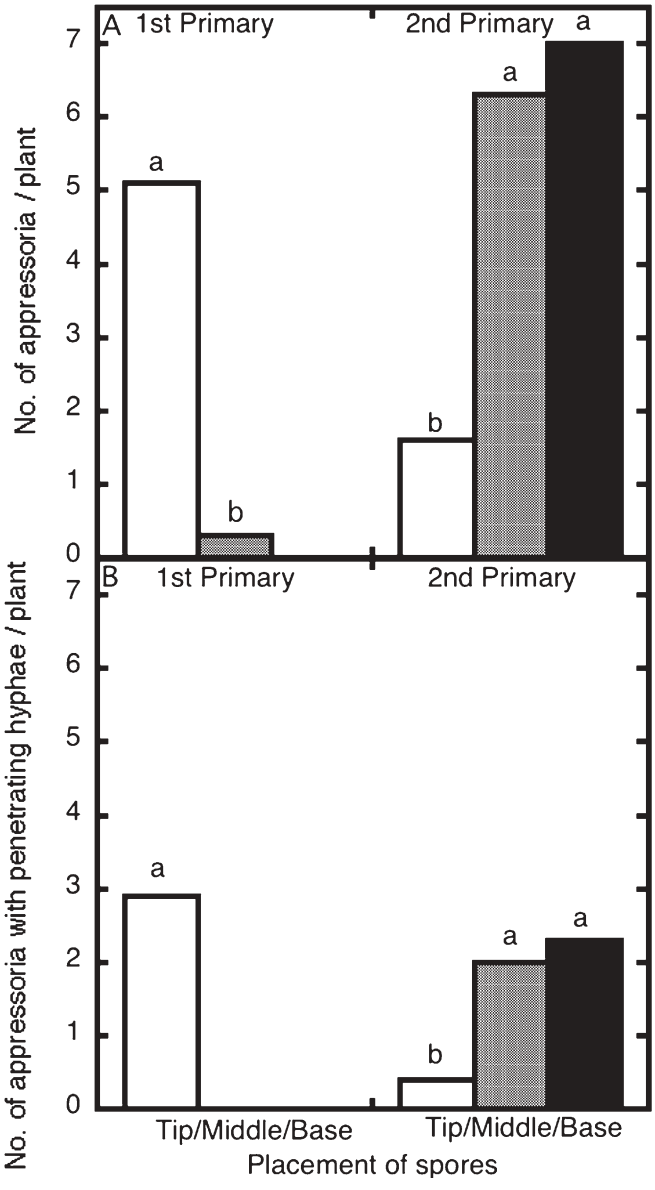

Fig. 2. Number of appressoria formed on the 1st primary and the 2nd primary root (**A**), and number of appressoria with penetrating hyphae into epidermal cells (**B**), 2 weeks after inoculation. *Bars with the same letter* are not significantly different ($P < 0.05$)

of the tip (the youngest part of the root) of the second primary root (see Fig. 1). Interestingly, in this case there was no appressoria formation on the first primary root (the oldest part of the root), but a large number of appressoria were formed on the second primary root, which was much younger than the first primary root.

A root age-dependent susceptibility to AM fungi has been reported previously (Amijee et al. 1993; Henry and Kosola 1999; Hepper 1981, 1985). In these studies, the whole roots were subjected to soil inoculum containing spores, external hyphae, and colonized root of AM fungi in their experiments. We inoculated two spores of *G. margarita* and traced the growth of external hyphae. We confirmed that the receptivity of onion roots to appressorium forma-

tion by *G. margarita* is different depending on the root age and that this fungus clearly prefers the younger root.

Attractive effects of root exudates on hyphal growth from spores of AM fungi have been shown in experimental whole root systems (Gemma and Koske 1988; Vierheilig et al. 1998). However, a specific root section for the attractive effect is not known. A root age-specific exudation pattern has been documented for different plants. More ninhidrin-positive compounds and reducing sugars were exuded from the root tip of broad bean (Pearson and Parkinson 1961; Schroth 1961), and in soybean the exudation of a number of flavonoids differed depending on the root section (Kape et al. 1992; Graham 1991). Thus, it is possible that some specific exudation around the root tip is responsible for the effect on appressoria formation we observed.

From an ecological point of view, our results on a root age-dependent susceptibility to AM fungi could mean that plant species or cultivars with high root branching and thus the formation of many root tips except for non-host plants may either achieve a higher degree of AM colonization or at least be colonized by AM fungi more rapidly. In a further study, this hypothesis will be elucidated.

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