

Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation

Alexandra Pinior, Urs Wyss, Yves Piché, and Horst Vierheilig

Abstract: The effect of root exudates from non-mycorrhizal and mycorrhizal cucumber (*Cucumis sativus* L.) plants colonized by one of three arbuscular mycorrhizal fungi (*Gigaspora rosea* Nicolson & Schenck, *Glomus intraradices* Smith & Schenck, or *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe) on hyphal growth of *Gi. rosea* and *G. intraradices* in axenic culture and on root colonization by *G. mosseae* in soil was investigated. Root exudates from non-mycorrhizal cucumber plants clearly stimulated hyphal growth, whereas root exudates from all mycorrhizal cucumber plants tested showed no stimulation of the hyphal growth of *Gi. rosea* and only a slight stimulation of the hyphal growth of *G. intraradices*. Moreover, root exudates from all mycorrhizal cucumber plants inhibited root colonization by *G. mosseae* compared with the water-treated controls. These results suggest that plants colonized by AM fungi regulate further mycorrhization via their root exudates.

Key words: Glomales, *Gigaspora rosea*, *Glomus intraradices*, *Glomus mosseae*, root exudates, regulation.

Résumé : L'effet des exsudats racinaires de plantules, mycorhizées ou non, du concombre (*Cucumis sativus* L.) par un des trois champignons endomycorhiziens arbusculaires (*Gigaspora rosea* Nicolson & Schenck, *Glomus intraradices* Smith & Schenck et *Glomus mosseae* Nicolson & Gerdemann) Gerd. & Trappe), sur la croissance in vitro des hyphes de *Gi. rosea* et *G. intraradices* de même qu'au niveau de la colonisation racinaire par *G. mosseae* a été étudié. Les exsudats des racines non mycorhizées du concombre stimulent nettement la croissance des hyphes tandis que tous les exsudats des racines mycorhizées du concombre montrent peu ou pas de stimulation chez les hyphes de *G. intraradices* et *Gi. rosea*, respectivement. De plus, les exsudats racinaires de toutes les plantules mycorhizées inhibent la colonisation des racines par *G. mosseae* en comparaison du témoin aqueux sans exsudat. Ces résultats suggèrent que les plantes colonisées par des champignons endomycorhiziens arbusculaires peuvent contrôler la mycorhization subséquente via leurs exsudats racinaires.

Mots clés : Glomales, *Gigaspora rosea*, *Glomus intraradices*, *Glomus mosseae*, exsudats racinaires, régulation.

Introduction

During the formation of the arbuscular mycorrhizal (AM) symbiosis, root exudates seem to have an important signaling function. They stimulate AM hyphal growth, as first reported by Hepper and Mosse (1975) and in many studies thereafter (recently reviewed by Vierheilig et al. 1998c), and exhibit an attractive effect on AM hyphal growth (Koske 1982; Gemma and Koske 1988; Koske and Gemma 1992; Suriyapperuma and Koske 1995; Vierheilig et al. 1998b).

Roots exudates are also known to be essential signals in the interaction between legumes and *Rhizobium*. Certain exuded (iso)flavonoids induce or repress the synthesis of the rhizobial lipo-chito-oligosaccharide signals (see reviews by Bladergroen and Spaink 1998; Cohn et al. 1998; Schultze and Kondorosi 1998). Xie et al. (1995) recently showed that co-inoculation of *Rhizobium* with the AM fungus *Glomus mosseae* (Nicolson & Gerdemann) Gerd. & Trappe resulted in an enhanced root colonization by the AM fungus, and it was suggested that this enhanced root colonization was due to the changed flavonoid content found in rhizobial root exudates and roots (Recourt et al. 1992; Dakora et al. 1993; Schmidt et al. 1994; Bolanos-Vasquez and Werner 1997).

As found in legume roots during the rhizobial symbiosis, in roots of mycorrhizal plants the content of phenolic compounds, e.g., flavonoids, was found to be different from those of non-mycorrhizal plants (Grandmaison et al. 1993; Harrison and Dixon 1993; Maier et al. 1995, 1997, 1999; Peipp et al. 1997). Therefore, it is tempting to speculate that changes of flavonoids or other compounds in root exudates of mycorrhizal plants could affect further root colonization by AM fungi.

To our knowledge, all studies on the effect of root exudates on AM fungi have been performed with root exudates collected from non-mycorrhizal plants (see Vierheilig et al.

Received December 8, 1998.

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1998c). However, no information is available about the effects of root exudates obtained from mycorrhizal plants on AM fungi. The objective of the present study was to compare the effect of root exudates from non-mycorrhizal cucumber plants with the effect of root exudates from mycorrhizal cucumber plants on in vitro AM hyphal growth and on root colonization by AM fungi.

Materials and methods

General conditions

AM fungi studied

Glomus mosseae (BEG 12; La Banque Européenne des Glomales, International Institute of Biotechnology, Kent, U.K.); *Glomus intraradices* Smith & Schenck (DAOM 197198; Department of Agriculture, Ottawa, Ont.); and a recently newly classified *Gigaspora rosea* Nicolson & Schenck (Bago et al. 1998), which was formerly wrongly classified as *Gigaspora margarita* Becker & Hall (DAOM 194757) were used.

Root exudates and plant analyses

Cucumber (*Cucumis sativus* L. cv. Straight Eight) seeds were surface sterilized in 50% commercial bleach for 5 min, rinsed several times in sterile distilled water, and germinated in autoclaved (40 min, 120°C) vermiculite. After 5 days the seedlings were transferred to a steam-sterilized (40 min, 120°C) mixture of silicate sand, TurFace® (baked clay substrate that is mechanically broken into particles with a diameter of 2–5 mm; Applied Industrial Materials, Corp., Buffalo Grove, Ill.) and soil (2:2:1, v/v/v). Plants were grown in a growth chamber (16 h light : 8 h dark; 22:20°C (day: night); RH 50%) using for AM fungal inoculation the system developed by Wyss et al. (1991) consisting of three compartments. The central compartment (20 cm long × 10 cm high × 2 cm deep) with beans (*Phaseolus vulgaris* L. cv. Sun Gold), contained the inoculum of the three AM fungi. It was separated from the lateral compartments by a nylon screen (60-µm mesh size), which can be penetrated by hyphae but not by roots. This design led to a rapid root colonization of the cucumber plants in the lateral compartments, which were subdivided into five small subcompartments (3.3 cm long × 10 cm high × 2 cm deep). As two lateral compartments were adjacent to each central-inoculum compartment, 10 plants could be inoculated at a time. In the non-mycorrhizal control treatment central compartments were filled with the steam-sterilized substrate, watered with a filtered (Whatman No. 1; Maidstone, U.K.) water suspension (10 mL) of the inoculum to introduce associated microflora (McAllister et al. 1997), and bean plants were grown.

Twenty days after joining the lateral compartment with the central compartments (i.e., inoculation with the AM fungi), cucumber plants were harvested, their roots were rinsed with tap water, and roots of whole plants were placed in the growth chamber in distilled water for 20 h to obtain root exudates.

Thereafter, the pH of the solutions (pH: distilled H₂O, 4.62; non-mycorrhizal roots, 5.0; roots colonized by *Gi. rosea*, 5.5; roots colonized by *G. intraradices*, 5.7; roots colonized by *G. mosseae*, 5.7), the colonization level (*Gi. rosea*, 69 ± 2 (mean ± SD); *G. intraradices*, 80 ± 2; *G. mosseae*, 66 ± 6) of the cucumber plants (method described below), and the root fresh weight were determined. For the analysis of the P content, dried roots (30 h, 65°C) were digested (Parkinson and Allen 1975), and the P content was determined by a Perkin Elmer model 40 emission plasma spectrometer. All experimental data were analyzed by a Tukey test ($P < 0.05$).

Determination of root colonization

Roots of each plant were cleared by boiling in 10% KOH and stained according to the method of Vierheilig et al. (1998a) by boiling in a 5% ink (Shaeffer; black) – household vinegar (5% acetic acid) solution. After staining, the percentage of root colonization was determined according to the method of Newman (1966) by quantifying the presence or absence of fungal structures within root at 100 intersections.

Axenic culture experiments

The effect of root exudates from *Gi. rosea* and *G. intraradices* colonized plants on the hyphal growth of *Gi. rosea* in vitro was compared to study intergeneric effects, whereas the effect of root exudates from *G. mosseae* and *G. intraradices* colonized plants on the hyphal growth of *G. intraradices* was compared to find intrageneric effects.

Production of spores

Spores of *G. intraradices* were routinely obtained from in vitro dual cultures with tomato (*Lycopersicon esculentum* Mill. cv. Vendor) roots (Chabot et al. 1992). Before use, *G. intraradices* plates were stored for several weeks at 4°C. Spores of *Gi. rosea* were isolated from a leek (*Allium porrum* L.) pot culture by wet sieving followed by gradient centrifugation (Furlan et al. 1980). Spores were surface sterilized (Bécard and Fortin 1988) and stored in a mixture of 0.02% (w/v) streptomycin sulfate and 0.01% (w/v) gentamicin sulfate solution at 4°C until used.

Treatment of root exudates and preparation of the media

Solutions were passed through filter paper (Whatman No. 1) and Millipore filters of different sizes (3.0, 0.8, and 0.22 µm), before concentrating exudates by lyophilization to a ratio of 1 g root fresh weight equivalent to 1 mL of exudate solution. After concentrating, the pH of the root exudates was adjusted to 5.5, and solutions were sterilized by passing them through a 0.22-µm Millipore filter. These sterile exudate solutions were stored at –20°C until use.

The experiments on hyphal growth were carried out in a modified minimal White's medium (Bécard and Fortin 1988), which was solidified with 0.3% Gel-Gro (ICN Biochemicals, Aurora, Ohio). The pH was adjusted to 5.5 before autoclaving at 121°C for 20 min. Exudate solutions were added aseptically to the warm culture medium.

Experimental conditions

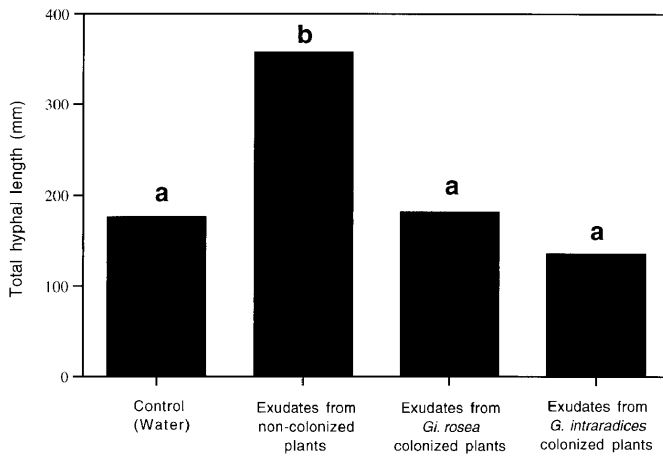
In a pre-experiment the effect of different concentrations (0.01, 0.05, 0.2, 0.5, and 1.0 mL) of the exudates of non-mycorrhizal cucumber plants on hyphal growth was determined. The lowest concentration exhibiting a clear stimulatory effect was chosen for further experiments with root exudates from non-mycorrhizal and mycorrhizal plants.

Eight to ten spores of *Gi. rosea* were placed in a row in a square Petri dish (15 × 90 × 90 mm) filled with 20 mL of the medium and mixed with 1 mL of the concentrated exudates or distilled H₂O as a control. As 1 mL of the concentrated exudates is equivalent to 1 g root fresh weight, after mixing with the medium the final ratio was 1 g root fresh weight equivalent to 21 mL.

Eight to ten spores of *G. intraradices* were placed in a circle with a diameter of about 2 cm in the center of a round Petri dish (15 × 90 mm) filled with 20 mL of the medium and mixed with 0.5 mL of the concentrated exudates or distilled H₂O as a control. As 0.5 mL of the concentrated exudates is equivalent to 0.5 g root fresh weight, after mixing with the medium the final ratio was 0.5 g root fresh weight equivalent to 20.5 mL.

The experiments were repeated twice with three Petri dishes per treatment. Petri dishes were sealed with parafilm and incubated at 27°C in the dark in a 2% CO₂-enriched environment according to

Fig. 1. Total hyphal length of *Gi. rosea* after application of root exudates of non-mycorrhizal cucumber plants and of cucumber plants colonized by *Gi. rosea* or *G. intraradices* after 40 days. Bars with the same letter are not significantly different according to the Tukey test ($P < 0.05$).



Poulin et al. (1993). The parafilm was perforated to allow a free exchange of CO_2 . Because of the negative geotropic growth of hyphae of *Gi. rosea* (Watrud et al. 1978), the square Petri dishes with *Gi. rosea* were kept in a vertical position.

Measurements

The hyphal development was followed under a Wild M3Z stereomicroscope and marked with coloured markers on the bottom of the Petri dish. The effect of the concentrated root exudate solutions on the total hyphal length of *Gi. rosea* and *G. intraradices* and on the hyphal growth pattern of *G. intraradices* was determined at the end of the experiment (40 days).

For quantification of the hyphal growth pattern, a modified measurement method as suggested by Juge et al. (1998) was performed. Circles were drawn around each spore and the radius of the circle that intersected the most distant hyphal tip was determined (see Fig. 3a).

Root colonization experiments

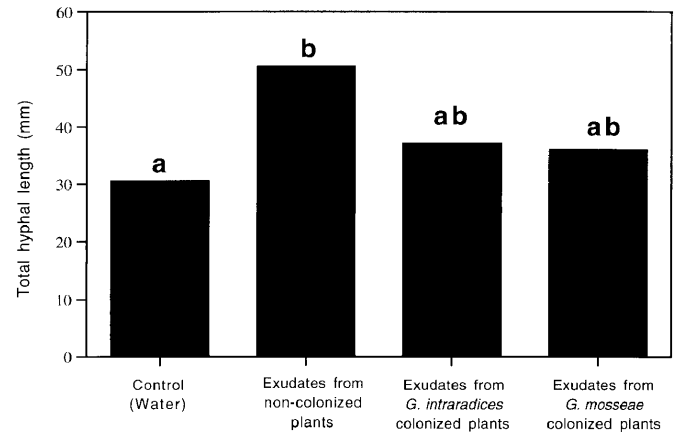
The effect of collected root exudates from cucumber plants on root colonization of cucumber plants by *G. mosseae* was tested in the above described compartment system. Root exudate solutions were adjusted to a ratio of 1 g root fresh weight equivalent to 22 mL of exudate solution by adding distilled water when necessary. This concentration is similar to the concentration used in the axenic culture experiment with spores of *Gi. rosea*. The pH was adjusted to 5.5 and exudates were stored at -20°C until use.

Bean plants in the central inoculum compartment were cut to prevent an interference with their root exudates. After 6 days, cucumber plants (5 days old) were transferred to the compartment system, and root exudate solutions or distilled H_2O as a control (5 mL/plant) were applied daily to each cucumber plant in the small lateral subcompartments. After 8 days, cucumber plants were harvested and root colonization was determined as described above. The experiment was repeated twice using five replicates per treatment.

Results

In axenic culture of *Gi. rosea*, root exudates from non-mycorrhizal plants clearly stimulated hyphal growth compared with the water control (Fig. 1). This stimulatory effect

Fig. 2. Total hyphal length of *G. intraradices* after application of root exudates of non-mycorrhizal cucumber plants and of cucumber plants colonized by *G. intraradices* or *G. mosseae* after 40 days. Bars with the same letter are not significantly different according to the Tukey test ($P < 0.05$).



was absent in the presence of root exudates from mycorrhizal plants, independently of the colonizing fungus (*Gi. rosea* or *G. intraradices*). Hyphal length in the presence of root exudates from mycorrhizal plants was similar to that of the water control.

In axenic culture of *G. intraradices*, root exudates from non-mycorrhizal plants showed a clear hyphal growth stimulating effect compared with the water control (Fig. 2) but not with exudates from mycorrhizal plants (colonized by *G. intraradices* or *G. mosseae*).

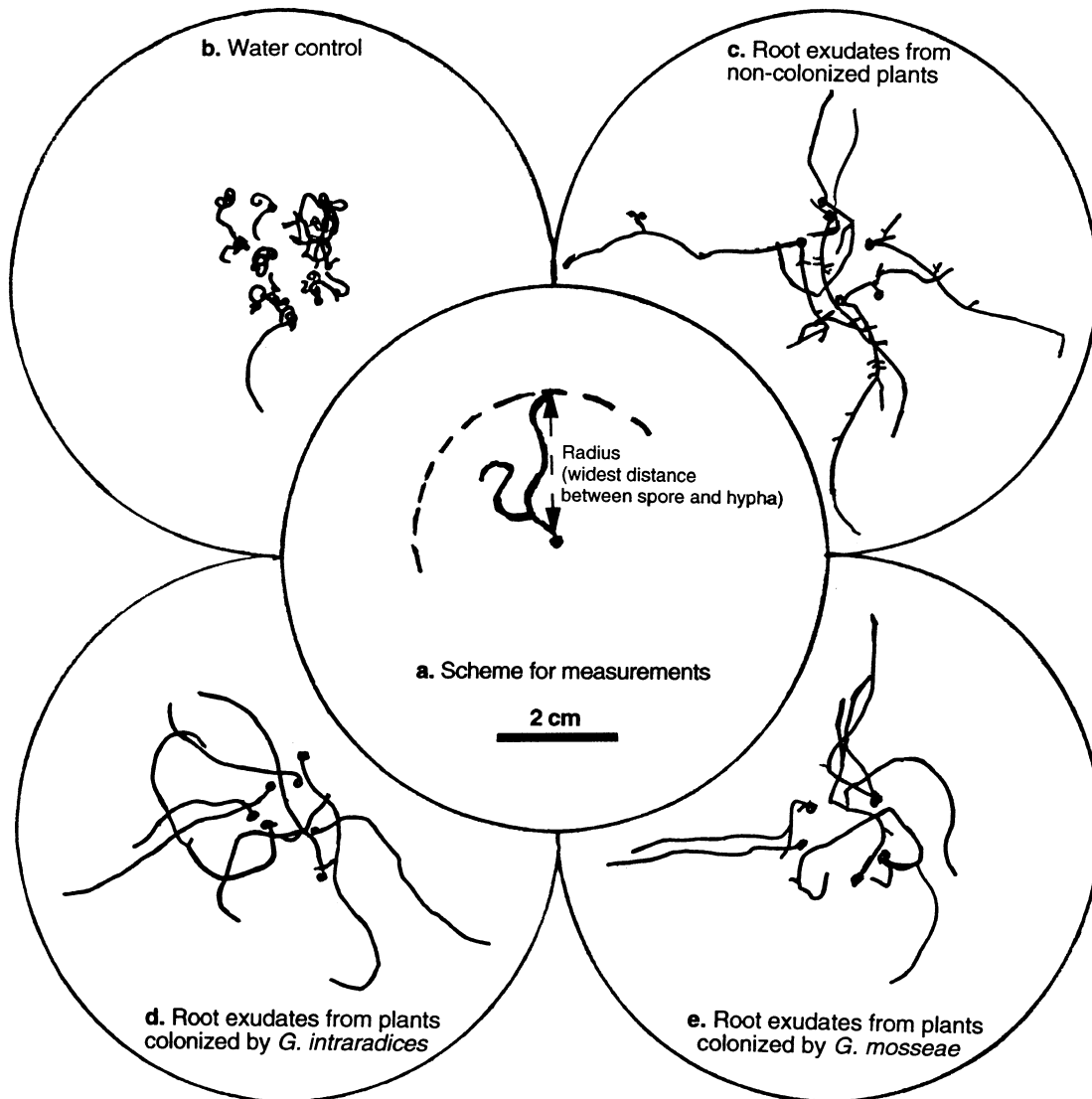
In all treatments with root exudates, the hyphal growth pattern of *G. intraradices* was changed. In the water control, hyphae grew spirally around roots, whereas in the presence of root exudates from non-mycorrhizal and mycorrhizal plants, long runner hyphae could be observed, growing away from the spore (Fig. 3). When the distance between the spore and the most distant hyphal tip was measured, it was significant that hyphae in the presence of all root exudates explored a greater surface area than in the water control (Fig. 4). The P content (Fig. 5) in roots of all mycorrhizal plants used for the collection of exudates was enhanced.

Root colonization (Fig. 6) was slightly, but not significantly, enhanced when root exudates from non-mycorrhizal plants were added to cucumber plants inoculated with *G. mosseae*. In contrast, in all the treatments with root exudates from mycorrhizal plants, independently of the colonizing fungus (*Gi. rosea*, *G. intraradices*, or *G. mosseae*), root colonization by *G. mosseae* was clearly reduced compared with the water control treatment.

Discussion

We have shown in this paper that root exudates collected from AM colonized cucumber plants show no AM hyphal growth stimulation (*Gi. rosea*) and an inhibitory effect on root colonization by AM fungi compared with root exudates from non-mycorrhizal plants and to a control treatment without root exudates. This is reminiscent of a feedback-regulated autoregulatory mechanism that suppresses the ex-

Fig. 3. (a) Scheme for measurement of the distance between the spore and the most distant hyphal tip. (b) Hyphal growth pattern of *G. intraradices* in the water control treatment. (c–e) Hyphal growth pattern of *G. intraradices* after application of root exudates of non-mycorrhizal cucumber plants (c) and root exudates of cucumber plants colonized by *G. intraradices* (d) or *G. mosseae* (e) after 40 days.



tent of the establishment of symbiosis in the legume–*Rhizobium* interaction (reviewed in Caetano-Anolles and Gresshoff 1991). However, it remains to be elucidated whether the unknown signals involved in the autoregulation of nodule formation also affect AM colonization and whether these signals elicit symbiosis suppressing defense responses in the host plant. The mycorrhizal root apparently reduces further colonization by AM fungi via its exudates. A similar mechanism has been reported from some AM non-host plants: roots exude compounds inhibitory to the formation of the AM symbiosis (El-Atrach et al. 1989; Schreiner and Koide 1993; Vierheilig et al. 1995, 1996).

The stimulatory effect of root exudates of AM host plants on AM fungal growth has been reported in many studies (see review Vierheilig et al. 1998a). In our study, root exudates of non-mycorrhizal plants clearly stimulated AM hyphal growth in axenic culture. This stimulatory effect of root exudates of plants with a low P status towards AM fungi has

been attributed to P-regulated permeability of membranes in roots, which depending on the P status of the plant, alters the root exudates qualitatively or quantitatively and hence affects further root colonization (Ratnayake et al. 1978; Graham 1982; Graham et al. 1981; Schwab et al. 1983; Elias and Safir 1987; Nagahashi et al. 1996; Tawaraya et al. 1994, 1996a, 1996b, 1998). Tawaraya et al. (1998) recently reported enhanced root colonization when root exudates of non-mycorrhizal onion plants were applied to onions (*Allium cepa* L.) inoculated with *Gi. margarita*. This stimulation of root colonization was attributed to phenolics in the root exudates. It is well known that certain phenolics applied to plants enhance root colonization by AM fungi (Siqueira et al. 1991a; 1991b; Xie et al. 1995; Fries et al. 1997; Vierheilig et al. 1998c; Junior and Siqueira 1998). The portion of AM stimulating phenolics in the root exudates seems to vary, depending on the P status of the plant. Roots of plants with a low P status seem to exude a higher portion of

Fig. 4. Distance between spores of *G. intraradices* and their most distant hyphal tip after application of root exudates of non-mycorrhizal cucumber plants and of cucumber plants colonized by *G. intraradices* or *G. mosseae* after 40 days. Bars with the same letter are not significantly different according to the Tukey test ($P < 0.05$).

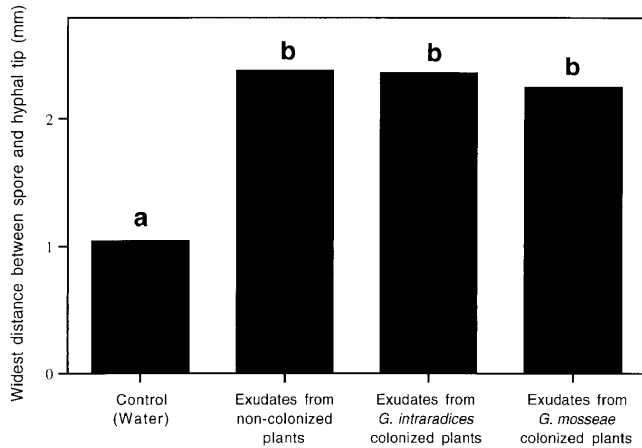
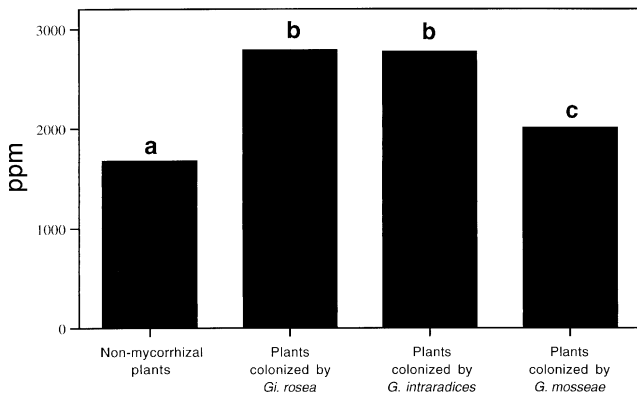


Fig. 5. P content of dried roots of non-mycorrhizal and mycorrhizal cucumber plants used for the production of root exudates. Bars with the same letter are not significantly different according to the Tukey test ($P < 0.05$).

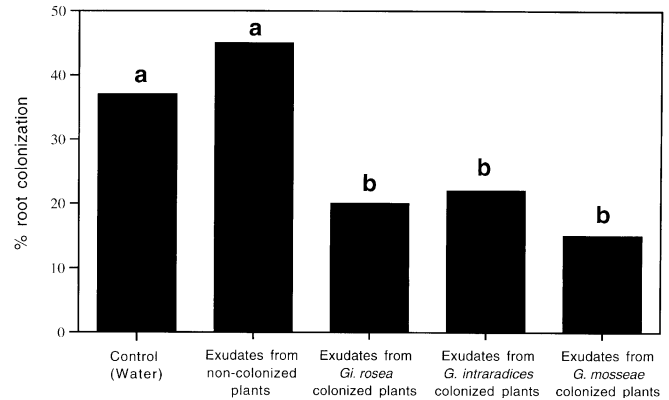


AM-stimulating phenolics than roots of plants with a higher P status (Tawaraya et al. 1998).

In our experiments, all mycorrhizal plants showed increased P levels in the roots. Root exudates from non-mycorrhizal plants, with a low P level in the roots, clearly stimulated hyphal growth, whereas root exudates from mycorrhizal plants, with a higher P level in the roots, showed no hyphal growth stimulation (*Gi. rosea*) or only a slight hyphal growth stimulation (*G. intraradices*). These results point towards a P-mediated effect in our hyphal growth experiments.

However, the results of our experiment on root colonization suggest a different mechanism. After application of root exudates of non-mycorrhizal plants to cucumber plants, a slight stimulation of root colonization was observed, confirming the findings by Tawaraya et al. (1998), but root exudates of mycorrhizal plants exhibited a clear inhibitory effect. This inhibition of root colonization argues against a simple P-mediated mechanism, as no inhibitory effects of

Fig. 6. Effect of root exudates from mycorrhizal (colonized by *Gi. rosea*, *G. intraradices*, or *G. mosseae*), from non-mycorrhizal cucumber plants and of water (control) on root colonization of cucumber plants 8 days after inoculation with *G. mosseae*. Bars with the same letter are not significantly different according to the Tukey test ($P < 0.05$).



root exudates from plants with an enhanced P status have been reported so far (Elias and Safir 1987; Nagahashi et al. 1996; Tawaraya et al. 1996a, 1998). Recently, an inhibitory effect on AM hyphal growth and spore formation in the vicinity of *G. intraradices* colonized roots has been observed in a monoxenic system, and this inhibition was attributed to high concentrations of bioactive substances around mycorrhizal and non-mycorrhizal roots (St.-Arnaud et al. 1996).

We suggest a combination of several effects to be responsible for the reduced root colonization in our experiment: (i) a P-mediated effect, as described above, resulting in a reduction or even elimination of hyphal growth stimulating compounds in the exudates, and (ii) the presence of compounds in root exudates of mycorrhizal plants, which block essential steps for further root colonization by AM fungi.

Vierheilg et al. (1998c) suggested that, apart from general signal requirements that are similar for all AM fungi, AM fungi also have genus- or even species-specific requirements for a successful establishment of the symbiosis. A recent work showed that alfalfa (*Medicago sativa* L.) could be susceptible to colonization by *G. intraradices* but resistant to *Gi. margarita* (Douds et al. 1998). Moreover, an AM fungal species-specific accumulation of some secondary plant compounds in mycorrhizal roots also points towards this hypothesis (Grandmaison et al. 1993). Our results clearly demonstrate that a possible accumulation of AM fungal genus- or species-specific compounds in roots does not result in the exudation of compounds specifically affecting other AM fungal genera or species. The observed inhibitory effect seems a general effect on all AM fungi. However, the mycorrhizal-induced alterations of the root exudates might be linked with the enhanced resistance of mycorrhizal plants towards soilborne plant pathogens as suggested by Graham and Menge (1982) and Bansal and Mukerji (1994).

In axenic culture, it was recently shown that hyphae emerging from fresh spores of *G. intraradices* grew spirally around spores, whereas after a cold treatment, hyphae grew straight and elongated and away from the spore (Juge et al. 1998). A similar role for this hyphal growth pattern was proposed (Juge et al. 1998) as by Graham (1982) who sug-

gested the enhanced hyphal branching of AM fungi increased the probability of hyphae encountering roots. Interestingly, the hyphal growth pattern of *G. intraradices* changed in the presence of root exudates. Spiral growth in the proximity of the spore was observed only without root exudates, whereas long runner hyphae developed in presence of root exudates of mycorrhizal and non-mycorrhizal plants. Apparently a factor in the root exudates signals the presence of a root to the AM fungus *G. intraradices*, thus changing its growth pattern. Interestingly, even when the hyphal length stimulating effect was less clear in the treatment with the root exudates from mycorrhizal plants, the effect on the growth pattern was maintained. This indicates at least two different signals in the root exudates: (i) a hyphal length stimulating signal, which decreases in root exudates of mycorrhizal plants and which is probably P mediated, and (ii) a signal for the hyphal growth pattern, which is present in root exudates of mycorrhizal and non-mycorrhizal plants.

To summarize, this study gives the first indication that, apart from being implicated in the precolonization phase as AM hyphal growth-stimulating and attractational factors, root exudates also play an important role after successful establishment of the AM fungus in the root, regulating further root colonization by AM fungi. Further studies are needed to identify the mechanisms and compounds involved in this regulation.

Acknowledgements

We thank C. Juge, Université Laval, and Dr. C. Staehelin, CNRS, Gif-sur-Yvette, France, for helpful discussion. This work was supported by grants of the Deutsche Forschungsgemeinschaft, Germany to H.V.; the Dr. Helmut Robert Foundation, University of Kiel, to A.P.; and the Natural Sciences and Engineering Research Council of Canada to Y.P.

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