

In vivo observations of the arbuscular mycorrhizal fungus *Glomus mosseae* in roots by confocal laser scanning microscopy

HORST VIERHEILIG^{1,3*}, ANNETTE BÖCKENHOFF², MICHAEL KNOBLAUCH²,
CHRISTINE JUGE³, AART J. E. VAN BEL², FLORIAN GRUNDLER¹, YVES PICHÉ³ AND URS WYSS¹

¹ Institut für Phytopathologie, Christian-Albrecht-Universität Kiel, Hermann Rodewald-Strasse 9, 24118 Kiel, Germany

² Institut für Allgemeine Botanik und Pflanzenphysiologie, Justus-Liebig-Universität, Senckenbergstrasse 17, 35390 Giessen, Germany

³ Université Laval, Faculté de Foresterie et de Géomatique, CRBF, Pavillon C.-E.-Marchand, Québec G1K 7P4, Canada

Confocal laser scanning microscopy was used for *in vivo* observations of autofluorescent fungal structures of *Glomus mosseae* in intact, living rye-grass roots. Clear images of hyphae and collapsed arbuscules could be obtained without staining or sectioning roots. As rye-grass roots are highly transparent, with only a few cell layers above the vascular cylinder, they provide an excellent *in vivo* system for microscopical confocal laser scanning studies of arbuscular mycorrhizal structures.

After staining and thus killing roots and fungi, confocal laser scanning microscopy (CLSM) has recently been used for the observation of fungal structures in ectomycorrhizas (Schelkle *et al.*, 1996) and endomycorrhizas (Czymmek, Whallon & Klomparens, 1994; Smith & Smith, 1997). In epifluorescence microscopical studies on autofluorescence of arbuscular mycorrhizal (AM) structures has been reported (Ames, Ingham & Reid, 1982; Jabaji-Hare, Perumall & Kendrick, 1984; Klingner *et al.*, 1995*a*), which may give prospect to CLSM *in vivo* studies. In CLSM it can be a problem to obtain clear images in unsectioned roots, having many cell layers (Czymmek *et al.*, 1994). The highly transparent roots of rye-grass with only a few cell layers above the vascular cylinder could provide a valuable system for CLSM *in vivo* studies of AM fungi in unsectioned roots.

MATERIALS AND METHODS

Biological material and growing conditions

Seeds of rye-grass (*Lolium perenne* L.), were surface-sterilized by soaking in 0.75% sodium hypochlorite for 5 min, rinsed with tap water and germinated in vermiculite. The seedlings (10 plants) were inoculated in a growth chamber (day/night cycle: 16 h; 22 °C/8 h; 20 °; r.h. 50%) with *Glomus mosseae* (T. H. Nicolson & Gerd.) Gerd. & Trappe (BEG 12; La Banque Européenne des Glomales; International Institute of Biotechnology, Kent, G.B.) in the compartment system developed by Wyss, Boller & Wiemken (1991) in a steam-sterilized (40 min, 121 °) mixture of sand and soil (1:1/v:v).

Six weeks after inoculation with *G. mosseae*, the 10 rye-grass plants were harvested. For microscopical observations

roots were washed with tap water and placed on slides in water. For observations with the water immersion objective, water droplets were placed onto the cover slips. Roots of the same plant were used for light microscopy, epifluorescence microscopy (FM) and CLSM.

Several roots of each plant were cleared by boiling in 10% KOH and stained according to the method of Vierheilig & Piché (1998) by boiling in a 0.05% trypan blue/household vinegar (= 5% acetic acid) solution. Stained roots were observed with the light microscope.

Microscopical studies

Light microscopical and epifluorescence observations were made using a Polyvar/Leica microscope (Leica, Heidelberg, Germany) equipped with a fluorescence condenser. Fluorescence was excited by a band pass blue filter combination (450–495 nm). Epifluorescence micrographs were taken with Kodak 400 ASA, light microscopical micrographs with Kodak 64 ASA film.

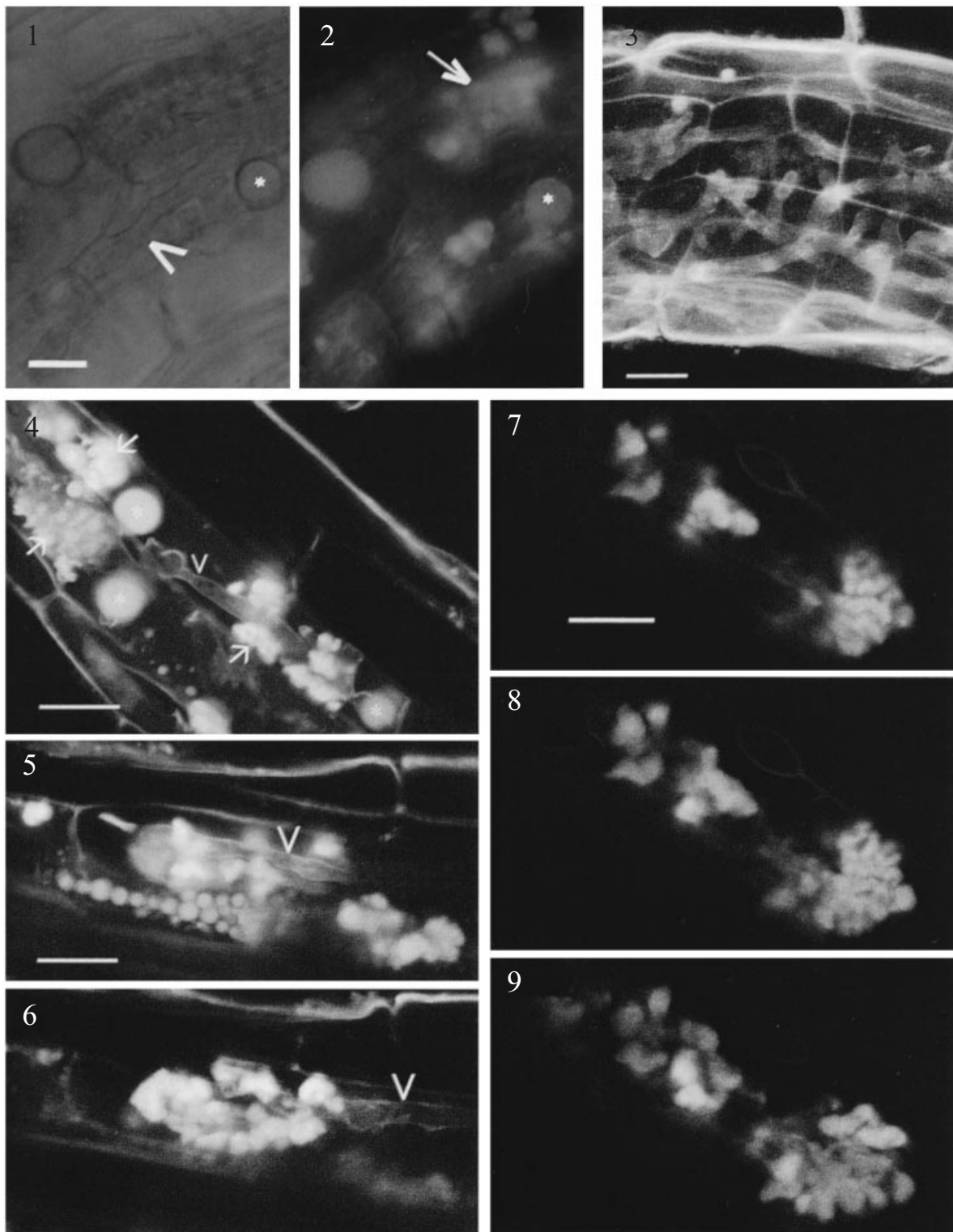
Confocal laser scanning microscopy was performed with a Leica TCS 4D (Leica, Heidelberg, Germany) at 488 nm produced by a 75 mW argon/krypton laser (Omnichrome, Chino, CA). The emission was observed with a high-magnification water immersion objective (PL APO × 63/1.20 W CORR, Leica) using a FITC bandpass filter.

RESULTS

Staining of rye-grass roots showed an intense colonization by *G. mosseae* 6 wk after inoculation. The fungus had formed intraradical hyphae and arbuscules. Arbuscules in different developmental stages were detected.

When intact, living AM-colonized roots were observed by

* Corresponding author.



Figs 1–9. **Figs 1–2.** *In vivo* micrographs of rye-grass roots colonized by *G. mosseae* (bar = 10 μ m). **Fig. 1.** (Light microscopy) An intraradical hypha (arrowhead) seems to branch into an arbuscule which can be seen in Fig. 2 (arrow). Large drops (asterisks) are visible. **Fig. 2.** (Epifluorescence microscopy; 450–495 nm). The drops and the arbuscules show a strong autofluorescence. **Figs 3–9.** *In vivo* observations by confocal laser scanning microscopy (488 nm) of rye-grass roots colonized by *G. mosseae* (bars = 20 μ m). **Fig. 3.** Root segment with coiled intraradical hyphae. **Fig. 4.** Root segment with arbuscules (arrows), an intraradical hypha (arrowheads) and drops (asterisks). **Figs 5, 6.** Successive layers of a root segment at different depths in the same location. **Fig. 5.** A hypha (arrowhead) grows towards an arbuscule which is located in vicinity of accumulated droplets; **Fig. 6.** The hypha (arrowhead) branches into an arbuscule, which is located above the cell with the droplets. **Figs 7–9.** Successive layers of an arbuscule at different depths. Parts of the arbuscule have a raspberry-like appearance with single drupels.

FM, many diffusely fluorescing arbuscules and fluorescing large drops were visible in the roots (Fig. 2). By FM it was not possible to elucidate whether the fluorescing arbuscules were clumped or finely branched. Light microscopy in combination with FM revealed intraradical hyphae, which did not seem to fluoresce (Figs 1, 2).

In contrast to FM, CLSM produced clear *in vivo* images of AM fungal structures in roots. Intraradical hyphae were only slightly autofluorescent (Figs 3–6), whereas arbuscules exhibited a strong autofluorescence (Figs 4–9).

Optical sectioning of a root segment by CLSM (Figs 5, 6) showed the accumulation of small autofluorescing droplets close to arbuscules. The droplets fluoresced less intensively than the neighbouring arbuscule (Fig. 5). Root cells were only partly filled by the droplets and the droplets showed Brownian movement. In Fig. 6 a weakly fluorescing hypha seemed to branch into a highly fluorescing arbuscule.

Optical sectioning of an arbuscule by CLSM (Figs 7–9) revealed the autofluorescence of the whole arbuscule. Arbuscules looked loosely clumped, like a *Rubus* inflorescence; branches or arbuscules looked like the drupels of a raspberry. No highly branched autofluorescing arbuscules could be observed by CLSM.

DISCUSSION

CLSM observations of AM fungal structures in roots have been performed recently (Czymmek *et al.*, 1994; Smith & Smith, 1997). The fungal structures were stained with fluorochromes, thus no *in vivo* observations were possible. In living roots by FM at low magnifications ($\times 160$) we detected many strongly autofluorescing spots which, at higher magnifications ($\times < 300$) without sectioning of the roots, could be identified as diffusely fluorescent arbuscules as reported by Ames *et al.* (1982), Jabaji-Hare *et al.* (1984) and Klingner *et al.* (1995a). This AM fungal autofluorescence enables excellent CLSM observations without prior staining. By CLSM, the same arbuscules, which diffusely fluoresced by FM, became clear, looking raspberry-like and loosely clumped, corresponding to collapsed arbuscular structures (Bonfante-Fasolo *et al.*, 1990). As we never found autofluorescing highly ramified arbuscules with fine branches, it seems that in our study the autofluorescence is limited to collapsed arbuscules.

By CLSM we also observed an autofluorescence of droplets accumulated in the proximity of arbuscules and of larger drops in the roots. The droplets, showing Brownian movement, have recently been described in AM colonized roots (Klingner *et al.*, 1995a). Until now they were found only in roots of the Gramineae and consist of a carotenoid, which, due to its yellow fluorescence has been named yellow pigment (Klingner *et al.*, 1995b). As the larger drops exhibited the same autofluorescence as the droplets a similar composition is likely. Klingner *et al.* (1995a) proposed a correlation between the enhanced resistance to fungal attack of AM-colonized plants and the formation of the yellow pigment.

Apart from the strong autofluorescence of the arbuscules revealed by FM, Jabaji-Hare *et al.* (1984) and Klingner *et al.* (1995a) also observed a weak autofluorescence of intraradical hyphae in sectioned root material. By contrast, using FM, we

and Ames *et al.* (1982) observed an autofluorescence specifically of arbuscules, but not of the hyphae. We assume that these contradictory observations can be attributed to the unsectioned, intact roots used by Ames *et al.* (1982) and ourselves. In intact roots the weak autofluorescence of the hyphae could be covered by the strong autofluorescence of arbuscules. Optical sectioning by CLSM confirms this hypothesis. CLSM observations showed varying fluorescence intensities of different AM structures in the intact root including a weak autofluorescence of the intraradical hyphae.

The reasons for the strong autofluorescence of the collapsed arbuscules are still unknown. Jabaji-Hare *et al.* (1984) attributed the observed fluorescence to the chitin of the fungal cell wall. This would explain the differing autofluorescence of the hyphae and the arbuscules, as chitin changes its integration into the fungal cell wall. Depending on the fungal structure, chitin is either encased in other cell wall components in the hyphae or located at the surface in the arbuscular phase (Bonfante-Fasolo *et al.*, 1990). With chitin on the fungal surface all arbuscules should fluoresce. Our CLSM study revealed, however that autofluorescing arbuscules were always collapsed and, as chitin is reduced in collapsed arbuscules (Bonfante-Fasolo *et al.*, 1990), it does not seem to be the fluorescing compound we observed.

Ames *et al.* (1982) proposed the autofluorescence of the arbuscules to be associated with the matrix or host plasmalemma rather than with the fungus itself. In our study, however, the active, finely branched arbuscules, which are surrounded by the host plasmalemma and the matrix, were not autofluorescent. Autofluorescence was only observed with clumped, collapsed arbuscules. The collapsing of an arbuscule results in its disintegration, probably releasing fungal cell-wall components. Morandi (1996) suggested that increased levels of phenolic compounds are elicited by these cell-wall components. As phenolic compounds show fluorescence in plant cells (Jahnen and Hahlbrock, 1988; Schröder, Hahlbrock & Kombrick, 1992) they might be responsible for the observed strong autofluorescence of the collapsing arbuscules.

According to Czymmek *et al.* (1994) shadowing can be a problem in CLSM. Shadowing refers to the dimming of images from deeper parts of a sample as a result of interference by overlying structures. In extreme cases an overlying structure can completely block a deeper one. AM fungi colonize the cell layers just above the vascular cylinder, forming inter- and intra-cellular hyphae, arbuscules and vesicles. In microscopical *in vivo* studies of AM colonization this can be a problem when thick, opaque roots with many cell layers above the vascular cylinder are used. In our study, possibly due to the morphology of the rye-grass roots, having only a few cell layers above the vascular cylinder and being highly transparent, all images were clear. Shadowing was, therefore, not a problem.

The present study shows the feasibility of *in vivo* observations of AM fungi in intact, living roots. CLSM provided for the first time clear *in vivo* images of *in planta* AM fungal structures. In combination with markers for *in vivo* labelling of different AM structures in roots, this technique could offer a great potential for further studies.

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