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# Chemotropism in the arbuscular mycorrhizal fungus *Glomus mosseae*

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**Abstract** In this work, we report the occurrence of chemotropism in the arbuscular mycorrhizal (AM) fungus *Glomus mosseae*. Fungal hyphae were able to respond to host-derived signals by reorienting their growth towards roots and to perceive chemotropic signals at a distance of at least 910 μm from roots. In order to reach the source of chemotropic signals, hyphal tips crossed interposed membranes emerging within 1 mm from roots, eventually establishing mycorrhizal symbiosis. The specificity of chemotropic growth was evidenced by hyphal growth reorientation and membrane penetration occurring only in experimental systems set up with host plants. Since pre-symbiotic growth is a critical stage in the life cycle of obligate AM fungal symbionts, chemotropic guidance may represent an important mechanism functional to host root location, appressorium formation and symbiosis establishment.

**Keywords** Chemotropism · Growth reorientation · Arbuscular mycorrhizal fungi · Host recognition · Root-derived factors

## Introduction

The establishment of symbiotic and pathogenic relationships is often realised through pre-contact attraction of biotrophs mediated by host signals (Currier and Strobel 1986; Jansson et al. 1988; Vande Broek et al. 1998; Hernandez et al. 1999). The involvement of host factors, either chemical or physical, ruling the fundamental steps of the life cycle of zoosporic fungi has been studied by many authors: host-derived signals can be perceived by motile spores, guiding and/or ruling chemotactic attraction (homing), encystment and adhesion and by hyphal tip receptors involved in germ tube growth orientation towards the infection site (Chi and Sabo 1978; Mitchell and Deacon 1986; Deacon 1996).

Chemoattraction represents a fundamental step in the establishment of symbiotic associations: for example, motile algal cells of *Platymonas convolutae* are chemotactically attracted by factors of the host egg capsule (Holligan and Gooday 1975), and *Rhizobium meliloti* shows chemotactic responses to nodulation signals (Dharmatilake and Bauer 1992). Host-specific chemotropic growth of the ectomycorrhizal fungi *Pisolithus tinctorius* and *Paxillus involutus* was evidenced by mycelial penetration through membrane filters overlying roots of *Eucalyptus globulus* (Horan and Chilvers 1990). The authors suggested that the perception of chemotropic signals could enable ectomycorrhizal fungal hyphae to locate host roots and to develop centripetally through and between the cap cells during sheath tissue formation. The establishment of arbuscular mycorrhizal (AM) symbiosis involves a coordinated sequence of recognition events, mediated by signal molecules released by plant host and fungal symbiont (Koide and Schreiner 1992; Giovannetti et al. 1994; Gianinazzi-Pearson 1996; Samra et al. 1997; Harrison 1999; Buée et al. 2000; Chabaud et al. 2002; Tamasloukht et al. 2003; Kosuta et al. 2003). The main host-specific morphogenetic event indicating the recognition of a host root surface by fungal hyphae is represented by the development of appressoria, whose differentiation is mediated by unknown signals, probably involving both chemo- and mechanosensing properties of hyphal tips (Garriock et al. 1989; Giovannetti et al. 1993a; Nagahashi and Douds 1997).

The contact between host and symbiont has been generally considered to occur by chance, although the questions as to whether and how hyphal tips of AM fungi are able to locate host roots remain to be answered (Mosse and Hepper 1975; Powell 1976; Miller-Wideman and

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Watrud 1984). Different studies showed that AM fungi are able to perceive host root factors (Giovannetti et al. 1993b, 1994, 1996; Balaji et al. 1995; Douds et al. 1996; Nagahashi et al. 1996, Nagahashi and Douds et al. 1999; Buée et al. 2000), and that such factors could exert an attractional effect on fungal hyphae (Vierheilig et al. 1995, 1998), although the experimental systems used did not allow the authors to discriminate unequivocally between attraction and growth enhancement effects and to detect hyphal reorientation toward roots. The only direct evidence of the ability of AM fungi to locate roots is represented by the occurrence of tropism toward host roots in *Gigaspora gigantea* aerial hyphae, which were able to perceive volatile attractant signals in an in vitro system (Gemma and Koske 1988).

In this work, a three-dimensional system, consisting of membrane filters interposed between plant roots and mycelium of the AM fungus *Glomus mosseae*, was used to investigate whether hyphal tips are able to locate roots by means of host-derived chemotropic factors. The experiments were aimed at assessing (1) hyphal chemotropism, (2) host specificity of chemotropic growth and (3) the radius of action of host-derived chemotropic factors.

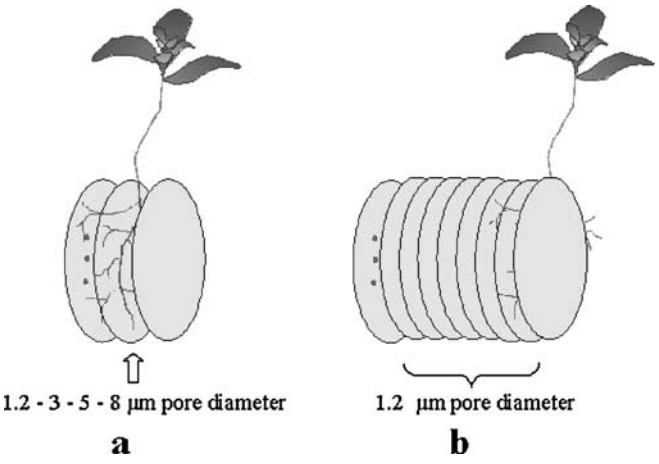


**Fig. 1** Scheme of the experimental system devised for the assessment of chemotropism and growth reorientation of arbuscular mycorrhizal mycelium. **a** Membranes of different pore sizes and different plant species were used to assess chemotropic growth of mycelium and chemotropism specificity, respectively. **b** One to seven superposed membranes were used to assess the radius of action of chemotropic factors

## Materials and methods

### Fungal and plant material

The AM fungus *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe (IMA 1), maintained in *Medicago sativa* L. pot cultures in the collection of the Department of Crop Plant Biology, University of Pisa, Italy, was used. Experiments were carried out with the following plant species: *Ocimum basilicum* L., *Vaccinium myrtillus* L. and *Eruca sativa* Lam. as host of arbuscular mycorrhizas, host of non-arbuscular mycorrhizas and non-mycorrhizal species, respectively.

### Detection of hyphal chemotropism

Surface-sterilized seeds of basil were germinated in sterile grit, and, 20 days after germination, the root systems of two plants were sandwiched between two 47-mm diameter membrane filters. A membrane filter bearing ten germinated sporocarps of *G. mosseae* was placed on this sandwich. Membrane filters of different pore sizes (0.45, 1.2, 3, 5 and 8 µm Millipore isopore filters) were used to separate plant roots and mycorrhizal fungi, and 0.45-µm filters were used as external membranes (Fig. 1a). Five replicates were set up, and the experiment was repeated twice. Sandwiched plants were placed in 10-cm diameter pots filled with sterile quartz grit, maintained in growth chamber (16–8 h light/dark cycle, 24°C day and 21°C night temperature) and harvested after 14 days. Sandwiches were opened by removing the outer membrane overlying the root system. The intermediate membrane, with plant roots maintained in situ, was stained with 0.05% trypan blue in lactic acid: the number of hyphae able to cross the membrane and to emerge on the root side was assessed under a dissecting microscope (Wild, Leica, Italy). The distance of emerging hyphae from nearby roots was measured in 10-mm root segments sampled from both absorbing and apical parts by using a micrometric slide with 10-µm scale. Five samples of each root zone were scored for each replicate membrane. After removing roots, membranes were mounted, root side up, on microscope slides, and the diameter of 50 emerging hyphae was measured for each membrane with a micrometric eyepiece using a Reichert-Jung (Vienna, Austria) Polyvar light microscope.

The length of mycelium on root and fungal membrane sides was measured according to the grid-line intersect method (Giovannetti and Mosse 1980) with a 47-mm diameter grid with lines 1 mm apart.

Plant roots removed from the membranes were stained according to Phillips and Hayman (1970), using lactic acid instead of lactophenol, to determine infected root lengths.

All data were submitted to analysis of variance, and means were compared using the Student–Newman–Keuls test.

Cross-sections of the membrane areas showing hyphal penetration were prepared by cryo-sectioning samples with a Leitz Kryostat 1720, mounted in 0.05% trypan blue in lactic acid and observed under the Polyvar microscope.

### Assessment of host specificity of chemotropic growth

Sandwiches were prepared as described above using membrane filters of 1.2-µm pore size (Fig. 1a). The host species *O. basilicum*, the non-host species *E. sativa* and *V. myrtillus* as well as dead host plants were tested for their ability to attract AM fungal hyphae. Five replicates were set up, and the experiment was repeated twice.

At harvest, the number of hyphae crossing the membrane and emerging at the root side and the length of mycelium on both fungal and root side of the membrane were recorded as described.

### Radius of action of host-derived chemotropic factors

Sandwiches were prepared as described or superposing two, three, four, five and seven membrane filters (1.2-μm pore size) to separate plant roots and mycelium (Fig. 1b). After 14 days, membranes and roots were stained as described, and number and diameter of emerging hyphae, as well as the number of entry points formed by *G. mosseae* on basil roots, were recorded.

## Results

### Detection of hyphal chemotropism

Membrane filters inserted between host roots and fungal mycelium were consistently crossed by hyphae, whenever pore diameter size was ≥1.2 μm. The number of hyphae able to cross the membranes and to emerge on the root side ranged from 391.4±34.6 (mean±SEM) to 463.4±30.8 per membrane and did not change with the increase in membrane pore size diameter (Table 1). Hyphal growth, both on fungal and on root side of the membranes, was similar on filters with different pore diameter sizes. Moreover, no differences were observed in the diameter of emerging hyphae (Table 1).

The evidence of tropism towards roots was represented by 3-D reorientation of hyphal tips, which were able to cross the membranes and to reach the host only in areas overlying plant roots, where differential morphogenesis also occurred (Figs. 2a, 4b). Microscopic analyses of membrane cross-sections revealed that most hyphal tips were able to plunge in the membranes, to cross them and to emerge near plant roots (Fig. 2b), following a linear pattern across the membranes (Fig. 2c).

Tropism towards roots was evidenced by measuring the distance between hyphae crossing the membrane and the nearby root. In fact, 96% of hyphae emerged at a maximum distance of 800 μm from the root, whereas no fungal hyphae were able to cross the membranes in areas more than 1 mm away from roots (Fig. 3a, b). Such finding is supported by regression curves showing logarithmic (Fig. 3a) and linear (Fig. 3b) relationships between the number of emerging hyphae and their distance from the nearby root, either in the absorbing region (Figs. 3a, 4c) or at root tip (Figs. 3b, 4c).

An additional evidence of fungal chemotropism is represented by hyphal ability to change growth direction closely following root development and direction on the membrane surface (Fig. 4a).

**Table 1** Ability of *G. mosseae* hyphae to cross membrane filters with different pore sizes in areas coinciding with host root systems

| Membrane pore diameter sizes (μm) | Mycelial length on fungal side of membrane (cm) | Number of hyphae emerging on root side of membrane | Diameter of emerging hyphae (μm) |
|---|---|---|---|
| 0.45 | 182.6±6.9 | 0 | 0 |
| 1.2 | 201.8±7.5 | 391.4±34.6 | 4.9±0.12 |
| 3.0 | 177.0±5.2 | 417.0±44.2 | 4.8±0.12 |
| 5.0 | 193.1±10.4 | 447.4±20.6 | 4.8±0.11 |
| 8.0 | 187.3±11.2 | 463.4±30.8 | 4.9±0.11 |

In columns, means±standard errors are not significantly different

### Assessment of host specificity of chemotropic growth

Host specificity of chemotropism was shown by results obtained with non-host plants. Neither penetration attempts on fungal side nor hyphae emerging on the root side were detected in membranes overlying dead host or non-host roots and in control membranes (Table 2). On the contrary, results obtained with living roots of the host plant basil were consistent with those obtained in experiment 1, showing both hyphal penetration on fungal side and emerging hyphae on root side.

### Radius of action of host-derived chemotropic factors

Pads consisting of superposed single membranes to obtain thickness of 260, 390, 520, 650 and 910 μm (calculated on the basis of supplier's information), inserted between plant roots and fungal mycelium, were crossed by hyphae of *G. mosseae*. During growth across the membranes, hyphae did not spread on their surfaces, i.e. in spaces between superposed filters, but they grew directly towards roots, following a linear pattern.

Also, in this experiment, hyphae were able to cross the membranes only in areas overlying roots, and no hyphae were observed to emerge far from roots even in sandwiches consisting of seven membranes (910 μm). AM fungal hyphae emerging on the root side of the proximal membrane were always able to infect basil plant roots whichever the number of superposed membrane filters, with an average number of entry points per plant of 6.4±1.4 (Fig. 2d).

## Discussion

This work represents a direct evidence of the occurrence of chemotropism in the AM fungus *G. mosseae*. Fungal hyphae are able to respond to host-derived signals by reorienting their growth towards roots and to perceive chemotropic signals at a distance of at least 910 μm from roots.

It was evidenced that only in the presence of living host plants *G. mosseae* hyphae were able to penetrate through membrane filters with pore sizes higher than 1.2 μm and to

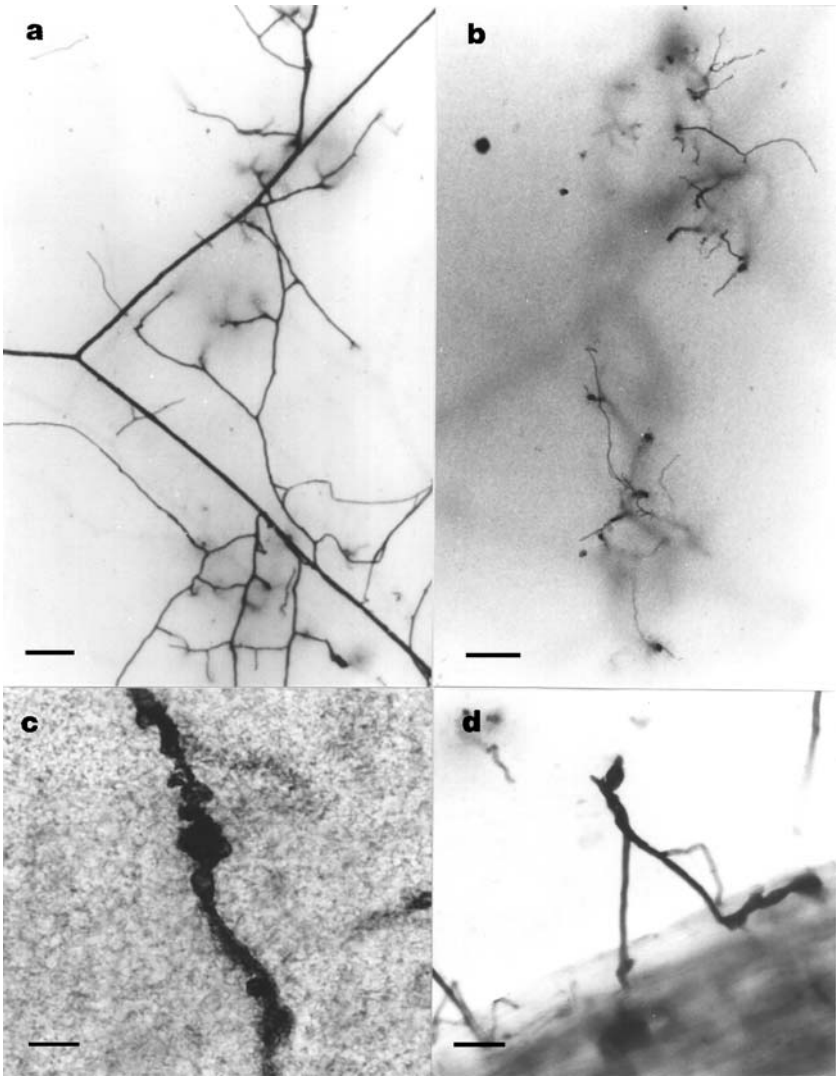


**Fig. 2** Micrographs of *G. mosseae* hyphae growing on the surface and across a membrane filter overlying *O. basilicum* roots. **a** Fungal side view of mycelium developing in an area overlying host root, showing many hyphal tips plunging in the membrane. *Scale bar*=80 μm. **b** View of hyphal tips crossing the membrane and emerging on the root side. *Scale bars*=80 μm. **c** Section of membrane area overlying roots showing development of a fungal hypha across the membrane. *Scale bar*=15 μm. **d** An emerging hypha which has developed appressoria and infected the nearby host root. *Scale bar*=25 μm

reach host roots. Both non-host and dead host plants failed to induce hyphae to cross the membranes. Moreover, chemotropic growth of *G. mosseae* hyphae did not show an even distribution on the surface of membrane filters, but it was localised in areas overlying roots, where host-specific differential morphogenesis occurred. Such localised fungal responses could be due to the presence, in host root exudates, of highly unstable compounds or to the need of a high concentration of host signals. Since host root tips have been suggested to be the main site of release of chemotropic signals (Mehrota 1972; Horan and Chilvers 1990), chemotropic responses occurring only in areas overlying roots could be the result of hyphal reorientation during root tip growth.

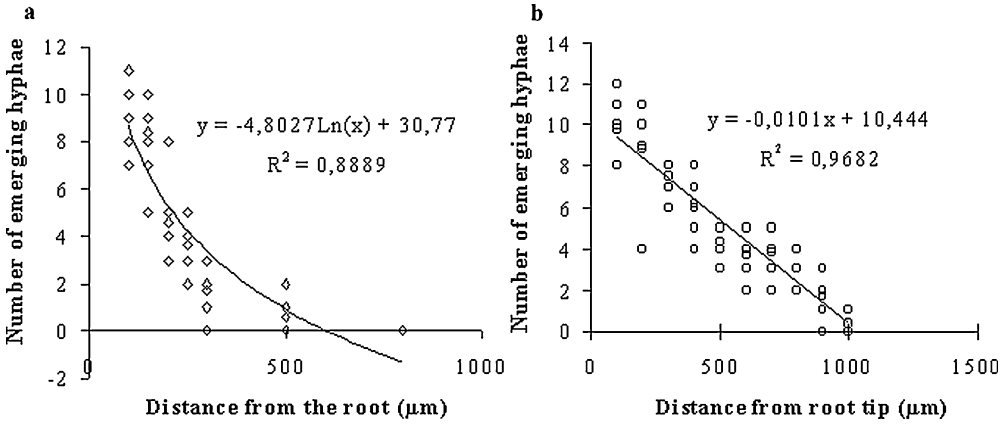


**Fig. 3** Regression analyses showing the relationships between the number of *G. mosseae* hyphae emerging from membrane filters overlying host roots and their distance from the nearby root. Number of hyphae and their distances were recorded both in absorbing (**a**) and apical (**b**) regions of root segments. Logarithmic (**a**) and linear (**b**) regression equations and $R^2$ indicated are both for $P<0.01$

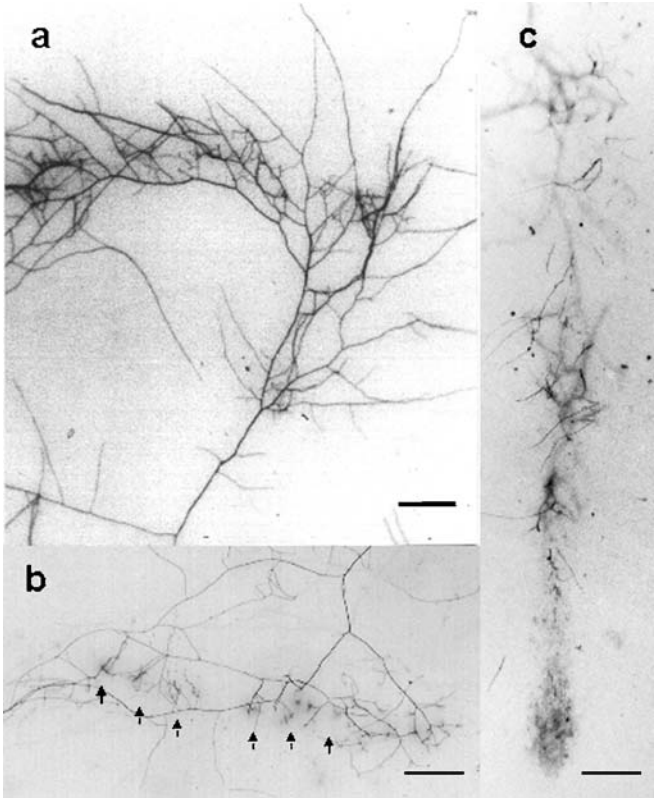


**Fig. 4** Micrographs of *G. mosseae* hyphae growing on the membrane filter overlying *O. basilicum* roots. **a** Main hypha and branches of *G. mosseae* closely following the direction of the roots growing underneath the membrane. *Scale bar*=150 μm. **b** Low magnification view of *G. mosseae* hyphae showing penetration points (*arrows*) along the axis of a root growing underneath the membrane. *Scale bar*=200 μm. **c***G. mosseae* hyphae emerging at the root side of the membrane along the root track (indicated by the imprint of the root apex in the lower part of the micrograph). *Scale bar*=300 μm

**Table 2** Ability of *G. mosseae* hyphae to cross membrane filters in areas overlying host or non-host plant roots

| Plant species | Mycelial growth on fungal side of membrane (cm) | Mycelial growth on root side of membrane (mm) | Number of hyphae emerging on root side of membrane |
|---|---|---|---|
| *O. basilicum* | 196.1±7.8[a] | 298.2±43.3 | 493.6±120.2 |
| Dead *O. basilicum* | 98.2±3.4[b] | 0 | 0 |
| *V. myrtillus* | 98.8±4.7[b] | 0 | 0 |
| *E. sativa* | 103.5±4.9[b] | 0 | 0 |
| Control without plant | 102.4±5.0[b] | 0 | 0 |

In columns, means±standard errors followed by different letters are significantly different for $P$=0.01

AM fungal hyphae were able to cross the membranes even when seven of them were interposed between mycelium and roots, and no lateral spread was detected on membrane surface. Since the thickness of membrane filters is about 130 μm, the radius of perception of the chemotropic attractant results to about 900 μm. *P. tinctorius* was able to contact *E. globulus* roots passing through two or three membrane filters (260- to 390-μm distance, Horan and Chilvers 1990). In the interaction between *Cochliobolus sativus* and *Hordeum* roots, chemotropic effects were confined within a distance of 1–2 mm from the root surface, whereas larger radius of action (over a distance of up to 11 mm) was reported for volatile signals perceived by aerial hyphae of the AM fungus *G. gigantea* contacting maize roots (Jansson et al. 1988; Koske 1982). On the contrary, chemoattractants involved in hypha–hypha interactions and compounds controlling hyphal fusion in other non-mycorrhizal fungi have been shown to act within 7–10 μm, with a calculated half-life of about 10 s, suggesting the presence of unstable compounds (Muller and Jaffe 1965; Raper 1952).

The exchange of pre-contact signals represents the earliest step in any plant–microbe interaction, and the detection of specific plant-derived molecules by microbes is functional to host recognition and colonization. Our results are consistent with this view since hyphal tips were able to follow root growth patterns on the overlying membrane, to cross it and to emerge within 1 mm from roots, eventually establishing the symbiosis.

It is generally accepted that plant hosts of AM fungi release compounds acting as recognition and growth-enhancing signals for symbionts, though their chemical nature is poorly understood[1]. Both water-soluble and volatile compounds exuded by host roots are able to increase hyphal growth and branching of AM germlings and to regulate the establishment of infection and fungal root colonization (Gemma and Koske 1988; Mosse 1988; Bécard and Piché 1989; Giovannetti et al. 1993b, 1994; Nagahashi and Douds 1999; Suriyapperuma and Koske 1995; Nagahashi et al. 1996; Tawaraya et al. 1996; Buée et al. 2000; Chabaud et al. 2002). Interestingly, other studies have reported, along with growth enhancement, an attractional effect of *Phaseolus vulgaris* root exudates on *G. mosseae* fungal hyphae growing in a soil compartment-membrane system. However, such an experimental model did not allow the direct detection of hyphal reorientation toward roots (Vierheilig et al. 1995, 1998).

A close relationship between chemoattraction and recognition clues has been evidenced in other experimental systems. For example, host root phenolics stimulating the expression of nodulation genes in rhizobia or those inducing virulence genes expression in *Agrobacterium tumefaciens* were shown to function also as chemoattractants for these microorganisms (Ashby et al. 1987, 1988; Aguilar et al. 1992; Dharmatilake and Bauer 1992). Results obtained with

[1] The chemical nature of branching factors has been recently described (Akiyama et al. Nature 635:826–827).

*Rhizobium* and *Azospirillum* strains indicate that nonchemotactic mutants maintain the ability to nodulate roots, though their efficiency and competitivity are reduced (Bergman et al. 1988; Caetano-Anolles et al. 1988; Vande Broek et al. 1998). The involvement of electric phenomena, acting with synergistic effect with chemotaxis-tropism, could also be important since the elongating region of roots produces an electric current, and electric fields are known to induce orientation and hyphal branching (McGillivray and Gow 1986; Berbara et al. 1995).

Since pre-symbiotic growth is a critical stage in the life cycle of obligate AM fungal symbionts (Tamasloukht et al. 2003), chemotropic guidance may represent an important mechanism functional to host root location, appressorium formation and symbiosis establishment.

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