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Coupling autotrophic *in vitro* plant cultivation system to scanning electron microscope to study plant-fungal interactions

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Abstract

The interactions of plants with pathogens and beneficial micro-organisms have been seldom compared on the same host and under strict controlled autotrophic *in vitro* culture conditions. Here, the life cycle of two plant beneficial (*Glomus* sp. MUCL 41833 and *Trichoderma harzianum*) and one plant pathogen (*Rhizoctonia solani*) fungi were described on potato (*Solanum tuberosum*) plantlets under autotrophic *in vitro* culture conditions using video camera imaging and the scanning electron microscope (SEM). (i) The colony developmental pattern of the extraradical mycelium within the substrate, (ii) the reproduction structures and (iii) the three-dimensional spatial arrangements of the fungal hyphae within the potato root cells were successfully visualized, monitored and described. The combination of the autotrophic *in vitro* culture system and SEM represent a powerful tool for improving our knowledge on the dynamics of plant-fungal interactions.

Additional key words: arbuscular mycorrhizal fungi, autotrophic culture system, potato, *Rhizoctonia*, scanning electron microscope, *Trichoderma*.

Resumen

Acoplamiento de sistemas autotróficos de cultivo de plantas *in vitro* con microscopía electrónica de barrido para estudiar interacciones planta-hongo

Raramente han sido comparadas las interacciones entre las plantas y microorganismos patógenos y/o beneficiosos utilizando el mismo hospedero en condiciones estrictamente controladas *in vitro* y usando sistemas de cultivo autotróficos. En este estudio se ha descrito el ciclo de vida de dos microorganismos beneficiosos (*Glomus* sp. MUCL 41833 y *Trichoderma harzianum*) y otro microorganismo patógeno (*Rhizoctonia solani*) usando como hospedero plántulas de patata (*Solanum tuberosum*) en condiciones autotróficas empleando imágenes de video-cámara y microscopía electrónica de barrido (SEM). Se ha podido visualizar, monitorear y describir (i) el patrón de desarrollo del micelio extrarradical, (ii) las estructuras de reproducción y (iii) el reordenamiento tridimensional de las células fúngicas en las células radicales de patata. La combinación del sistema autotrófico *in vitro* y la SEM constituye una poderosa herramienta para el progreso de nuestro conocimiento sobre las interacciones entre hongos y plantas.

Palabras clave adicionales: hongos formadores de micorrizas arbusculares, microscopio electrónico de barrido, patata, *Rhizoctonia*, sistema de cultivo autotrófico, *Trichoderma*.

Introduction

The rhizosphere (*i.e.* zone of soil influenced by plant roots and characterized by an important microbiological activity) represents a highly dynamic region governed by a complex mosaic of interactions between plants and micro-organisms (Kennedy and De Luna, 2004). Some micro-organisms inhabiting this zone are benefi-

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Abbreviations used: AM [arbuscular mycorrhizal (fungi)], BAS (branching absorbing structures), GINCO (Glomeromycota *in vitro* collection), MS [Murashige and Skoog (medium)], MSR [modified of Strullu-Romand (medium)], MUCL (Mycothèque de l'Université catholique de Louvain), PDA [potato dextrose agar (medium)], PPF (photosynthetic photon flux), RH (Runner hyphae), ROC (root organ culture), SDWP (sterile distilled water-peptone), SEM (scanning electron microscope).

cial to plants (*e.g.* increase soil fertility and structure, control root pathogens) (Barea *et al.*, 2005), while others are detrimental (*i.e.* pathogenic) causing considerable damage to economical important crops (Higa and Parr, 1994). One primary objective in studies of the biology of this soil-root interface is to improve the interactions between the plant and the plant-beneficial micro-organisms (*e.g.* arbuscular mycorrhizal (AM) fungi, *Trichoderma* species), whilst controlling the harmful effects of those which are phytopathogenic.

Host-pathogen interactions as well as host-beneficial micro-organisms interactions are multi-step processes that involve adhesion to the host surface, infection/colonization of root tissues, which provokes cell damage and disease or help the plants to acquire nutrients and withstand stress conditions. To study these processes several *in vivo* and *in vitro* systems have been set up that includes both the host and the pathogenic or beneficial micro-organisms (Friese and Allen, 1991; Olivain and Alabouvette, 1999; Vleeshouwers *et al.*, 1999; Declerck *et al.*, 2005; Huang *et al.*, 2005). Recently, an autotrophic culture system was developed for the *in vitro* mycorrhization of micropropagated potato (*Solanum tuberosum*) plantlets (Voets *et al.*, 2005). This system provides unique visualization of the AM fungal development and the possibility to conduct time-course non-perturbed three-dimensional morphological observations.

The potato has been described to form beneficial associations with AM fungi (Duffy and Cassells, 2000) and *Trichoderma* spp. (Harman *et al.,* 2004), while being severely affected by *Rhizoctonia solani* (Sneh *et al.*, 1996). Arbuscular mycorrhizal (AM) fungi and fungi from the genus *Trichoderma* are well known to develop beneficial associations with plants (Harman *et al.*, 2004; Smith and Read, 2008). Members of both groups increase plant growth and productivity and have been shown to behave as bio-control agents against a wide range of important soil-borne plant pathogens (Harman *et al.*, 2004; Barea *et al.*, 2005). Certain members of the *Rhizoctonia* genus (*i.e., R. solani*) (Sneh *et al.*, 1996) are known to be pathogens causing serious damages to some of the most important economical crops throughout the world.

Although the life cycles of many soil-borne fungi have been studied *in vitro*, most have not been compared *in vivo* on a single host plant species (*e.g.* potato) that is susceptible to a large number of pathogenic (*e.g. R. solani*) and beneficial (*e.g.* AM fungi and *Trichoderma*) microorganisms.

This difficulty was partly related to the obligate symbiotic nature of AM fungi which, *in vitro*, was most often studied on transgenic excised roots, *i.e.* without photosynthetic tissues (see Declerck *et al.*, 2005). Hence, the objective of this work was to describe *in vitro* the life cycle of three major fungi (*i.e. Glomus intraradices*, *T. harzianum* and *R. solani*) on autotrophic potato plantlets as a single host. For the three fungi, the hyphal extension, colonization of the substrate, root infection/colonization and reproduction structures were described under autotrophic *in vitro* culture conditions (Voets *et al.*, 2005), combining the video camera imaging (De la Providencia *et al.*, 2005) and the cryo-electron scanning microscope (cryo-SEM) (Read and Jeffree, 1991).

Material and methods

Biological material

Propagation and maintenance of potato plantlets stock

In vitro propagated potato plantlets (*Solanum tuberosum* L. cv. Bintje) were supplied by the Station de Haute Belgique in Libramont, Belgium. Plantlets were micro-propagated every five weeks by placing nodal cuttings in sterile culture boxes (90 × 60 × 50 mm, 20 cuttings per box) (see Voets *et al.*, 2005), filled with 50 mL of 4.41 g L^{-1} sterilized (121°C for 15 min) Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 20 g L^{-1} sucrose, 3 g L^{-1} Gel Gro™ (ICN, Biomedicals, Inc., Irvine, CA, USA) and adjusted to pH 5.9. Plantlets were kept in a growth chamber at 20/15°C (day/night) and illuminated for 16 h d^{-1} under a photosynthetic photon flux of 15 µmol m^{-2} s^{-1}.

Culture, propagation and maintenance of Glomus *sp. MUCL 41833*, Trichoderma harzianum, *and* Rhizoctonia solani

The AM fungus used was *Glomus* sp. MUCL 41833, formerly identified as *Glomus intrarradices* and presently reclassified in a clade that contains the recently described species *G. irregular* Blaszk Wubert, Renker and Busco (Stockinger *et al.,* 2009) was supplied by Glomeromycota *IN vitro* Collection (GINCO) (http://www.mbla.ucl.ac.be/ginco-bel). The spores were

extracted by solubilization of the gellan gel (Doner and Bécard, 1991) and an approximate of 100 were placed close to actively growing transformed carrot (*Daucus carota* L.) roots (approximately 70 mm length) on Petri plates (90 mm diam.) containing the Modified Strullu-Romand (MSR) medium (Declerck *et al.*, 1998 modified from Strullu and Romand, 1986) solidified with 3 g L^{-1} Gel Gro (ICN, Biomedicals, Inc., Irvine, CA, USA) (Declerck *et al.*, 2005). The Petri plates were incubated during 3 months in the dark in an inverted position at 27°C. Several thousand spores and extensive mycelium were produced within this period.

A culture of *Trichoderma harzianum* Rifai MUCL 29707 was supplied by the Mycothèque de l'Université catholique de Louvain (MUCL) (http://bccm.belspo.be/about/mucl.php). A plug of gel containing several conidia and mycelium was placed into a sterile 1.5 mL glass tube filled with 0.4 ml of 1% sterile distilled water-peptone (SDWP) (Duchefa, The Netherlands). The plug was swirled in the SDWP with a vortex mixer for 15 s and the suspension was serially diluted to 10^{-2}. From the 10^{-2} dilution, 50 µL was spread on potato dextrose agar (PDA) (Scharlau Chemie S.A, Barcelona, Spain) Petri plates and further incubated at 25°C for seven days. The Petri plates were subsequently maintained at 4°C until needed.

A culture of *Rhizoctonia solani* Kuhn MUCL 49235 was supplied by MUCL. A plug of gel containing several bulbils (or sclerotia, hard pieces of mycelium tissue) (Sneh *et al.*, 1996) and mycelium was placed on PDA Petri plates that were then incubated at 25°C for seven days.

Experimental set-up

Ten days old potato plantlets were used to study the life cycle of the three fungal organisms under *in vitro* autotrophic culture conditions. One potato plantlet was inserted in each Petri plate on the MSR medium, with the roots placed on the surface of the medium and the shoot extending outside through the hole made at the base and the lid of the Petri plate (for details, see Voets *et al.*, 2005).

Spores of the AM fungus cultures were isolated by solubilization of the MSR medium (Donner and Bécard, 1991) and maintained in deionized sterilized (121°C for 15 min) water before inoculation. Approximately 50 spores were placed in the vicinity of the roots of potato plantlets in each Petri plate. The Petri plates were then closed with parafilm™ (Pechiney, Plastic Packaging, Chicago) and the hole cautiously plastered with sterilized (121°C for 15 min) silicon grease (VWR International, Belgium) to avoid contaminations (Voets *et al.*, 2005).

The inoculation with the *T. harzianum* strain was performed using 50 µL of fungal suspension from a 10^{-2} dilution containing several conidia and mycelium (see above). The suspension was spread on Petri plates containing PDA and incubated at 25°C for 24 h. A single-germinated conidium was transferred to the root vicinity of potato plantlet and the Petri plates were closed as above.

The inoculation with *R. solani* was performed with a 3-mm-diameter plug removed with a sterile cork-borer from a seven-day old culture on PDA medium. The plug containing abundant mycelium and bulbils was placed in the vicinity of roots of potato plantlets and the Petri plates were closed as above.

Whatever the fungus, all the Petri plates were covered with an opaque plastic bag and incubated horizontally in a growth chamber set at 22/18°C (day/night) with 70% relative humidity, a 16 h photoperiod and a photosynthetic photon flux (PPF) of 300 µmol m^{-2} s^{-1}. Ten ml of sterilized (121°C for 15 min) MSR medium lacking sucrose and vitamins (cooled to 40°C in a water bath) was added every two weeks in the Petri plates containing the AM fungus to maintain an adequate level of MSR medium and to supply the plantlets with renewed nutrients. For each fungus, five replicates were examined. In addition, five un-inoculated potato plants were used as control.

Observations and description of the fungal development

For the three fungi, the hyphal extension, colonization of the substrate, root infection/colonization and reproduction structures were described. Anastomosis, that is *«a mechanism by which different branches of the same or different hyphae fuse to constitute a mycelial network»* (Kirk *et al.*, 2001) formed during the colony development of the three organisms was further categorized following the detailed description performed by Buller (1933). For the AM fungus the Petri plates were checked weekly during 6 weeks following the method described in Declerck *et al.* (2001). For *T. harzianum* and *R. solani* strains the development of the fungal colony was followed daily during 2 weeks

due to the high speed of mycelium development. All the measurements were conducted under a dissecting microscope (Olympus SZ40, Olympus Optical GmbH, Hamburg, Germany) (magnification x6.7 to x40) and compound bright-field light microscope (Olympus BH2-RFCA, Olympus Optical GmbH, Hamburg, Germany) (magnification x50 to x250). Images of the different stages of the life cycle of the three fungi were captured with a digital camera (model Leica DFC320; Leica Microsystems Ltd.) coupled to a compound bright-field light microscope Olympus BH2-RFCA and displayed on a 15-inch hp pavilion ze4500 computer using the image manager software: Leica IM50, version 4.0 Leica Microsystems Imaging solutions Ltd., Cambridge, UK.

Newly produced spores of *Glomus* sp. MUCL 41833, conidia of *T. harzianum* and mycelium and bulbils of *R. solani*, isolated from autotrophic *in vitro* cultivation system after 6 weeks and 12 days, respectively, were tested for their capacity to germinate and ability to produce the fungal life cycle. Spores of the AM fungus were isolated by solubilization of the MSR medium (see above) and placed in the vicinity of roots of potato plantlets in Petri plates (90 mm diam.) containing the MSR medium without sucrose and vitamins (see above). The Petri plates were incubated in the same condition that described above. Germination was observed under stereo microscope at 10-40x magnification after 10 days. A plug of gel containing several conidia and mycelium of *T. harzianum* and a plug of gel containing several bulbils and mycelium of *R. solani* were placed on PDA and MSR Petri plates and further incubated at 25°C for three days. The regrowth of *T. harzianum* and *R. solani* was observed under stereo microscope at 10-40x magnification.

Tissue preparation and cryo-fracturing electron scanning microscopy (cryo-SEM)

Six weeks after inoculation with the AM fungus and 6 and 12 days after inoculation with *T. harzianum* and *R. solani*, some of the roots developing on the surface of the MSR medium and in contact with the fungal mycelium were gently removed from the gel with forceps without damaging the whole root system and disturbing the fungal colony development. Six days old, 12 days old and 6 week old un-inoculated roots of potato plantlets were also gently removed from the gel and used as control for comparing the level of damage caused by the pathogens with the normal process of root aging. The roots were excised to 1-cm length and put into sterilized distilled water. The root pieces were subsequently transferred to Press-to-Seal™ (Molecular Probes) silicone chambers and pressed onto a dry microscope slide 24 × 60 mm (Menzel-Glazer®). Sterilized water-agar 3% (0.6 mL) (Oxoid, Basingstoke, Hampshire, England) was added to the chamber to fix and immobilize the root pieces, and thus to create a thin layer in which they were embedded. Subsequently, the silicone isolator chamber was removed and the root pieces embedded in the solid water-agar were transversally cut with a sterile scalpel. For SEM observations (SEM Phillips XL20, Amsterdam, The Netherlands), roots were flash frozen (–212°C) in liquid nitrogen under vacuum for cryo-SEM (Oxford CT1500 cryosystem, Oxford, UK), transferred to the preparation chamber and then to the SEM chamber where the frozen samples were sublimated (–80°C) to remove ice particles. Samples were viewed under 2-5 kV at –170°C to –190°C.

Results

Glomus sp. MUCL 41833 development

Germination of the AM fungus spores was observed two days after inoculation. Straight, coenocytic and hyaline germinating hyphae arose through the subtending hyphae of spores and developed on the surface of the medium. Vigorous 10-15 µm wide hyphae branched at angles of approximately 45° and extended over short distances on the MSR medium. Following contact with the host root (within one week) several appressoria were formed (Fig. 1a). Subsequently, multiple hyphae developed intercellularly within the cortex (Fig. 1b) extending root colonization beyond the site of penetration. In parallel, runner hyphae (RH) developed extensively in the growth medium, and ramified repeatedly into hyphae bearing numerous branching absorbing structures (BAS). Interconnections of hyphae by means of hyphal fusions (anastomosis) were often observed in all parts of the colony. Fusions between hyphae were observed through tip-to-tip or tip-to-peg fusions to form an intermingled network of mycelium. The first daughter spores appeared four weeks post-inoculation and their formation was observed mainly on the primary hyphae (*i.e.* RH). After extensive colonization of the substrate (*i.e.* eight weeks post-inoculation) hundreds of spores of 92.7 ± 2 µm diam. were produced.

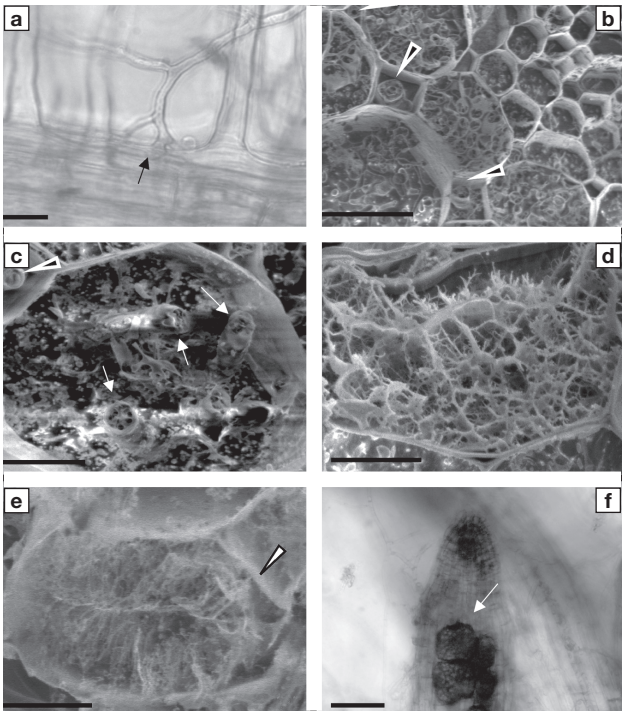

Figure 1. Description of the development of *Glomus* sp. MUCL 41833 under autotrophic conditions in association with *Solanum tuberosum*. (a) Appressorium formation on the surface of the host root (black arrow), bar = 100 µm. (b) Intercellular development and spreading of hyphae (white arrowhead). Note that the sequestered solutes during freezing give the striated appearance to the fully turgescent root cells (black arrow). Some cells are empty caused by the aging process of cell tissue (white arrow), bar = 50 µm. (c) Sectioned root cell showing the development of the 1st order branches of arbuscule within the cortical cell (white arrows) as well as the intercellular hyphal spreading of the fungi (white arrowhead), bar = 30 µm. (d) Full developed and highly branched Arum-type arbuscule (black arrow). Note that the arbuscule is occupying the entire volume of the root cell, bar = 40 µm. (e) Senescing arbuscule (white arrowhead), bar = 40 µm. (f) Formation of vesicle and spores a few millimetres behind the apex root zone (arrow), bar = 60 µm.

Numerous vesicles and intraradical spores were observed within roots. Some of them were observed a few millimetres behind the root apex (Fig. 1f). Observation of the root under cryo-SEM six weeks post-inoculation revealed the structural details of the development of the Arum-type arbuscule, intercellular hyphae penetrate cell walls at the inner root cortex and ramify in the apoplast finally forming one highly branched tree-like structure (Smith and Read, 2008) (Fig. 1c-e). Sectioned root segments showed the 1st order branches of arbuscules (Fig. 1c), highly branched mature arbuscules, occupying the entire cell volume (Fig. 1d) and degraded arbuscules (Fig. 1e). Root cells in intimate contact with the AM fungus did not show any signs of cellular degradation, but fully turgescent cells were often observed showing striations which are sequestered solutes formed during freezing (Fig. 1b). Cryo-SEM analysis after six weeks of culture also showed empty root cells caused by the aging process of the root cell tissues (Fig. 1b).

The observations of spore germination showed that 93% of the newly produced spores germinated within 10 days. Hyphae growth was observed emerging through the lumen of the subtending hyphae. The spores were able to form associations with the roots of the plantlets in the autotrophic *in vitro* culture system and produce new spores.

Trichoderma harzianum MUCL 29707 development

The development of *T. harzianum* was marked by a rapid germination of conidia. Highly branched hyaline germinating hyphae, branched at angles from 10 to 90°, extended rapidly on the MSR medium. During the first 24 h, hyphae (3-10 µm diam.) extended radially and a dense network of interconnected hyphae was observed in all parts of the colony where anastomoses were observed through to tip-to-tip, tip-to-peg and peg-to-peg fusions. Forty-eight hours later (72 hours post-inoculation) the entire Petri plates (*i.e.* roots and MSR medium) were covered with the fungal mycelium (Fig. 2a). Contacts with roots were observed (Fig. 2b) at that time. Aerial mycelium with arachnoid's appearance was coiled to the aerial roots (Fig. 2c). However, the direct and scrupulous observations of roots under bright-field light and electron scanning microscopes did not reveal any structure resembling appressorium. Five days after inoculation, conidiogenous cells (phialides) producing single phialidospores at the tip of each phialide were observed (Fig. 2d). Conidiophores were gathered in loose aggregates scattered on the surface of the medium or forming compact tufts along and attached to the roots (Fig. 2e). The distribution of the conidiophores along the root axis was not correlated with the age of the root, since these structures were observed along the main root as well as in secondary and lower order branches. Gradual changes in the colour of the aggregates or tufts from white to green and finally greyish-green, following production and maturation of the conidia, were observed. No chlamydospores were detected during the experimental period.

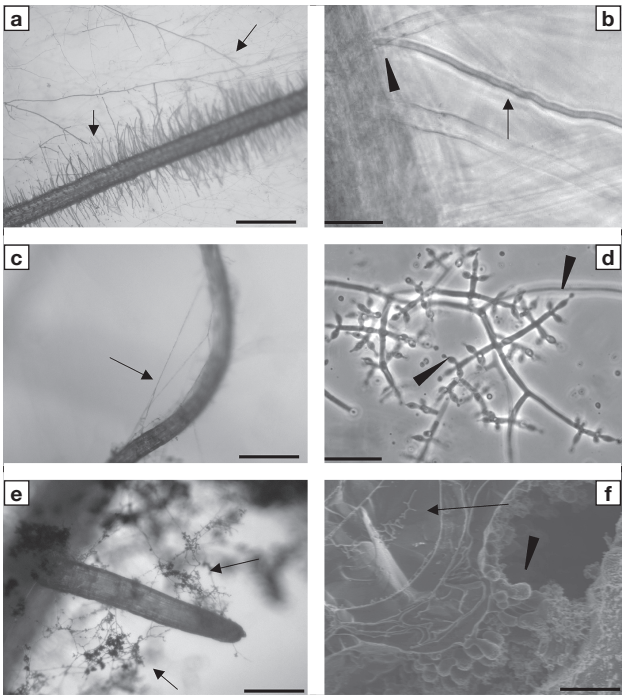

Figure 2. Description of the development of *Trichoderma harzianum* MUCL 29707 under autotrophic conditions in association with *Solanum tuberosum.* (a) Highly ramified mycelium (black arrows) spreading and colonizing the substrate (MSR medium), bar = 250 µm. (b) Growth of the hypha towards the root (arrow). Note the contact with the surface of the root (arrowhead), bar = 30 µm. (c) Aerial mycelium coiled around the roots (arrow), bar = 250 µm. (d) Conidiogenous cells producing single phialidospores at the tip of each phialide (black arrowheads), bar = 25 µm. (e) Conidiophores forming compact tufts along and attached to the roots (arrows), bar = 200 µm. (f) Sectioned root segment showing an intact cortical cell (arrow). Note the presence of a mature conidiophore attached to the root surface (arrowhead), bar = 20 µm.

No root cells invasion was observed under SEM after 6 days or after 12 days of roots contact. Hyphal development as well as conidiophores formation was confined to the epidermis root zone (Fig. 2f). The root cells did not show any signs of degradation caused by the presence of *T. harzianum*. Full content striated cells caused by cryo-preservation process were observed.

The mycelium and conidia of *T. harzianum* produced in the autotrophic culture system were able to regrowth and to produce new conidia.

Rhizoctonia solani MUCL 49235 development

Within the initial 24 hours following inoculation several hyaline hyphae emerged from the plug. Hyphae close to the inoculation plug presented a negative geotropism (hyphae growing upward, contrary to the gravity) and were highly ramified. The mycelium consisted of septate, hyaline hyphae, branching at 45-90° angles. The hyphal width varied from 5 to 12 µm. The mycelium was growing over the surface of the medium and no contact with roots was observed within this period. Forty-eight hours later (72 hours post-inoculation) the mycelium developed extensively and hyphae were growing into the medium. Hyphal anastomoses involving tip-to-tip and tip-to-peg fusions were clearly observed in all parts of the colony. Long hyaline hyphae having numerous lateral branches were growing towards the adjacent roots and colonizing the substrate (Fig. 3a). Hyphal swelling and coiling forming a right angle branch called «T-shaped branch» and giving rise to short appressorial structure or infection pegs, were observed on several points of the root surface (Fig. 3b). No formation of infection cushions was observed after inoculation during the whole experiment. The mycelium was firmly attached to the root surface. Subsequently, hyphae penetrated the epidermal tissue and developed both inter- and intracellularly, parallel to the root vertical axis (Fig. 3c). Necrosis of the root hairs near the infection points was the first sign of fungal damage. However the observations under bright-field light and scanning electron microscopes did not reveal fungal infection via the root hairs nor fungal occupation in this root zone. Particularly, the cryo-SEM observations of the roots 6 days after inoculation showed hyphal invasion of cortical cells. Total degradation of cell cytoplasm caused by the pathogen was observed (Fig. 3e). However deterioration of the root tissue was not extended to the whole root system. Undifferentiated aggregations of thick-walled melanized cells called bulbils, that represent the survival structures of *R. solani*, were observed after seven days of inoculation (Fig. 3d). These structures were observed mainly superficially (Fig. 3d) or detected attached to the roots. The cryo-SEM observations twelve days after inoculation revealed severe damage to the entire root system. Even when the cytoplasmic content of the root cells was totally degraded at that time (Fig. 3e), our observations showed well-preserved cell walls (*i.e.* without deformation or distortion in their structure). Fungal hyphae were easily observed within the root cells (Fig. 3e) and in the intercellular spaces. In addition, hyphae were mostly aligned with the vertical root axis (Fig. 3f).

The mycelium and conidia of *R. solani* produced in the autotrophic culture system were able to regrow and to produce new bulbils.

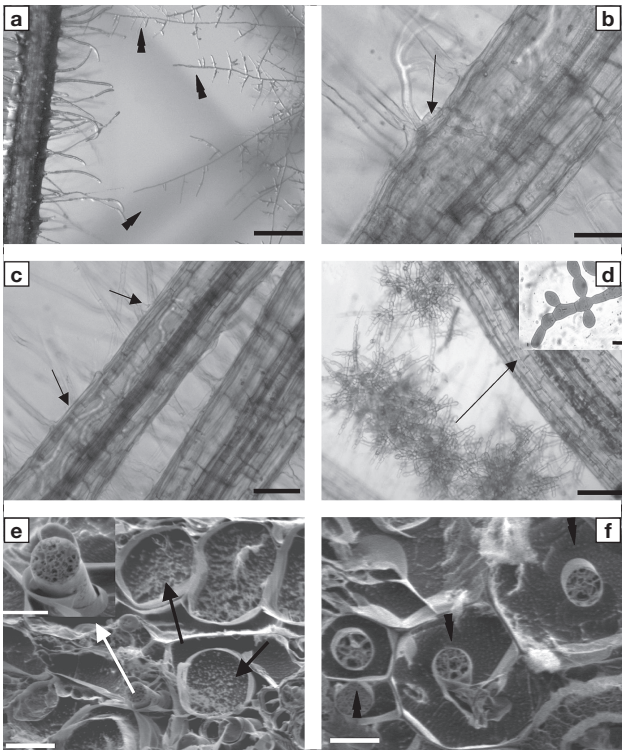

Figure 3. Description of the development of *Rhizoctonia solani* MUCL 49235 under autotrophic conditions in association with *Solanum tuberosum.* (a) Highly branched hyphae growing towards the roots (double arrowheads), bar = 250 µm. (b) Contact with root and formation of a T-shaped branch" appressorial structure at the surface of the root (arrow), bar = 50 µm. (c) Intracelullar spreading of hyphae. Note that the spreading of hyphae is parallel to the root vertical axis (arrows), bar = 200 µm. (d) Formation of compact tufts of bulbils (reproduction structures) close to the roots, bar= 200 µm. Inset: Closer view of bulbils, bar = 10 µm. (e) Highly deteriorated cortical cells (black arrow) due to the presence of *R. solani* hyphae, bar = 15 µm. Inset: Closer view of intercellular hypha, bar = 5 µm. (f) Well-preserved root cells being infected by *R. solani* hyphae (double arrowheads). Note that the root cells did not collapse due to the presence of intracellular hyphae, bar = 5 µm.

Discussion

The development of appropriate *in vitro* autotrophic plant cultivation systems on which several fungi with different relationships to root cells (*i.e.* obligate symbiont, non-obligate plant-beneficial fungi, and pathogen) can develop represents an important tool to study plant micro-organisms interactions. Here, we successfully monitored, visualized and described the life cycle of *Glomus* sp. MUCL 41833, *T. harzianum* MUCL 29707 and *R. solani* MUCL 49235 on potato plantlets grown *in vitro* under autotrophic culture conditions. The system allowed for easy visualization of the fungal structures by means of cryo-SEM analysis performed on root cells in direct contact with these fungi.

The life cycle of *Glomeromycota* (Schüßler *et al.*, 2001) has been described for several species (Fortin *et al.,* 2002). The development of root organ culture (ROC) (see Declerck *et al.*, 2005) improved significantly the description of the life cycle, among others for the vesicles-forming *Glomus* species (Chabot *et al.*, 1992; Strullu *et al.*, 1997). However, the use of ROC to study AM fungal symbiosis has some limitations, particularly the absence of photosynthetic tissues necessary to have a normal hormone balance and physiological source-sink relationships (Fortin *et al.*, 2002). To overcome this limitation, Voets *et al.* (2005) developed a system in which the plant roots were intimately associated to an AM fungus. In this system culture plant roots grew on a gelled medium under strict *in vitro* conditions, while the photosynthetic shoot developed under open-air conditions. Using this system we observed spore germination, appressorium formation, root colonization, and spore production. In addition, SEM observations of the root cells revealed, for the first time, the inter- and intraradical locations of hyphae and arbuscules development under *in vitro* conditions, supporting earlier observations made on *in vivo*-based experiments (see Smith and Read, 2008).

Arbuscules has been acclaimed as the major site for the exchange of minerals (in particular phosphorus) and carbohydrates between both partners in the AM symbiosis (Rosewarne *et al.*, 1999; Rausch *et al.*, 2001). Using a similar *in vitro* autotrophic cultivation system, Dupré de Boulois *et al.* (2006) demonstrated the transport of P from a hyphal compartment to a root compartment and subsequently to the plant shoot cells via the fungal interface, while the transport of carbohydrates from plant to the AM fungus was investigated by Voets *et al.* (2008). However, strict attention was not paid to either the development or presence of arbuscules. Therefore, the observations in our experiment of the presence, development and senescence of Arum-type arbuscule under cryo-SEM coupled with the transport of P and C observed by Dupré de Boulois *et al.* (2006) and Voets *et al.* (2008), respectively, supported the role of arbuscule as an exchange interface and the adequacy of the autotrophic *in vitro* culture system to sustain this essential attribute of the symbiosis. The presence of vesicles and intraradical spores just behind the apex further demonstrated that AM fungi are able to penetrate the root at the apex zone (Bago and Cano, 2005) and also that intraradical structures such as ve-

sicles may be located in this zone. These data supports the validity of the system used here to study AM fungal symbiosis under *in vitro* conditions.

The time-course monitoring of the life cycle of *T. harzianum* showed that the development of this strain was successfully completed under *in vitro* autotrophic culture conditions (*i.e.* from conidium germination to the formation of the reproduction structures). Numerous hyphae coiled around the roots and firmly attached to them with the formation of several mature conidiophores along the root system were observed. However, the scrupulous observations of roots under bright-field light microscope and cryo-SEM analysis on the root samples did not reveal any sign of fungal invasion in the root cortex.

Several studies have shown that root associated-*Trichoderma* strains resulted in increased levels of defense-related plant enzymes (see Harman *et al.*, 2004). These studies have related this phenomenon to the plant internal root colonization. However, currently only two reports have visualized the hyphal penetration of the roots by *Trichoderma* strains (Yedidia *et al.*, 1999; Chacón *et al.*, 2007). Yedidia *et al.* (1999) studied the induced defense mechanisms in cucumber plants (*Cucumis sativus* L.) following contact with *T. harzianum* T-203 (formerly re-described later as *T. asperellum* (Kullnig *et al.*, 2001). The electron microscopy analysis of ultrathin sections from *Trichoderma*-treated roots revealed penetration of *T. asperellum* hyphae into the roots mainly restricted to the epidermis and outer cortex. However, in some localized areas, the hyphae were observed also in the endodermis (Yedidia *et al.*, 1999). Working with *T. harzianum* CECT 2413 in tobacco (*Nicotiana benthamiana*) and tomato plants (*Lycopersicum esculentum*) Chacón *et al.* (2007) demonstrated the ability of this fungus to colonize roots and to stimulate plant growth in seedlings of both species. Despite that our observations did not reveal fungal penetration and invasion with *T. harzianum* MUCL 29707 into the potato roots, our results are not in contradictions with the studies of Yedidia *et al.* (1999) and Chacón *et al.* (2007) since plants and fungal species/strain are different. Furthermore, *Trichoderma* species are not obligate biotrophs of plants but they are mostly saprotrophic on soil and dead plant material, but can also be opportunistic plant root colonizers (Harman *et al.*, 2004). Therefore, root colonization is not an essential attribute of this group of fungi. Nowadays, little is known about the mechanisms which govern this colonization. Further works should be addressed to study the effects of different host on the *«symbiotic behaviour»* of some *Trichoderma* strains (*e.g. T. asperellum* T-203, *T. harzianum* T-22 and T-39) that are largely known to produce defense responses in plants (Yedidia *et al.*, 1999, 2003; Harman *et al.*, 2004) and also the combination of both factors.

Studies of plant-pathogen interactions are often hampered by difficulties in obtaining undisturbed internal views of infected material (Refshauge *et al.*, 2006). The traditional methods used to study plant-pathogen interactions drastically damage the arrangements of plant and fungal cells (see Refshauge *et al.*, 2006), particularly when highly damaged tissue are sampled and prepared for microscopy observations and analysis (Chi and Childers, 1964; Ruppel, 1973). In our study, the life cycle of *R. solani* associated to potato plantlets was successful monitored and described. The system used here allowed for easy visualization of the spreading of the colony in the gelled medium and further monitoring of hyphae within roots. Hyphae were seen to propagate into the roots cells mainly following the direction of the root vertical axis. The same behaviour was earlier described in the root of cereals (Wenzel and McCully, 1991; Refshauge *et al.*, 2006). These authors suggested that the propensity of hyphae to colonize roots following the direction of the vertical axis is the result of a long, tubular structure of the cortical cells that provide the pathogen a lower resistance pathway for infection. The detection of hyphae of *R. solani* inside and between potato root cells as early as 6 days post-inoculation were similar to the findings of Refshauge *et al.* (2006) and Hall (1987). These authors detected hyphae of *R. solani* within roots of wheat (*Triticum aestivum*) after six and seven days respectively. Cryo-SEM analysis of highly damaged root cells caused by the necrophytic action of *R. solani* helps to overcome the difficulties to obtain good quality internal views of the delicate root system.

The autotrophic *in vitro* plant cultivation system and cryo-SEM analysis are adequate and important tools to study plant-fungi interactions, either beneficial or pathogenic. The ability to couple and to integrate both tools offers the possibility of deepening our understanding on how soil-borne micro-organisms and plants behave during their interactions, giving a complete picture of the structural arrangements of the fungal cells within the root tissue during their whole life cycle. Hence, they are not limited to the study of plant-fungi but could be extended to other soil-borne micro-organisms that are largely known to develop beneficial and/or

harmful associations with plants or to interactions studies combining multiple organisms (beneficial or harmful) on the same host.

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