7 Breaking Myths on Arbuscular Mycorrhizas in Vitro Biology

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1 Introduction

"For a critical study of the effects of vesicular-arbuscular mycorrhiza on plant growth, typical infections must be produced under controlled microbiological conditions". With those initial words, Mosse published in 1962 a research article in which the establishment of "vesicular"-arbuscular mycorrhizas under "aseptic" conditions was first reported. Mosse, together with Hayman and some other early mycorrhizologists, were pioneers in suggesting the importance of this mutualistic symbiosis (Mosse 1953, 1956, 1957; Mosse and Hayman 1971; Hayman and Mosse 1971, 1972). Soil microbiologist and phytopathologist as she was, Mosse was conscious of the necessity of fully understanding the biology of a given micro-organism, in order to obtain some indication for its subsequent manipulation and use in particular situations.

Mosse's first "aseptic" mycorrhizal cultures were, in fact, dixenic (i.e. the AM fungus plus two foreign organisms; Williams 1992) cultures between the AM fungus known at that time as *Endogone* sp., sterile-raised seedlings of different plant species, and *Pseudomonas* sp. The author claimed that the presence of such soil bacteria was necessary for the symbiosis to be established (Mosse 1962; see also Mugnier and Mosse 1987). It was not until 1975 that Mosse and Hepper reported the first in vitro co-culture between a root organ and a contaminant-free inoculum from a glomalean species (*Endogone mosseae*). However, and perhaps due to the difficulty of maintaining such dual cultures, this in vitro technique was almost forgotten for more than a decade.

In 1986 and 1987, Strullu and Romand initiated a series of papers demonstrating the high potential of mycorrhizal root pieces and isolated intraradical vesicles for the establishment of monoxenic cultures. Both papers pioneered the use of this intraradical fungal material for in vitro culturing, nowadays used for some high-number vesicle-forming *Glomus*

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Soil Biology, Volume 4
In Vitro Culture of Mycorrhizas
(ed. by S. Declerck, D.-G. Strullu, and A. Fortin)

species (Diop et al. 1994; Simoneau et al. 1994; Declerck et al. 1996, 1998, 2000). Great, but unsuccessful, efforts were made in the meanwhile, mainly by Hepper and coworkers, to produce AM fungi in axenic cultures from spores (Hepper and Smith 1976; Hepper 1979, 1981, 1983, 1984, 1987; Hepper and Jakobsen 1983). As a result of these studies, certain physicochemical conditions, such as a 2% CO_2 atmosphere, the addition of some organic acids, amino acids, vitamins, sulphur compounds, root exudates (revised by Hepper 1984; Azcón-Aguilar et al. 1998) or even suspension-cultured plant cells (Carr et al. 1985) to the culture media were found to enhance germ-tube development, but only to a very limited extent. Nowadays, we still have no choice but to consider glomalean fungi as obligate biotrophs (Bago and Bécard 2002).

In 1988, Bécard and Fortin reformulated the experimental conditions for AM monoxenic cultures to be easily maintained, using the idea of Mugnier and Mosse (1982) of using Ri T-DNA transformed carrot roots as the host. Unfortunately, little attention and much disbelieve were given to this major contribution when it was first discussed at the 8th North American Conference on Mycorrhizas (Jackson Hole, WY, USA; Bécard and Piché 1990). As a proof of this, few reports of the use of AM monoxenic cultures were published between 1988 and 1996 (Bécard and Piché 1989a, b, 1992; Chabot et al. 1992; Diop et al. 1992, 1994; Simoneau et al. 1994; Elmeskaoui et al. 1995; Mathur and Vyas 1995; Nuutila et al. 1995), most of them produced by the same research groups and being just modifications on the experimental systems published earlier. In particular, the Diop et al. (1994) paper could be considered as the first report of thousands of *Glomus* spores produced in vitro (with root pieces as inoculum), and the first demonstration of the daughter spores being able to re-establish in vitro and in vivo mycorrhizas.

A crucial date in the widespread use of AM monoxenic cultures was 1996, when an important improvement in this experimental system was reported – by means of bi-compartmented Petri plates, St-Arnaud et al. (1996) achieved the physical separation of the AM fungal extraradical mycelium (ERM) from the host root and its immediate environment. This resulted in the possibility of obtaining large amounts of AM fungal material in one compartment (the "hyphal compartment", HC), especially spores, which nevertheless maintained a symbiotic nature (i.e. the fungus was still connected to the host root, but spatially separated from it). First reports on AM physiology carried out in vitro were published immediately by using that system (Bago et al. 1996; Villegas et al. 1996), and other descriptions on AM fungal cell cycle, ERM morphogenesis and AM fungal colony developmental dynamics promptly followed (Declerck et al. 1996a, b, 1998, 2000; Bago et al. 1998a, b, 1999). Later on, and combining AM monoxenic cultures with other powerful techniques, important advances in our

knowledge of AM fungal cytology, biochemistry and physiology have been achieved (Jolicoeur et al. 1998; Pfeffer et al. 1998, 1999; Bago et al. 1999a, b, 2000, 2002, 2003, 2004a, b; Declerck and Van Coppenolle 2000; Hawkins et al. 2000; Joner et al. 2000; Koide and Kabir 2000; Declerck et al. 2001; Lammers et al. 2001;Nielsen et al. 2002; Tiwari and Adholeya 2002). More recently, molecular biologists have realized that AM monoxenics produced the optimal fungal material to carry out their studies. Indeed, the first successful amplification of ribosomal DNA from an AM fungus was done by using monoxenically produced spores (Simon et al. 1993) and, after bi- and multi-compartmental monoxenics development, an increased number of AM molecular biologists adopted these systems (e.g. Lammers et al. 2001; Maldonado-Mendoza et al. 2001; González-Guerrero et al. 2004). In summary, a change of mood has occurred in mycorrhizologists quite recently with respect to AM monoxenic cultures – from profound skepticism to general acceptance, and from a residual to a widespread use. The danger of all this consists in researchers using AM monoxenics just as a tool to get large amounts of fungal material, paying little or no attention to the type (i.e. developmental stage) and quality of material they are collecting. Indeed, the unadvertised misuse of AM monoxenics could result in poor or inappropriate AM material and, consequently, in inaccurate results and interpretations.

This chapter aims to warn about such misuses, and to address some of the questions, skepticisms and myths raised by AM monoxenic culturing. Final take-home messages should be that (1) AM monoxenics are far more than just a routine technique, (2) easy, but strict protocols should be followed for success and, most importantly, (3) some training/expertise on AM establishment, fungal colony development and hyphal morphogenesis under such conditions is mandatory for researchers aiming to use this technique, to be able to certify the quality of the material obtained and, consequently, the reliability and accuracy of the results obtained.

2
Questioning AM Monoxenic Cultures

2.1
Are AM Monoxenic Cultures Devices Too Artificial to Trust?

One of the most frequent criticisms against AM monoxenic cultures is that these are too artificial to trust any results obtained with them. While recognizing the limitations of the system, which will be addressed below, it is at least curious that most researchers making such statements use whole plants growing in pots in highly controlled environments, which have

little or nothing to do with natural conditions. There are certainly some differences in AM fungal development when growing in Petri plates versus pot microcosms and in nature (see below), but these could be minimized if one is aware of them. Fortunately, the benefits obtained by using AM monoxenic cultures (in terms of increase in our knowledge of AM fungal biology) have by now convinced quite a few of these former skeptics.

The most convincing proof supporting the suitability of monoxenic cultures for AM research is the fact that AM fungi form, both intra- and extraradically, typical symbiotic structures (i.e. appressoria, entry points, intercellular hyphae, arbuscules, intraradical vesicles and spores; Fig. 1a–c), and that they successfully complete their life cycle under these conditions by producing new and infective propagules. It is important to stress here that a given co-culture of a root organ + an AM fungus should not be considered a symbiotic monoxenic culture (SMC) unless the fungal life cycle is completed and new spores, able to establish new AM symbiosis under either monoxenic or soil conditions, are obtained. This is an important rule which should be strictly followed to preserve AM culturing credibility. The second rule is that any report of a new SMC should be presented in a peer-reviewed article and deposited in an appropriate in vitro bank (e.g. the International Bank of Glomeromycota BEG, Colección Iberoamericana de Micorrizas Arbusculares CIMA, Estación Experimental del Zaidín EEZ, Glomeromycota In Vitro Collection GINCO, and Mycothèque de l'Université catholique de Louvain MUCL), and given a deposit code in order to be validated. By following these two simple rules, one could avoid confusion/uncertainty about the AM fungal species/ecotypes actually maintained in SMC, a situation reflected in the following example. When carefully considering the list of glomalean species claimed to be maintained in monoxenics (Fortin et al. 2002), we get some uneasy numbers: only 15 of the 27 cultures listed (55.6%) have been published; to the best of our knowledge, of these 15 at least two (*G. caledonium*, *G. versiforme*) are no longer maintained in monoxenics, and one has serious doubts about the *symbiotic* status of some other such cultures (e.g. *G. etunicatum, G. mosseae, Gi. gigantea, Gi. rosea*) after carefully considering their associated publications. The latter thought is frightening, since tests performed in a culture claimed to be symbiotic, but which is not, will most probably produce misleading results of unpredictable consequences.

Another frequently made comment about AM monoxenic cultures is that while they may be useful to study the extraradical phase of the mycorrhiza formed in vitro (i.e. the fungal extraradical mycelium), this is probably not the case for its intraradical phase, especially at the level of the symbiotic plant–fungal interfaces. We may expect that roots in monoxenic culture have an altered way of acquiring carbohydrates compared to normal roots – their vascular cylinder should be partially or

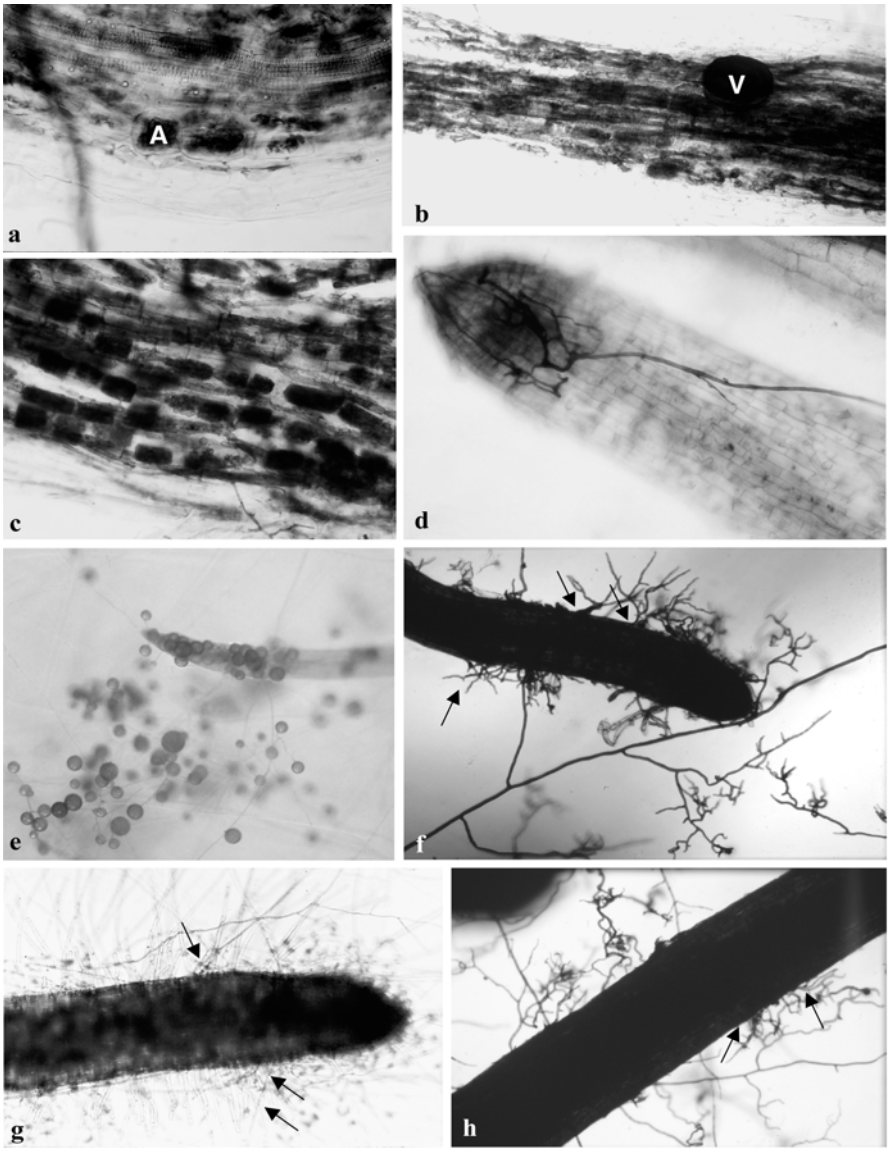


Fig. 1a–c. Intraradical features of three AM fungi grown under monoxenic conditions with a carrot Ri T-DNA transformed root organ culture (ROC, DC-2 clone). **a** *Glomus intraradices* DAOM 181602. **b** *Glomus proliferum* MUCL 41827; **c** *Glomus* sp. DAOM 227023. Colonization develops quite normally in all the species tested, and characteristic fungal structures such as entry points (*EP*), intercellular hyphae, coils, arbuscules (*A*) and intraradical spores/vesicles (*V*) are usually formed. **d–g** Apical colonization of a DC-2 ROC by *Glomus* sp. DAOM 227023 (**d**), *G.* sp. CIMA10 (**e**) or *Gigaspora margarita* CIMA 11 (**f**). **f–h** Pictures showing hyphae exiting different zones of a carrot ROC (DC-2 clone): *Gi. margarita* CIMA 10 (**f**, **h**, *arrows*) and *G. intraradices* DAOM 181602 (**g**, *arrows*)

totally useless, since sucrose is provided to the monoxenic culture homogeneously through the agar medium. Once this has been taken into account, there is no further indication that any physiological, metabolic or genetic process in root organ cells is affected. In a recent work, the cell structure of root organ cultures (ROC) from a tomato variety (c.v. 76R) and its reduced mycorrhizal colonization mutant (rmc, Barker et al. 1998; Gao et al. 2001) have been compared to the corresponding whole-plant roots growing in soil – no remarkable differences were found (Bago et al. 2004a). Also, ROC from roots expressing a Nod^- Myc^- genotype maintained such a phenotype when monoxenically cultured (Balaji et al. 1994). Finally, we should consider the strict requirements of AM fungi to become symbiotic: firstly, a series of pre-symbiotic dialogues between the two partners should occur (Bago and Bécard 2002); secondly, the newly formed interfaces must be fully functional for symbiosis to be established. It seems therefore clear that unless the physiology and functioning of ROC epidermal and cortical cells are preserved, the AM fungus would never acquire its symbiotic status and, consequently, complete its life cycle. In conclusion, while it is crucial to consider case by case the suitability of using AM monoxenic cultures for particular research purposes, there do not seem to be enough reasons to discard by default the use of such experimental systems in the study of AM intraradical/interfacial functioning.

2.2 Are Transformed Root Organs a Good Host Material to Study AM Fungal Biology?

Most of the monoxenic cultures reported to date in mycorrhizal research use Ri T-DNA transformed root organs as hosts (Table 1). These naturally transformed plant roots are obtained by the insertion of the Ri T-DNA plasmid from the ubiquitous soil bacterium *Agrobacterium rhizogenes* into a given plant tissue, which is then induced to morphologically develop as a root (a condition known as "hairy roots"; Giri and Narasu 2000). Transformed root organs usually present a greater growth potential than non-transformed ones, probably due to their modified hormonal balance (Fortin et al. 2002). Ri T-DNA transformed roots show greater AM intraradical colonization and sustain higher extraradical hyphal development than non-transformed ROC, which has led mycorrhizologists to preferentially use these roots rather than the less known non-transformed cultures. However, it has not yet been investigated if transformation could somehow affect AM fungal behaviour, and this is a possibility which should be taken into account (Bago 1998). Due to the increased secondary metabolite produc-

Table 1. Root organ cultures used to date as hosts for AM monoxenic cultures

Plant species	Ri T-DNA transformed	Clone or cv	First citation
Daucus carota L.	Yes	DC1	Bécard and Fortin (1988)
Daucus carota L.	Yes	DC2[a]	Bécard and Fortin (1988)[b]
Medicago truncatula L.	Yes	Jemalong	Boisson-Dernier et al. (2001)
Lycopersicon esculentum Mill.	Yes	–	Simoneau et al. (1994)
Pisum sativum L.	Yes	Lincoln	Balaji et al. (1994)
Pisum sativum L.	Yes	Sparkle E135[c]	Balaji et al. (1994)
Pisum sativum L.	Yes	Sparkle R25[c]	Balaji et al. (1994)
Pisum sativum L.	Yes	Sparkle R72[c]	Balaji et al. (1994)
Fragaria x Ananassa Duch.	Yes	Senga sengana	Nuutila et al. (1995)
Trifolium repens L.	Yes	New Zealand White	De Souza and Berbara (1999)
Linum usitatissimum L.	Yes	Atalante	Karandashov et al. (1999)
Tagetes patula L.	Yes	nana	Karandashov et al. (1999)
Althaea officinalis L.	Yes	–	Karandashov et al. (1999)
Trifolium pratense L.	No	S123	Mosse and Hepper. (1975)
Fragaria ananassa Duch	No	ananassa	Strullu et al. (1986)
Solanum lycopersicon Mill.	No	Saint-Pierre	Strullu and Romand (1987)
Medicago sativa L.	No	Europe	Strullu et al. (1989)
Lycopersicon esculentum Mill.	No	Vendor	Chabot et al. 1992
Lycopersicon esculentum Mill.	No	76R	Bago et al. (2004a)
Lycopersicon esculentum Mill.	No	rmc[c]	Bago et al. (2004a)
Helianthus annus L.	No	HES	Bago et al. (unpublished data)

[a]There might be further Ri T-DNA transformed carrot root clones used for AM monoxenic cultures, but this is not specified in the paper
[b]There is no clear mention in this paper on whether the carrot ROC used corresponds to what it is today known as DC1 or DC2 clones; however, since both clones came from the same authors/laboratory, we use this as first citation
[c]Mutant clones usually impaired for AM symbiosis

tion of transformed root organs (Giri and Narasu 2000), one may expect the final composition of culture media containing such transformed cultures to be different to those containing non-transformed root organs of the same plant species. Moreover, by growing different plant root organs in initially similar culture media, such media will become different in composition,

since each ROC produces specific compounds (see Giri and Narasu 2000, Table 1). Rigorous studies comparing the effects of transformed versus non-transformed root organs on AM fungal development are lacking. Such studies would be important to perform in order to evaluate and better understand the basis of AM symbiosis, and even mandatory in assessments of aspects such as fungal symbiotic gene expression.

2.3 The Downfall of Two Colonization Myths

AM in vitro cultures, in general, and monoxenics, in particular, are exceptional tools for studying the pre-symbiotic and symbiotic steps of the colonization process (Bago and Bécard 2002). Observations difficult to carry out in soil are easily performed in monoxenics, and important information has been acquired as a result of this (Schreiner and Koide 1993; Bago 1998; Bago et al. 1998a, b, 2004b; Jolicoeur et al. 1998; de Souza and Berbara 1999; Declerck et al. 2000, 2001; Nielsen et al. 2002). Taking advantage of this, we want to address very briefly two old "myths" traditionally accepted by mycorrhizologists as true, which become now challenged by direct observation with monoxenic cultures:

1. "*Primary colonization by AM fungi occurs in young roots, but the actual root apices are rarely if ever colonized*" – it has been thought for a long time that preferential colonization of roots by AM fungi occurred in subapical zones (0.5 to 1.5 µm from the root tip; Harley and Smith 1983; Smith and Read 1997), where the root is growing most actively and its cell walls are still loose, and that a sort of "exclusion zone" for AM colonization was formed at the root apical level (see Plate 3, Harley and Smith 1983). Based on colonization modelling, Smith and coworkers (2001) found that such exclusion zones were minimal or zero in most cases. Monoxenic culture observations of different AM fungi confirm these theoretical results, as is clearly shown in Fig. 1d–g. Even more, we could say that apical colonization is quite frequent. Although one may think at first that the homogeneous environmental conditions encountered by both root and fungus in AM monoxenic cultures could make root tips more prone to colonization, the observation of root tips developing in soil also suggests that apical colonization is not a rare event (C. Cano, pers. observ.), and that AM fungi have the potential to colonize root apices with minimal interference on its meristematic growth.
2. *Do hyphae exit the root after symbiosis setup?* – mycorrhizal "lore" establishes that, after symbiosis setup, and synchronously with in-

traradical colonization spreading, the AM fungus acquires a sort of hyphal "invigoration" which makes it possible to develop and explore the soil surrounding the root. In other words, this "lore" states that whereas AM fungal hyphae have the mechanisms to penetrate the root, they are unable to exit it, using instead the already established hyphal penetrating network to develop towards the soil. In contrast, a more intuitive mechanism for AM soil colonization was suggested by Friese and Allen (1991), who referred to "exiting hyphae" when describing hyphal spread in the soil from a host root; such a suggestion has caused some controversy. Monoxenic cultures confirm that AM hyphae are indeed able to exit the root to explore the surrounding media (Fig. 1f–h), and that this is a quite common event. This is in fact no big surprise, since the same mechanisms (both mechanical and enzymatic) described to be used by AM fungi to penetrate host roots (Cox and Sanders 1974; Kinden and Brown 1975; Holley and Peterson 1979; Gianinazzi-Pearson et al. 1981; García-Romera et al. 1990, 1991) could be easily used by the mycobiont to develop in the opposite direction.

2.4 Are Branched Absorbing Structures (BAS) Commonly Formed by all Glomalean Fungi? Are They Artifacts Formed Only Under in Vitro Conditions?

In the first report of the in vitro co-culture of a root organ and a glomalean species (Mosse and Hepper 1975), "... a form of branching strongly reminiscent to arbuscules ..." was already described. Since then, different authors have mentioned such "arbuscule-like structures" (ALS) formed on extraradical runner hyphae of different AM fungi (Mosse 1988; Bécard and Fortin 1988; Chabot et al. 1992; Declerck et al. 1996; Bago et al. 1998a). Strullu et al. (1997) suggested such structures are a thallus emerging from the intraradical mycelium of AM fungi during the saprophytic phase, thus being different from the spore-produced pre-symbiotic hyphae. In 1998, Bago et al. (1998b) studied ALS in depth, finding striking morphological, cytological and developmental similarities between these and intraradical arbuscules. ALS were suggested to be preferential sites for nutrient uptake by the extraradical mycelium of AM fungi (Bago et al. 1998b), and homologous to arbuscules in the context of AM fungi being highly bipolarized organisms (Bago 1998). Following the advice of the two referees revising the paper, Bago et al. (1998b) had to rename arbuscule-like structures to "branching absorbing structures" (BAS): both referees felt that such structures were (1) not similar enough to arbuscules, (2) different in function,

and (3) perhaps artificial structures produced by the AM fungus *Glomus intraradices* under highly controlled in vitro conditions.

Since then, the question of BAS being artificial structures has been raised in different scientific meetings and paper reviewing processes. At the same time, more and more mycorrhizologists using monoxenic cultures realized the omnipresence of BAS on AM extraradical runner hyphae (de Souza and Berbara 1999; Declerck et al. 2000; Nielsen et al. 2002). In this section, we present graphic evidence of (1) the occurrence of BAS in all of the different AM fungal isolates successfully cultivated by us in monoxenics (Fig. 2) and (2) the occurrence of such structures under natural conditions (Fig. 3a–j). Finally, we will show first evidence confirming the hypothesized homology between arbuscules and BAS (Fig. 3k, l).

In Fig. 2, the morphology of extraradical hyphae of 12 different AM fungal isolates is shown. The most striking feature in all of these is the presence of BAS at regular intervals on the runner hyphae. In some of the species/isolates studied, BAS were more difficult to distinguish, due to the frequent anastomoses suffered by extraradical hyphae, which masked BAS occurrence (Fig. 2b, *G. etunicatum*). In all cases, BAS show their typical, slender morphology, with a dichotomous branching pattern and a hyphal tip thickness of approx. 1.5 μm. BAS were slightly different in morphology from one species/isolate to another; this could be of use in the future as a taxonomic character. In agreement with first descriptions (Bago et al. 1998b), BAS were ephemeral (5–7 day life span) in all isolates tested, except for those undergoing spore formation events ("spore-BAS", Bago et al. 1998b; see Fig. 2, insets). BAS are developed not only by *Glomus* species, but also by members of the *Gigaspora* (Fig. 2j–l), *Scutellospora* (de Souza and Declerck 2003) and *Acaulospora* (Dalpé and Declerck 2002) genera tested to date. We can now say that BAS are not just artificial structures formed in vitro by AM fungi – they are also formed under soil ex-vitro conditions, although in such situations they are extremely difficult to observe (Fig. 3a–j). Bago (1998) proposed that, besides the putative physiological role of BAS as preferential soil nutrient scavengers, these structures would also be implicated in maintaining soil structure and aggregate formation, as their thin branches would grow between soil particles, holding them together. This could be the reason why they are usually hidden, and their presence overlooked. Likewise, these structures might also be involved in the excretion of substances possibly involved in the establishment of a microbial mycorhizosphere. Such hypotheses, although not sustained at present, would merit in-depth investigation.

Photomicrographs in Fig. 3a–j illustrate extraradical hyphal structures formed by two AM fungi as they develop in soil containing vermiculite particles. The latter semi-transparent substrate is organized in thin layers, which allows the fungus to develop three-dimensionally between them.

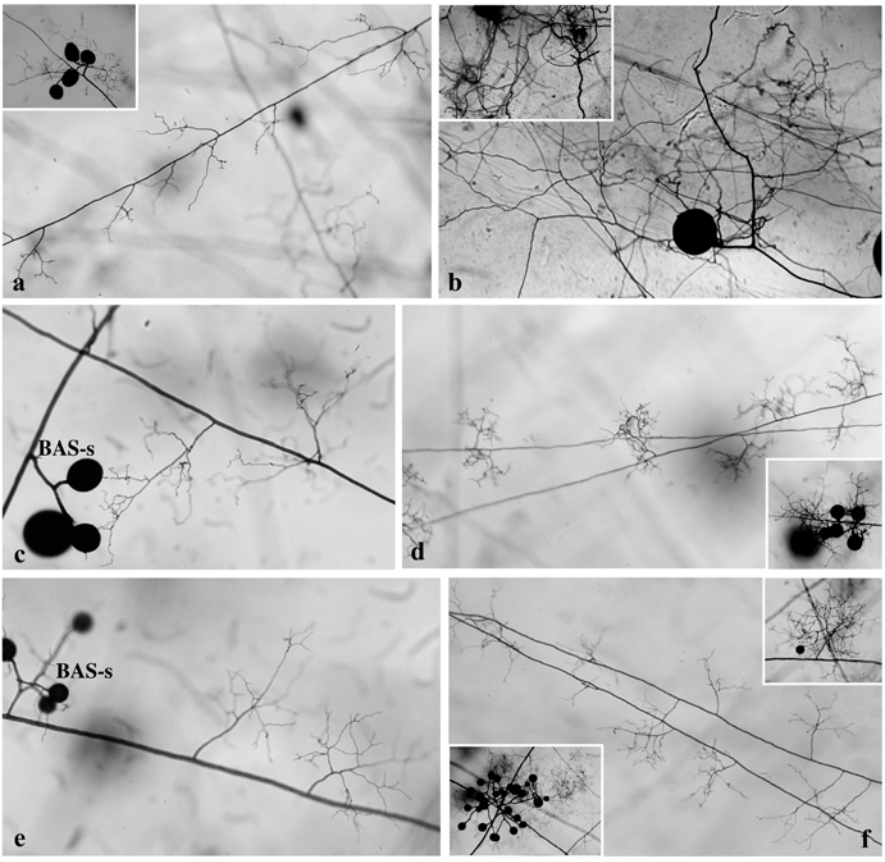


Fig. 2a–l. Branched absorbing structures (BAS, BAS-s) formed by extraradical hyphae of six different AMF grown under monoxenic conditions with a carrot Ri T-DNA ROC (DC-2 clone). **a** *Glomus intraradices* DAOM 181602. **b** *Glomus etunicatum* CIMA 07. **c** *Glomus* sp. MUCL 43195. **d** *Glomus proliferum* MUCL 41827. **e** *Glomus cerebriforme* DAOM 227022. **f** *Glomus* sp. DAOM 227023. **g** *Glomus* sp. CIMA 09. **h** *Glomus* sp. CIMA 10. **i** *Glomus* sp. CIMA 12. **j** *Gigaspora margarita* BEG34. **k** *Gi. margarita* CIMA 05. **l** *Gi. margarita* CIMA 11. While presenting similar general morphogenetic and developmental features (i.e. ephemeral short structures with dichotomous branching pattern formed by runner hyphae at regular intervals), BAS from different fungi differ from each other, which might be of taxonomic interest. In all the cases studied, spore-BAS were observed (**a**, **d**, **f**–**h**, *insets*; **c**, **e**, **i**, *BAS-s*), except for the *Gi. margarita* isolates, where auxiliary cells preferentially developed at the BAS trunk (**j**, *arrow in inset*; **k**, **l**, *arrows*). *G. etunicatum* CIMA 07 presented the most differential developmental pattern (**b**) in which BAS were difficult to observe due to frequent anastomoses of thin branches (**b**, *inset*) (*continued on next page*)

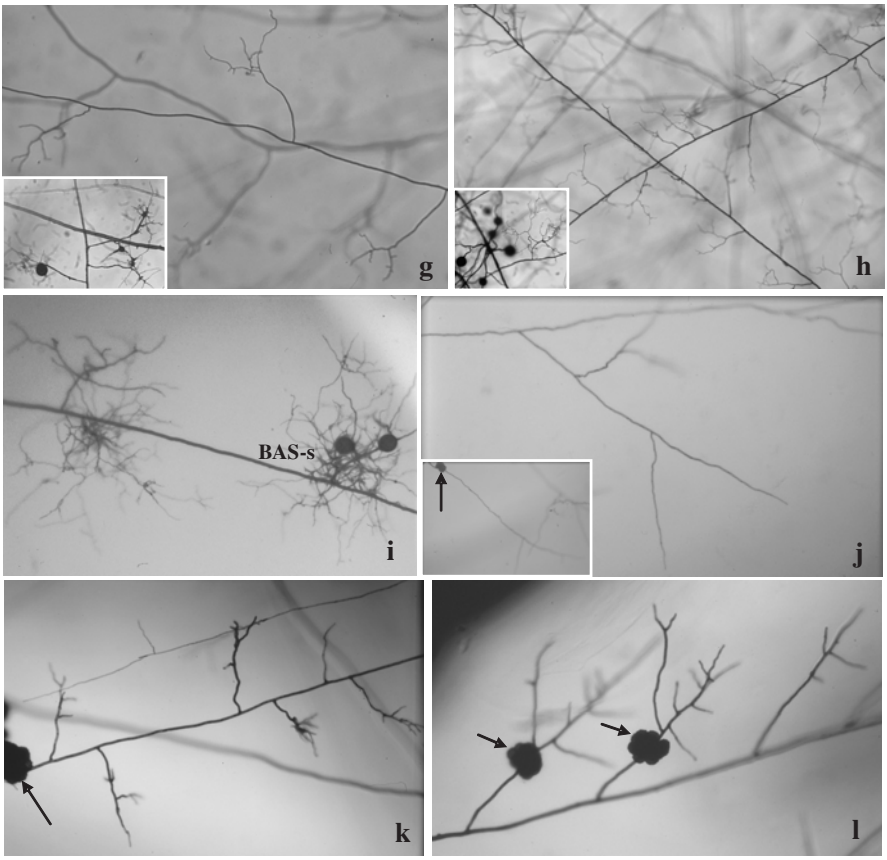


Fig. 2a–l. (*continued*)

Such layers seem to be suitable for BAS to be formed, thus allowing us to show, for the first time, their actual occurrence under ex vitro conditions. Interestingly, preferential sporulation between vermiculite layers has been observed (Vidal et al. 1990; C. Cano, pers. observ.; Fig. 3f, g), suggesting that some or most BAS formed there could, in fact, be spore-BAS.

Arbuscules and BAS do share some features (Bago 1998; Bago et al. 1998b), and this leads us to propose (and this may appear scandalous to some mycorrhizologists) that they are homologous structures, and bipolar extremes of one and the same AM fungal colony; if this were true, then some arbuscules should reflect intraradically the occurrence of spore-BAS extraradically, by supporting vesicle formation on their branches. This is indeed the case, as shown, also for the first time, in Fig. 3k. Further observations should confirm the frequency for such "spore-arbuscules" to occur within the root; nevertheless, their actual presence gives some additional

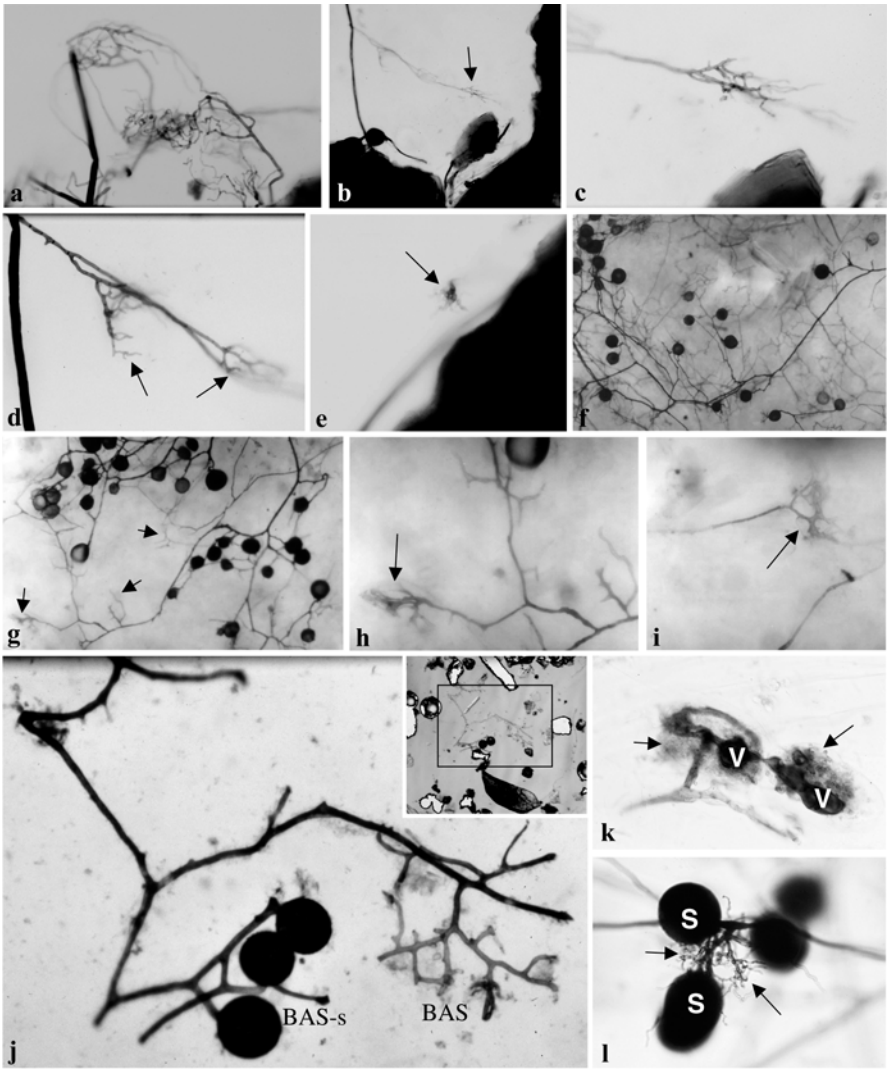


Fig. 3a–l. Occurrence of branched absorbing structures ex vitro, in either soil, or soil-like substrates and under greenhouse or natural conditions (photographs in **f–i** were taken by C. Cano together with C. Azcón-Aguilar). **a–e** *Glomus intraradices* DAOM 181602 grown in soil:vermiculite:sepiolite (1:1:1). **f–i** *G. viscosum* EEZ 34 in sand:vermiculite (1:1). **j** *G.* sp. CIMA 12 extraradical hyphae isolated from natural soil. In all cases, BAS are indicated by *arrows*. **k–l** Comparison between arbuscule-forming vesicles (**k**) and spore-BAS (**l**). Note the striking morphological and developmental similarities of both structures, suggesting their homologous origin and confirming once more the AM fungal colony bipolarity. *Arrows* Arbuscule or BAS thin branches, *V* vesicle, *S* spore

validation to the above-mentioned hypothesis (cf. Fig. 3k, intraradical, with Fig. 3 l, extraradical). Moreover, it is interesting to note here that members of the Gigasporaceae family typically do not form intraradical vesicles; in parallel, BAS of such isolates never support spore formation. However, many of the BAS produced by the *Gigaspora* species formed auxiliary cells at the BAS trunk (Fig. 2j, inset, Fig. 2 l). A recent study has shed some light on the formation and development of auxiliary cells in the Gigasporaceae family (Declerck et al. 2004). More research is needed to reveal their real physiological role, and to understand their possible relationship to BAS.

2.5 Are There Any Differences in AM Fungal Development in Monoxenics Versus Soil?

While insisting that AM monoxenic cultures are valid experimental systems to study AM fungal biology, we cannot deny that highly controlled in vitro conditions could somehow affect fungal development. This is in fact the case for all in vitro-cultured micro-organisms, as they develop in nutrient-supplied, homogeneous agar media under optimal environmental conditions. Concerning AM fungi, observations by Pawlowska et al. (1999) and Dalpé (2001) indicate that monoxenically produced spores may be smaller and less pigmented than soil-borne spores (Fortin et al. 2002). We have also observed that a differential response to Melzer's staining usually occurs in soil versus monoxenically raised spores (Fig. 4a, b, e, f). Perhaps related to this, an important reduction in spore wall thickness is noted under monoxenic conditions (Fig. 4c, d, g, h). The latter could be observed at first as a frightening result, since one may think that monoxenically produced spores are weaker than those obtained from soil. However, this is absolutely not the case – monoxenically produced propagules have been shown to be even more effective in colonizing either seed-raised or micropropagated plants under greenhouse conditions than soil-raised inoculum (Vimard et al. 1999; Filion et al. 2001; Declerck et al. 2002; Jaizme-Vega et al. 2003; C. Cano, pers. observ.), and such material was shown effective to improve growth of plants under greenhouse conditions. Interestingly, tests performed in our laboratory indicate that AM fungi adapt to existing environmental conditions surprisingly quickly. Plants inoculated with monoxenically obtained, thin-walled spores and cultured in soil under greenhouse conditions produced a new generation of thick-walled AM fungal spores (C. Cano and B. Bago, unpubl. data). Such a result indicates once more the incredible plasticity of fungi; AM fungi in particular have been demonstrated to quickly adapt to a changing environment while preserving the integrity of the fungal colony (Bago et al. 2004b).

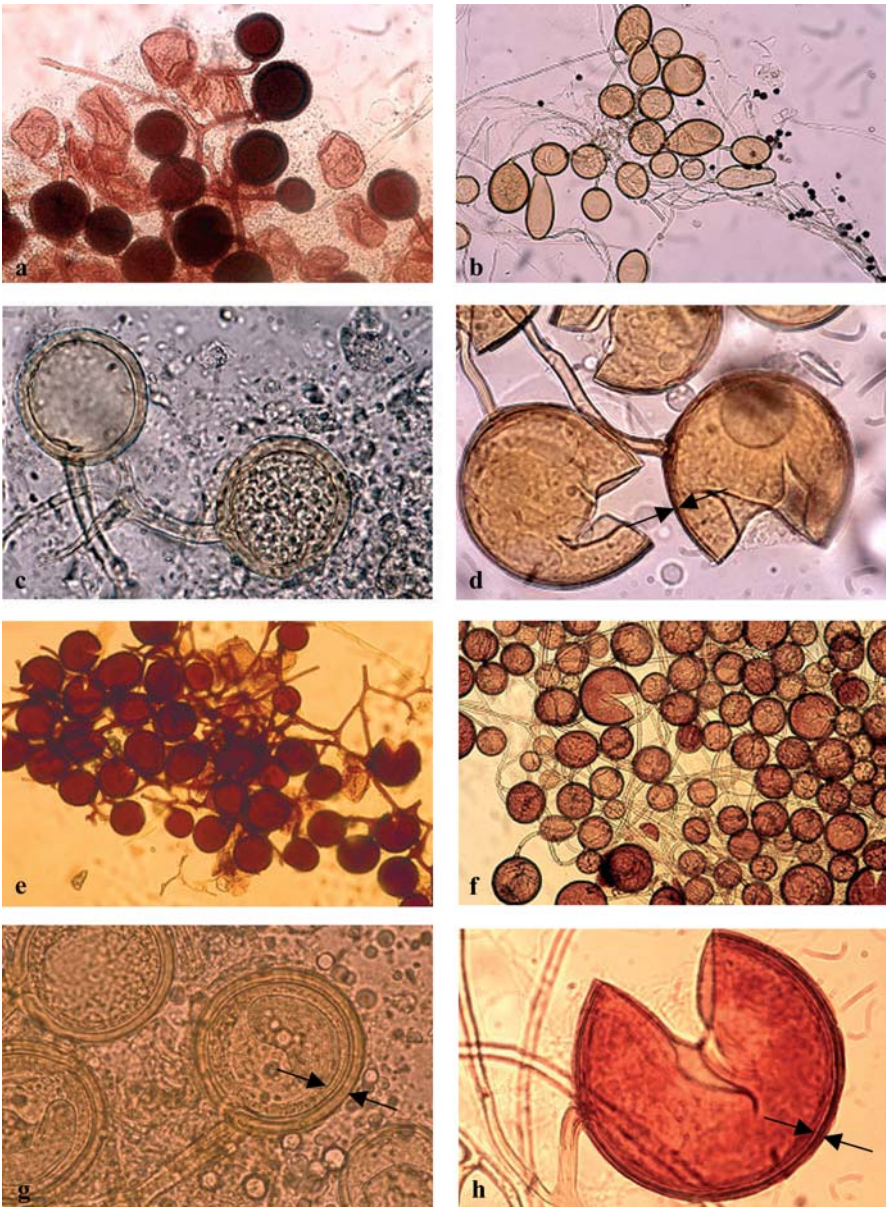


Fig. 4a–h. Comparison between spores of *Glomus intraradices* DAOM 181602 (**a–d**) and *G.* sp. CIMA 12 (**e–h**) produced either in soil (**a, c, e, g**) or under monoxenic cultures (**b, d, f, h**). Spores were extracted from the substrate and treated with either Melzer's reagent (**a, b, e, f, h**) or PVLG (**c, d, g**). Note the intense Melzer's staining of soil-borne spores compared to those obtained from monoxenic cultures (**a** vs. **b**, and **d** vs. **e**). This could be due at least partially to the dramatic reduction in cell wall thickness of soil- compared to monoxenically raised spores (**c** vs. **d**, and **g** vs. **h**, *arrows*)

Thus, careful observations of AM fungal developmental features should be performed in monoxenics in parallel to any physiological or molecular tests we may want to carry out. Fungal morphogenesis is a good indicator of any fungal response, and it is important to have eyes wide open during data interpretation to avoid misleading conclusions. Only on this basis will monoxenic cultures be of value for the advance of our understanding of AM symbioses.

2.6 Are AM Monoxenic Liquid Cultures Accurate?

As indicated above, more and more researchers now have a different perception of AM monoxenic cultures, and consider this experimental system as an extremely useful tool to work with, especially for fine research such as biochemistry and molecular biology of the AM symbiosis. Consequently, modifications of the original monoxenic culture described by Bécard and Fortin have been developed. A clear example of this is the bi-compartmented system (St-Arnaud et al. 1998), allowing the physical separation of extraradical hyphae from the influence of roots, or the multi-compartmented system (Bago et al. 2004b), which allows the testing of the physiological abilities of different parts of a single AM fungal colony growing on spatially heterogeneous media. Another modification of the system, which is now being widely used, is the replacement of the solid medium in the hyphal compartment by a liquid medium. Such a modification was first reported by Maldonado-Mendoza et al. (2001), and is extremely useful for pulse-chase experiments.

Nevertheless, and taking into account the warning notice expressed at the end of the previous section, when observing morphogenesis of extraradical hyphae growing in liquid culture, dramatic morphological changes are noted (Fig. 5). Indeed, the regular pattern of development, with runner hyphae extending from the fungal colony radially, and producing BAS at regular intervals (Fig. 5a, b), is lost in liquid cultures, in which runner hyphae are predominant (Fig. 5c, d) and BAS are scantly and appear much less branched (Fig. 5e). Zones of fine hyphal networks, which could be the consequence of multiple BAS anastomoses, appear instead (Fig. 5f), and on these preferential sporulation seems to take place (Fig. 5g, h). Liquid-growing fungal hyphae are extremely fragile, and it is common to inadvertently stop development of extraradical mycelia simply by slightly moving the Petri plate.

It would be important to make sure that the important morphological changes induced by liquid media do not affect the extraradical fungus either cytologically or functionally. For instance, one may think that under liquid

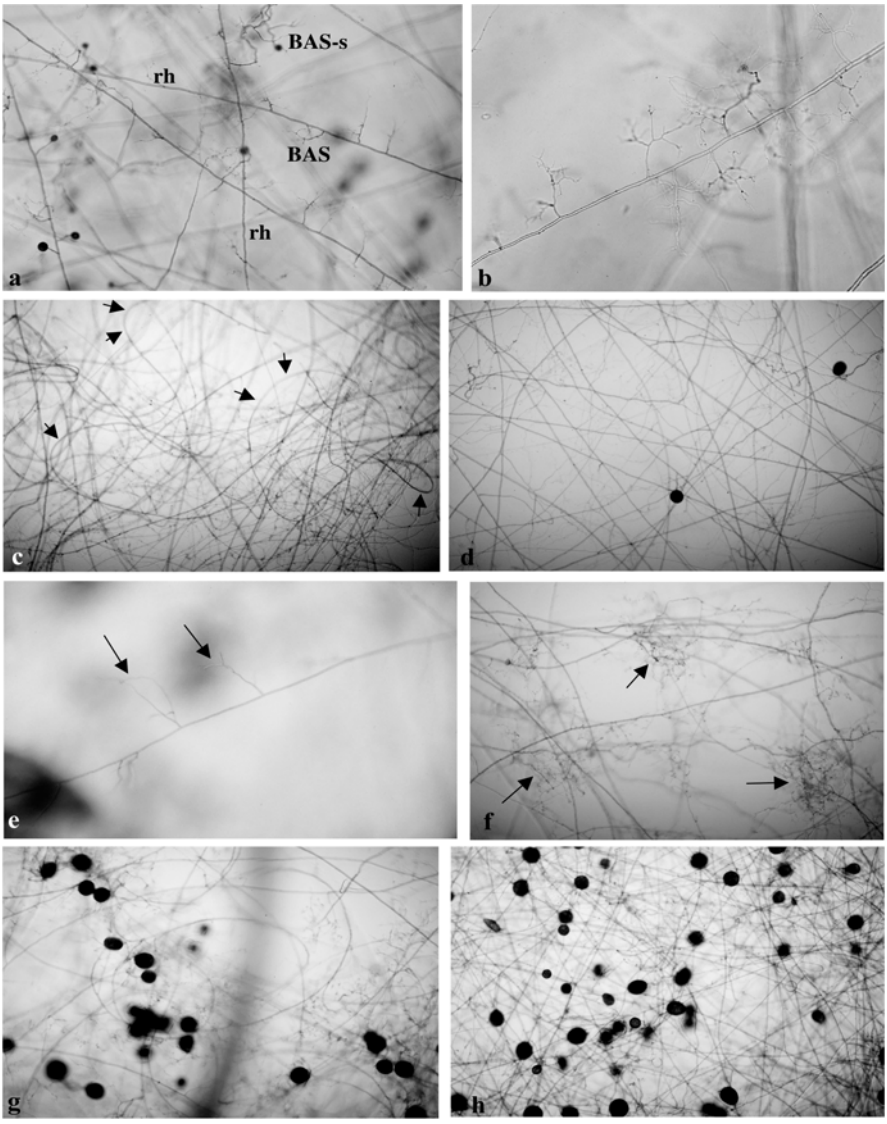


Fig. 5a–h. Developmental features of the extraradical mycelium of *Glomus intraradices* DAOM 181602 growing monoxenically in hyphal compartments containing either solid (**a**–**b**) or liquid (**c**–**h**) M-C culture media. When developing in solid medium, the extraradical AM fungal mycelium consists of straight, leading runner hyphae (*rh*) supporting differentiated *BAS* or *BAS-s* at regular intervals. However, the liquid medium modifies such patterns: runner hyphae become more generalized and prominent (**c**, **d**), and bend easily in the liquid medium (**c**, *arrows*). BAS are formed, but they are less profusely branched (**e**, *arrows*). Disorganized branching events become frequent in the extraradical mycelium (**f**, *arrows*), and seem to be preferential sites for spore formation under these special conditions (**g**). Sporulation is in no case affected by the liquid state of the medium (**h**)

conditions transport proteins would be distributed differently, that cell wall structure would adapt to the new environment, and that gene expression could be consequently affected. We are not challenging the validity of liquid cultures at all; we just warn researchers once more about the importance of considering their working material before just collecting and using it. Much additional information could be obtained, and many misleading results avoided by following this simple practice. On the other hand, the application of cutting edge techniques such as suppression subtractive hybridizations (SSH, Diatchenko et al 1996; Gianinazzi-Pearson et al. 2004), to test whether morphological changes in AM fungi indeed reflect changes in their gene expression, would be of great use.

2.7 The Danger of Contamination in AM Monoxenic Cultures

As is the case for all in vitro cultivation techniques, undesired contamination (either of fungal or bacterial origin) is the most important handicap for AM monoxenic culturing. Adequate manipulation knowledge, exhaustive contamination screening, and immediate removal of the affected plates are sine qua non conditions for any laboratory using AM monoxenic cultures. Even so, the occurrence of some contamination is unavoidable, but this should never exceed 5% as a maximum to ensure reliability of the manipulator. Our experience indicates that, in most cases, just one or two types of contaminating fungi, and two or three different contaminating bacteria appear in AM monoxenic cultures. Preliminary fatty acid profiling analysis of these (Larsen, Cano and Bago, unpubl. data) resulted in the identification of two bacterial isolates, a *Paenibacillus* sp. and a *Bacillus subtilis*. Interestingly, both of these bacteria are well-known PGPRs; moreover, another *Paenibacillus* isolate (*P. validus*, Hildebrandt et al. 2002) has been recently pointed out as an important inducer of morphological differentiation in *G. intraradices*. More research is needed because of the consistent presence of these bacteria in AM monoxenic cultures, which is linked somehow to the Mugnier and Mosse (1987) claim that the presence of some "helper bacteria", may be necessary to succeed in culturing certain AM fungi. In any case, the development of molecular kits for early detection of bacterial contaminations in AM cultures would probably be of great interest, especially for high-quality, certified in vitro AM inoculum production.

2.8 What Else Have Monoxenic Cultures to Offer on the Study of AM Fungal Biology?

Figure 6a shows a general view of AM extraradical mycelium as it develops monoxenically on a hyphal compartment. Here, it is easy to distinguish two phases in the development of extraradical hyphae (separated by the dashed line in the photograph). During the "absorptive phase" (Fig. 6a, ABS), BAS are the predominant fungal structure. This phase is at the forefront of the fungal colony, and it is easy to relate it with the substrate-scavenging abilities shown by AM fungi. Behind this absorptive phase, and probably formed after a given signal which might well be the arrival of storage lipids at important rates (Bago et al. 2002, 2003), we found the sporulative phase (Fig. 6a, SP). On this, the most frequent structures found are spore-BAS and spores which are formed in sporulation waves, according to sporulation dynamics described for AM and most other fungi (Bago et al. 1998; Declerck et al. 2001). It is important to stress here that genes of each of those two developmental phases (i.e. absorptive and sporulative) probably express differentially, according to the stage of hyphae, and therefore according to the younger versus older parts of the colony. As a consequence, caution should be taken when using extraradical hyphae for molecular biology studies – one must be sure of the physiological and developmental situation of the extraradical mycelium in order to avoid a mixture of gene expressions which may lead, once more, to misinterpretation of the obtained results.

Undoubtedly, there are still many surprises reserved for us concerning AM fungal biology. Monoxenic cultures could well be ideal tools for further advances in such knowledge. To conclude this chapter, we would like to show some "strange" growing patterns usually found in monoxenic cultures, but rarely reported due to the difficulty in identification and/or explanation.

2.8.1 Interwoven Hyphae of *Glomus Intraradices* Forming Sporocarp-Like Structures

Such a morphological event (Fig. 6b–f) occurs quite frequently at late developmental stages of monoxenically grown *G. intraradices*. It consists of a sudden re-growth of thin, newly formed hyphae emerging from a given point of a hypha on which, quite frequently, an anastomosis has taken place (Fig. 6b arrow). Such long, thin and tortuous hyphae interweave, giving rise to what we could name "hyphal knots" (Fig. 6c), which grow more compact (Fig. 6d) to support sporulation at latter stages (Fig. 6e), being at the end extremely reminiscent of sporocarps (Fig. 6f). Since the occurrence

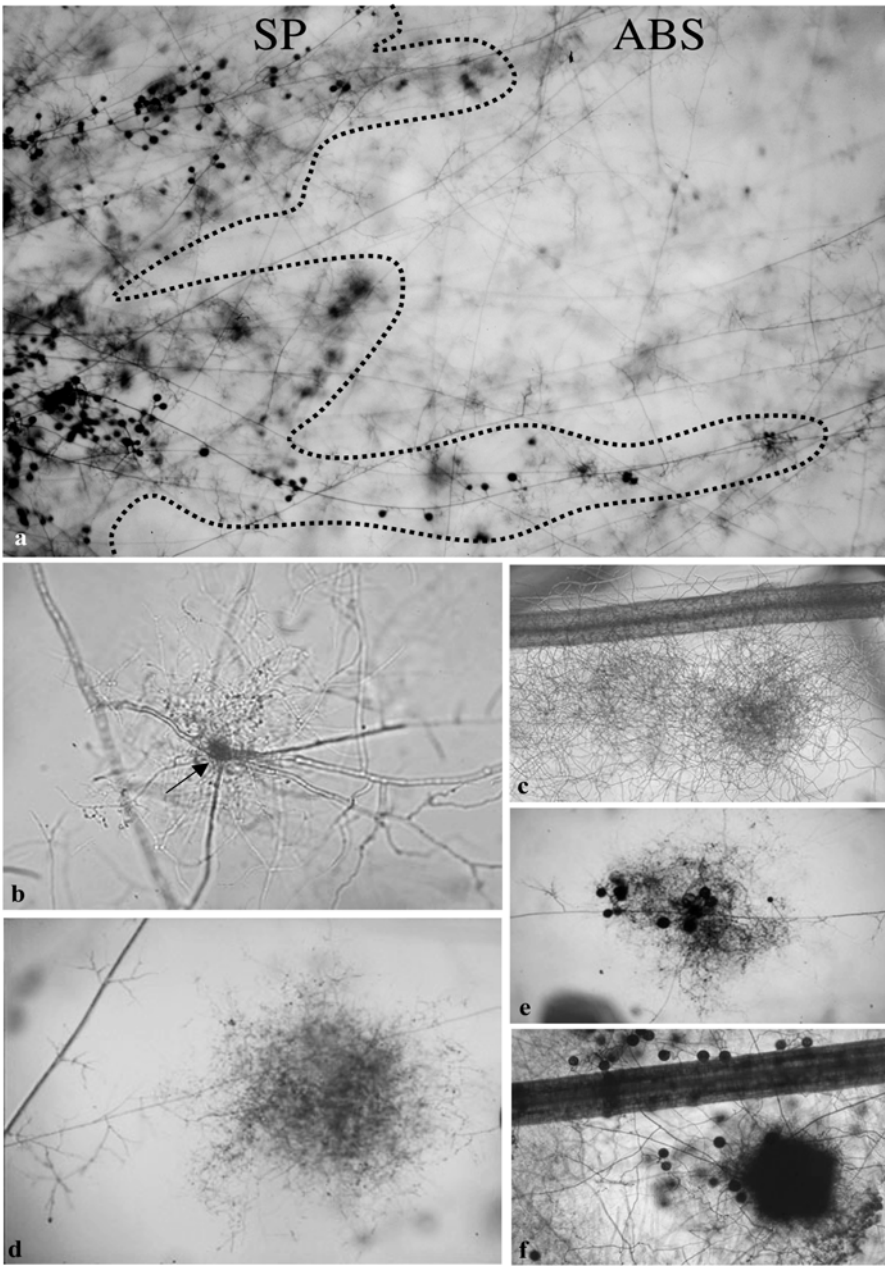
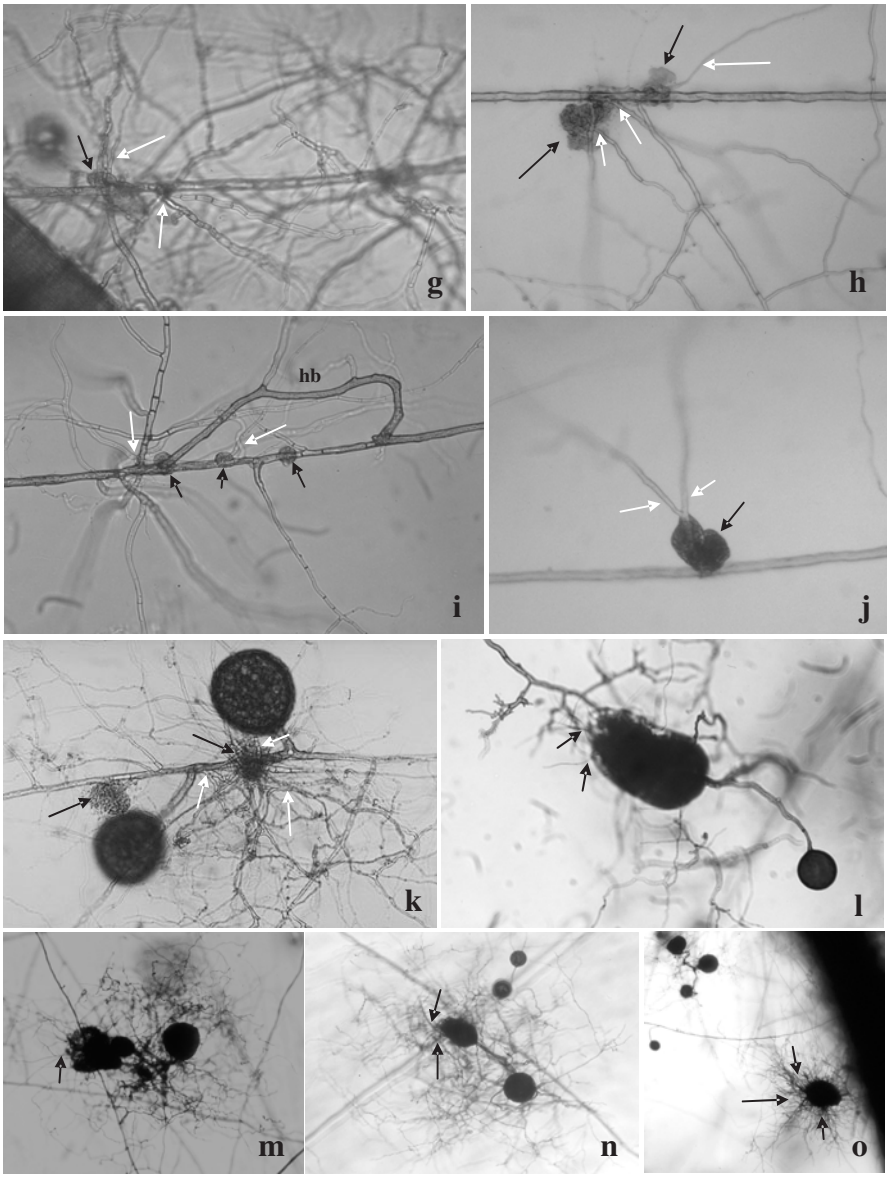


Fig. 6a–o. Morphogenetic events of interest in mature (**a**) and late (**b–o**) stages of AM fungal extraradical mycelium as revealed by monoxenic cultures. **a** Transition from absorptive (*ABS*) to sporulative (*SP*) phases. Note the well-differentiated limit between both developmental stages, which allows one to easily follow the "sporulation wave" undergone by the fungal colony. **b–f** Hyphal knots formed by the extraradical mycelium, usually after an anas-



tomosis event (**b**, *arrow*): hyphae interweave and curl progressively (see transition from **b** to **f**) to finally form a sporocarp-like structure in which preferential sporulation occurs (**f**). **g–k** "Protrusion-and-re-growing" events in old extraradical AM hyphae. Hyphae burst at given points with no apparent cause, and frequent cytoplasmic protrusion occurs (*closed arrows*); from these same points, newly formed, thin hyphae re-grow radially (*open arrows*). Occasionally, hyphal bridges are formed to bypass the affected zone (**i**, *hb*). **l–o** "Protrusion and re-germination" events in mature spores. Spores burst at their distal pole with cytoplasm loss, while newly formed, thin hyphae re-grow from the exploded sites (*arrows*)

of sporocarps in *G. intraradices* has never been described under either greenhouse or natural conditions, we may conclude that (1) the observed structure has a different function than sporocarps, and simply resembles them, or (2) *G. intraradices* has the potential to form sporocarps, but such a potential is rarely used under the experimental/natural conditions studied up to now.

2.8.2 Protrusion and Re-Growing Events

These processes usually occur in older parts of the fungal colony as well. They consist of a burst of a runner hypha in non-apical zones, with protrusion of cytoplasmic material (Fig. 6g–k, closed arrows), which is sometimes followed by "hyphal bridging" (Gerdemann1955; Mosse 1988) events (Fig. 6i). Either from, or at the protruded portion of hyphae, new thin hyphae re-grow (Fig. 6g–k, open arrows), usually in a quite unorganized manner. The most extreme situation of these protrusion and re-growing events is exploded spores (Fig. 6l–o). In this case, it is the distal pole of a given spore which bursts, liberating parts of its content from which new, thin, interwoven hyphae develop (Fig. 6l–o, arrows). This protrusion and re-growth in spores has been noted in all the AM fungal isolates revised, but its real cause/significance remains absolutely obscure to us. It is important to stress here that there is no indication that the bursting and protrusion of either hyphae or spores are a consequence of contamination of the monoxenic cultures by exogenous bacteria – contaminated plates are routinely discarded and never used in our studies.

2.8.3 Are There Sexual Processes Waiting to Be Described in AM Fungi?

Unfortunately, we have no pictures to answer this controversial question; it is, nevertheless, tempting to speculate that, similarly to the fact that there are morphogenetic processes which had never been described to occur in AM fungi before, there might be other significant processes waiting to be described. Could it be that the hyphal knots formed from an anastomosis are in fact the result of a genetic exchange of nuclei within a given hypha? Reports by several authors (Bago et al. 1999a; Giovannetti et al. 1999) seem to support such a possibility since, in very close hyphal tips, nuclei seem to sense and attract each other. Nevertheless, recent reports suggest that no sexual exchange occurs in AM fungi (Sanders 1999; Pawlowska and Taylor 2002), although such observations remain to be fully confirmed. Hopefully, new powerful techniques (molecular/microscopy) will answer

such questions – no doubt monoxenic cultures will be, once again, the selected experimental system to carry out such studies.

3 Conclusion

In this chapter, we tried to give a brief overview on subjects of potential interest for those using, or willing to use, AM monoxenic cultures. This experimental system has already opened, and can further open in the future, new avenues in our knowledge and understanding of AM symbiosis; it has also uncalculated potential as an inoculum source for either scientific or commercial purposes. Nevertheless, only by following some simple common sense rules, monoxenic cultures will retain liability and appropriateness for these purposes. Therefore, caution is needed not to misuse a system which, we could say, has promoted a quiet revolution in the fascinating research field of arbuscular mycorrhizas.

Acknowledgements. We thank the following researchers for providing the different AM monoxenic mother cultures indicated: Julie Samson for *Glomus cerebriforme* DAOM 227022 and *G.* sp. DAOM 227023; Stéphane Declerck for *G. proliferum* MUCL 41827 and *G.* sp. MUCL 43195; and Guillaume Bécard for *Gigaspora margarita* BEG34.

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