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Application of a membrane-gel procedure to determine the effects of root exudates on the growth of the extraradical hyphae of the arbuscular mycorrhizal fungus *Glomus etunicatum*

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Abstract
A technique was developed to allow *in vitro* studies of extraradical hyphae of the arbuscular mycorrhizal fungus *Glomus etunicatum*. Mycorrhizal root pieces were placed onto dialysis membrane overlying transparent high purity agarose gel. This restricted the hyphae to a two-dimensional growth form and permitted visualisation and measurement of hyphal proliferation, using light microscopy coupled with image analysis, in the presence of point sources of host plant root exudates. Fractal geometry was employed in mycelial description. Data showed that root exudates only affected the growth (length and fractal dimension) of the mycelium when positioned heterogeneously, as a point source.

Keywords: Morphology, branching, fractal

1. Introduction

Arbuscular mycorrhizal fungi (AMF) live in the roots of host plants, promoting the ability of the root to acquire nutrients from the soil, and in turn obtaining carbon compounds from host plant photosynthesis. Promotion of nutrient uptake occurs primarily as the result of increased absorptive capacity of the plant-fungal system, in which the extraradical mycelium plays a critical role. The relative uptake capacity of a particular mycorrhizal fungal species depends on the size and rate of spread of the mycelium, and on its uptake and translocation capacities (Gianinazzi-Pearson and Gianinazzi, 1983; Graham et al., 1982; Jakobsen et al., 1992). The mycelium also has an important role in colonising new roots. Colonising mycelium can arise from (i) spores (in some species), (ii) a living root attached to a host plant, or (iii) from fragments of roots that have become detached from the root system through interactions with soil animals, the physical processes in soil or root senescence; senescence itself affected by colonisation (Hooker et al., 1995). The growth of the latter (root fragments), which form an important source of inoculum for colonising (non-colonised) roots in a soil (Biermann and Linderman, 1983), are the focus of this paper.

Understanding how environmental factors influence the growth and development of mycelium from these fragments will contribute to a better understanding of the ecology of the extra-radical mycelium arising from root fragments, and their role in colonising living, non-colonised roots.

AMF hyphae are generically able to respond to a variety of environmental stimuli by changes in growth patterns. Their growth occurs in two or three dimensions to achieve maximum efficiency by branching to cover large volumes, and to allow communication and translocation between regions. While it is generally understood that hyphal growth of fungi will respond to localised variation in resource availability (Prosser, 1983), little is known about responses of AMF to environmental stimuli or the mechanisms by which spatial distribution of the hyphae within the network is determined (Trinci, 1984). Thus, although the processes by which AMF colonise the plant root are widely studied at morphological, physiological and molecular levels, little is known about their extraradical phase and in particular that associated with root fragments.

This is largely due to the difficulties of studying and

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observing fungi non-destructively within a soil environment, compounded by the fact that AM fungi are natural symbionts that defy attempts to culture them in isolation from their host plants (Sylvia, 1992). Consequently there have been relatively few attempts to study hyphal growth and morphology in detail, or to explore further observed hyphal responses to their environment.

Notable exceptions can be found in studies showing that the extraradical hyphae of AM fungi exhibit plasticity of form in response both to nutritional heterogeneity in their growth medium (St. John et al., 1983a,b) and to plant root exudation (Nair et al., 1991; Giovannetti et al., 1993b). Specialisation in form and function has also been observed (Friese and Allen, 1991). In the past, mechanisms relating to hyphal growth and development have generally been inferred from indirect evidence. A recent trend has seen the adoption of two-dimensional systems for the observation of hyphae *in situ*, used in experiments examining the effects of environmental influences on spore germination and early hyphal growth in AM fungi (Gianinazzi-Pearson et al., 1989; Giovannetti et al., 1993a,b, 1996, 2004). More recently, mycorrhizal root segments have been used as inoculum in direct studies of the effects of selected factors on growth of vegetative hyphae of AM fungi (Gryndler et al., 1998).

In describing hyphal growth, the heterogeneity of natural systems means that it is not always possible to express meaningful averages. Euclidean geometry is motivated by the desire for simplicity and order, which in nature inevitably results in approximations and caricatures. To assume that nature must be either complex or simple creates an artificial dichotomy that is unrealistic. In such cases the concept of fractal dimension can be used to bridge the gap that this dichotomy creates. Natural patterns can be very complex but they also appear to be scale invariant, remaining statistically unchanged over a wide range of scales. The essential feature of fractals is the way in which the material composing them is distributed in space, clustered heterogeneously but not randomly in such a way that the structure looks statistically identical, independent of the scale at which it is viewed. The key underlying concept is that of self-similarity, which is the ability to decompose an object into smaller copies of itself.

Thus, the structure can be rebuilt from magnified portions of itself. Natural patterns, particularly in ecosystems, frequently appear irregular, complex and hard to measure even at the small scale. Self-similarity forces the complexity of the object into the building blocks of which it is composed, allowing description through simple power laws (Mandelbrot, 1977, 1982). Fractal geometry has been seen as a tool for studying dynamics in complex systems, and has been adopted in studies of plant roots and fungal hyphae (Tatsumi et al., 1989; Ritz and Crawford, 1990; Crawford et al., 1993).

The aim of this research was to develop and demonstrate the utility of a simple experimental system for the spatio-temporal observation and measurement of extraradical hyphae, and apply it to establish the effects of root exudates on the growth and form of the mycelium of the AMF *Glomus etunicatum*. Root pieces were used because the intention here was to develop a system for studying extra-radical phases of the mycelium from root fragments. A specific experimental system is required because large physiological differences have been shown to exist between extra-radical and germ-tube hyphae (Hepper et al., 1996; Saito, 1995; Shachar-Hill et al., 1995; Gryndler et al., 1998). Hyphal response was described in terms of physical measurements of morphology, and fractal analysis was applied in interpretation of hyphal distribution.

2. Materials and Methods

Root exudates

Fungal cultures and plant material

The AM fungus used in the study was *Glomus etunicatum* Becker and Gerd. (isolate S329; INVAM; International Collection of Arbuscular and Vesicular-arbuscular Mycorrhizal Fungi, USA) maintained in pot cultures of cucumber.

Stock cultures of mycorrhizal cucumber plants were established by inoculation of seedlings with roots colonised by *Glomus etunicatum*. Root inoculum was chopped into small pieces and mixed thoroughly at a ratio of 1:6 with autoclaved potting medium (Seed and Cutting Compost, John Innes, UK). Cucumber seeds were surface sterilised in 10% (vol./vol.) Domestos (5% available chlorine, Lever Ltd., UK) and rinsed in sterile water before planting. Cucumber was used as the preferred stock plant because of its ability to produce abundant white roots in which mycorrhizal structures were easily visible without staining.

Preparation of inoculum

All procedures were carried out using mycorrhizal root pieces as inoculum. White (non-lignified) roots were collected from mycorrhizal pot cultures and washed thoroughly under running water to remove soil residues, then placed in Petri dishes containing sterile distilled water and examined under a binocular light microscope (Wild M10, Leica, UK). Root sections containing large numbers of vesicles, clearly visible in unstained roots, were identified and cut into transverse sections of approximately 1–1.5 mm in length using a sterile scalpel.

Protracted attempts were made to sterilise root pieces but this could not be achieved without significantly compromising the viability of the inoculum, so non-sterilised root pieces were used.

Experimental system

A technique for studying hyphal growth in the absence of the host plant was developed. Each mycorrhizal root piece was placed onto the centre of a 5×5 cm square of dialysis membrane (Medicell International Ltd., UK) overlying transparent high purity agarose gel (4–5 mm depth) to prevent membrane desiccation, in a disposable Petri dish (Bibby Sterilin Ltd., UK). Dialysis membranes were prepared by boiling twice for 30 minutes each time in distilled water, and sterilised by autoclaving at 121°C and 15 psi for 20 minutes. The membrane restricted the hyphae to a two-dimensional growth form. This facilitated observation using microscopy and image analysis techniques, and enabled morphological measurements to be made without the added complexity of three-dimensional growth. The Petri dishes were sealed with Nescofilm (Bando Chemical Industries, Japan) and incubated at 25°C in the dark.

Methods for both heterogeneous and homogeneous application of exudates were developed. Heterogeneous application used plugs of agarose as point stimuli, placed on the membrane at a distance of 5 mm from the root pieces. This distance was determined through preliminary experimentation, which showed that hyphae were able to grow approximately 5 mm in a given direction within 21 days. Plugs had been derived from agarose medium in which cucumber had been growing for 14 days. To test homogeneous application of a test substance, the agarose substrate was uniformly impregnated with the exudate. In this case cucumber plantlets were grown in low melting point agarose for 14 days and then removed. The gel was then re-melted, mixed and the membrane and root pieces applied.

Observation of mycorrhizal hyphae

Living AMF hyphae growing from root pieces were observed non-destructively using a binocular light microscope with continuous zoom. Images of the hyphae were captured by linking the microscope directly to an image analysis system (Quantimet 600, Leica, UK) via a 3 CCD colour video camera (Sony, Japan). The method produced high-definition images that provided accurate visual records of mycelium development over time.

Measurement of hyphal branching characteristics and fractal dimension

Captured images were edited using grey level processing techniques to help overcome contrast problems between hyphae and background. Images acquired in this way were then traced manually onto acetate sheets to provide high-contrast maps of mycelial growth, which were then recaptured via a macro-viewer and digitised for further analysis. Measurement of hyphal branching characteristics and fractal dimension was carried out using a dedicated program that extracted the necessary information from the images for characterisation of mycelial geometry (Ralph, 1997).

Characterisation of mycelial geometry using the program gave both whole network measurements, such as total hyphal length, and more detailed measurements including number and length of branches. Global characterisation of the network was calculated by the estimation of Fractal Dimension. The program was calibrated for each image using the magnification at which it was captured, giving a mm to pixel conversion. A minimum of four and a maximum of 16 replicates were used for optimisation of methodology and 5 replicates for experimental procedures. Because the branching data were highly variable and did not follow the Normal Distribution statistical analyses of these data were carried out using the Mann-Whitney test for comparisons of pairs. Fractal Dimension data was analysed using Analyses of Variance. Tests were carried out using the Minitab for Windows statistical package (Version 10; Minitab Inc., USA).

3. Results

Mycorrhizal root pieces were successfully utilised as inocula, and the membrane-gel experimental system developed permitted the non-destructive observation of the spatio-temporal development of the AMF mycelium (Fig. 1). In most cases, 100% of root pieces produced hyphal growth. However, length of hyphae showed considerable variation. The experimental system permitted the non-destructive observation of the spatio-temporal development of the AMF mycelium (Fig. 1), but further processing steps were required. The use of light microscopy allowed visualisation of the hyphae on the membrane surface via a video link, but hyphae often appeared thin and blurred, resulting in images composed of variable intensities. To improve contrast, grey level processing was performed and it proved necessary to trace the hyphae manually onto acetates before image analysis to record the full complexity of the mycelium (Fig. 2). Moreover, because hyphal growth often exceeded the microscope field of view, this usually required that a composite image of the whole mycelium be constructed from numerous images, each of which represented a single field of view (Fig. 3).

When a root exudate was applied homogeneously in the agarose below the membrane, there were no effects on either the length of the mycelium relative to the controls (Table 1), or the number of branches or the Fractal Dimension (Table 2). However, when the root exudate was placed heterogeneously as a point source there was a marked difference in the length of mycelium, with only 20% of the growth of the controls (Table 1). Effects on branching were not significant. However, changes in the Fractal Dimension were measured, but only when root exudates were

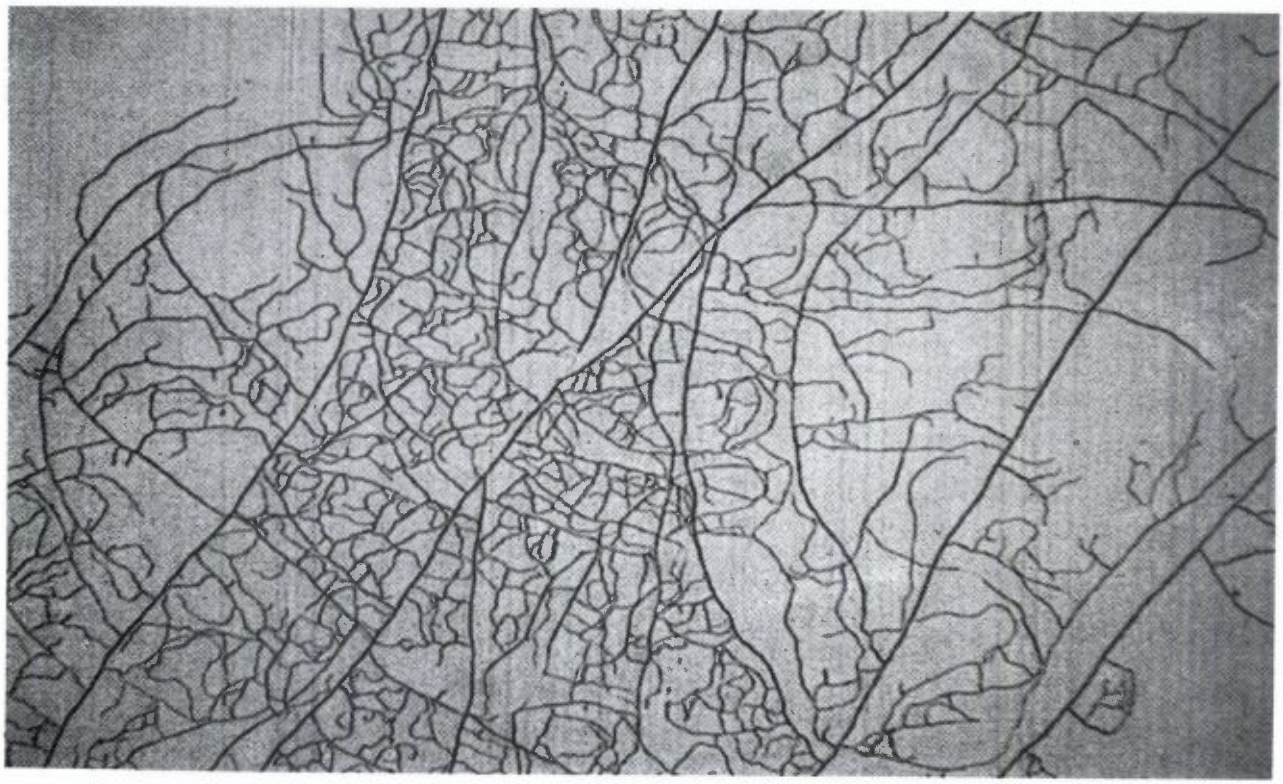

Figure 1. Extraradical hyphae of the arbuscular mycorrhizal fungus *Glomus etunicatum* growing on dialysis membrane from a colonized root piece.

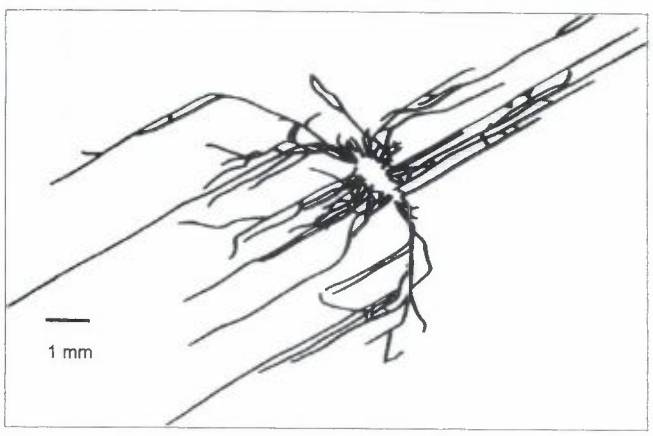

Figure 2. Example of a manually traced image on acetate film of extraradical hyphae of the arbuscular mycorrhizal fungus *Glomus etunicatum* growing from a colonized root piece.

positioned heterogeneously, as a point source, as might represent the situation in a natural soil. In this case the Fractal Dimension of the mycelium was 1.4, compared to 1.0 in the controls.

Table 1. Effect of host root exudate, and placement, on hyphal length in the mycelium of *Glomus etunicatum*.

Exudate	Placement	
	Homogeneous	Heterogeneous
No exudates (control)	19.4a	15.8a
Host exudates	43.7a	3.1b

Data are length of the mycelium (mm) after 7 days growth and are means of 5 replicates. Data in a column and treatment group, i.e. homogeneous or heterogeneous, followed by the same letter are not significantly different from each other.

Table 2. Effect of a source of host root exudates, and placement, on the hyphal branching and Fractal Dimension of the mycelium of *Glomus etunicatum*.

Exudate/placement	Number of branches	Fractal Dimension
Homogeneous		
No exudates (control)	0.54a	1.1a
Host exudates	0.74a	1.0a
Heterogeneous		
No exudates (control)	0.81a	1.4a
Host exudates	0.56a	1.0b

Data were from plates after 7 days growth and are means of 5 replicates. Data in a column and treatment group i.e. homogeneous or heterogeneous followed by the same letter are not significantly different from each other. Branching data was analysed using the Mann-Whitney Test and Fractal Dimension data using ANOVA. There were no significant differences in the length of the mycelium.

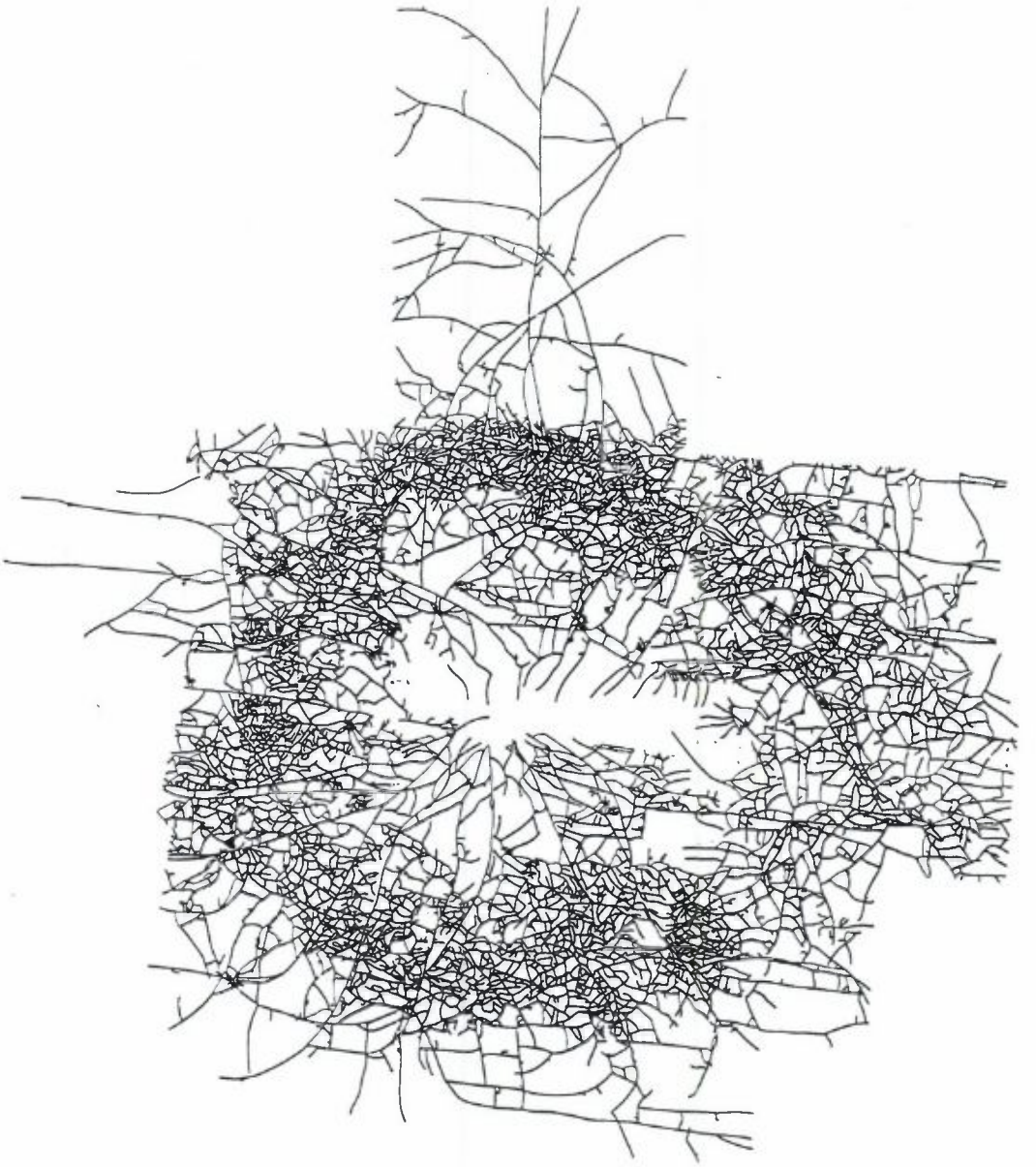

Figure 3. Example of a composite image of the whole mycelium of *Glomus etunicatum* constructed from numerous images growing from a colonised root piece, indicated by the blank area at centre.

4. Discussion

The present study shows that it is possible to obtain extraradical hyphae of the AM fungus *Glomus etunicatum* from mycorrhizal root pieces in simple experimental systems. The issue of variability implies that both uniformity of inoculum and level of replication needs to be considered. However, it should be recognised that this may represent natural variability, that could result from a variety of factors, including the availability of nutrients, including carbon, to the hyphal unit and the physical structure of the root itself and, in particular, the degree of lignification.

The most recently published, and comparable, work with root fragments is that of Gryndler et al. (1998) who similarly recognised the need to separate the environmental responses of the pre-symbiotic and symbiotic phases of AM fungi and used root pieces as inoculum for the proliferation of vegetative hyphae of AM fungi. Gryndler et al. (1998) incubated mycorrhizal maize root pieces in droplets of liquid medium and measured hyphal growth using the grid line intersect method. Hyphal length after 5 days appeared to be generally less than that observed in the present work. In addition, the percentage success rate of hyphal proliferation was generally low. The level of variation was not discussed, but may have arisen in terms of the presence or absence of hyphal development rather than in hyphal length itself.

In the present study, root piece sterilisation gave rise to problems that occurred largely because of the difficulties associated with removing internal contaminants without adverse effects on the AM fungus itself. As with *in vitro*

techniques in general, methods of sterilisation routinely applied in plant culture systems were found not to be directly transferable to AM fungi, and the study was ultimately carried out using non-sterile inoculum. Such a system does not allow discrimination between actual host-derived signals and metabolic products of micro-organisms associated with hyphae or roots, suggesting that hyphal responses to signals also need to be demonstrated under sterile conditions. However, Giovannetti et al. (1996) observed that the degree of sterility of an *in vitro* system did not influence the response of AM fungi to host derived signals, indicating that it was factors derived directly from roots that elicited the recognition responses. Gryndler et al. (1998) attempted surface decontamination of root pieces using a combination of antibiotics and sodium hypochlorite, but still found contamination in up to 55% of root pieces. The use of sterilants may have suppressed hyphal growth and compromised mycorrhizal viability, thereby potentially explaining the smaller hyphal lengths measured and the generally lower success rate in hyphal regeneration.

As has been described for plant root architecture, the explicit spatial configuration of a root system is important to its function (Nielsen et al., 1997). Likewise the spatial deployment of extraradical hyphae of AM fungi will also be important, given the scarcity and heterogeneous nature of the resources in the soil environment. However, hyphal architecture of AM fungi is little understood because of difficulties involved in observation, quantification and interpretation of a complex and dynamic growth form in a heterogeneous and opaque medium. The methodology described here presents an opportunity to begin to understand the factors important in regulating the growth of the extra-radical mycelium from root pieces in the soil. One feature that is likely to be important is the degree to which the mycelium grows and branches, as increases in either will increase the opportunities to encounter new roots to colonise. However, the potential to do so is limited by the reserves available to the mycelium, and these are likely, but not necessarily, all obtained from the host root fragment. Any directional response to the presence of particular signals, such as host root exudates, would bring advantage, particularly when the number of host roots was low. The propensity of fungi to branch has some genetic basis, which puts constraints on any postulated mechanisms for adaptation of branching patterns according to environment (Ritz and Crawford, 1990). The use of fractal geometry is an approach to the description of hyphal growth, and has not been applied previously in studies of arbuscular mycorrhizal mycelium. However, fractal geometry is likely to offer an improved technique for quantifying and encoding mycelial complexity and yielding ecological and physiological insights into the functional relevance of specific architectural patterns (Nielsen et al., 1997). Because there have been relatively few attempts to examine the fractal geometry of fungal mycelia (Ritz and Crawford, 1990; Crawford et al., 1993), there is little reference material against which to interpret results and relate them to natural conditions. However, recent measurements of the Fractal Dimensions of ectomycorrhizal fungi reported by Donnelly et al. (2004) were similar to those reported here.

To conclude, in the present study, fractal analysis of the AM fungal mycelium demonstrated that changes to the growth form of the mycelium did occur in response to environmental signals in host root exudates provided as a point source. This is of interest in its own right, but also highlights the value of using fractal analysis as a tool in interpretation of the growth form of the mycelium in AM fungi.

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