

Chapter 2

Functional Significance of Anastomosis in Arbuscular Mycorrhizal Networks

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Abstract Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs (Glomeromycota), which live symbiotically in the roots of most land plants and facilitate mineral nutrition of their hosts. Their spores are able to germinate in the absence of host-derived signals, but are unable to complete the life cycle without establishing a functional symbiosis with a host plant. Such behaviour did not represent a selective disadvantage, as a result of diverse survival strategies allowing them to compensate for the lack of host-regulated germination and to overcome their obligate biotrophic state. The ability to form hyphal fusions (anastomoses) between compatible mycelia may represent an important mechanism evolved by AMF to increase their chances of survival, since fungal germlings can plug into pre-existing extraradical mycelial networks, thus gaining immediate access to plant-derived carbon before asymbiotic growth arrest. In fusions between hyphae of the same or different individual germlings of the same isolate, perfect anastomoses occur with the highest frequency and are characterized by protoplasm continuity and complete fusion of hyphal walls. High anastomosis frequencies are also detected between extraradical mycelial networks produced by the same isolate, spreading from plants of different species, genera and families. Pre- and post-fusion incompatibility are often observed in hyphal interactions between asymbiotic and symbiotic mycelium and between genetically different germlings belonging to the same isolate, while pre-fusion incompatible responses, hindering hyphal fusions, occur between germlings of geographically different isolates. The analysis of vegetative compatibility/incompatibility during hyphal fusions represents a

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valuable tool for genetic studies of AMF, which are recalcitrant to axenic cultivation. Molecular analyses of the progeny of mycelium derived from nonself vegetative fusions of genetically different germplings of *R. irregularis* showed that genetic exchange occurs, despite low anastomosis frequencies and post-fusion incompatible responses, suggesting that anastomosis between genetically different mycelia may represent a recombination mechanism in the absence of an evident sexual cycle.

Keywords Mycorrhizal networks • Network nutrient transfer • Hyphal anastomosing ability • Hyphal recognition • Non-self hyphal compatibility

2.1 Introduction

The majority of land plants establish mutualistic symbioses with arbuscular mycorrhizal (AM) fungi (AMF), a group of beneficial soil microorganisms fundamental for plant nutrition and ecosystem biodiversity and productivity, affecting the composition of plant communities in terms of survival, competition and diversity of plants (van der Heijden et al. 1998; Wardle et al. 2004; Smith and Read 2008). AMF belong to the Glomeromycota, are obligate biotrophs and colonise the roots of their host plants obtaining sugars, which they are not able to synthesize. In exchange, the host plants receive mineral nutrients, absorbed and translocated through a fine network of extraradical hyphae extending from the roots to the surrounding soil. Such belowground networks represent the key structure for soil nutrient uptake and transfer to the roots, and are thought to have played a fundamental role in land colonisation by early terrestrial plants, which, lacking an extended root system, could have been facilitated by the AMF symbionts functioning as an auxiliary absorbing structure (Pirozynski and Malloch 1975). Fossil records and molecular data have supported such a view, considering AMF as evolutionarily successful living fossils, having survived 460 to possibly 980 million years (Simon et al. 1993; Remy et al. 1994; Phipps and Taylor 1996; Redecker et al. 2000; Blair 2009).

Mycorrhizal networks spreading from colonised roots into the surrounding soil represent the structure where the flow of nutrients is realized. Such a flow consists of a bidirectional flux of mineral nutrients, mainly P, N, Cu, Fe, K, Zn (Smith and Read 2008), from the soil to the host plant, and of sugars acquired by intraradical hyphae and transferred to other fungal structures in the soil, i.e. mycelium and spores. Moreover, mycorrhizal networks are of fundamental importance for plants, since they can grow indefinitely in every direction, foraging for soil nutrients far from the roots with high efficiency, given the very fine dimensions of hyphae (5–10 μm diameter).

Data on the mechanisms of absorption of mineral nutrients, in particular phosphate, confirmed the key role played by mycorrhizal networks in plant nutrition. Phosphorus can be absorbed in the soil-plant interface by both root hair and

epidermal cells and in the soil-fungus interface by fungal hyphae that transfer P to root cells in the root cell-fungus interface (Karandashov and Bucher 2005). Molecular studies show that genes for phosphate uptake are differentially expressed in extraradical hyphae (Harrison and van Buuren 1995; Maldonado-Mendoza et al. 2001; Casieri et al. 2013), and that the mycorrhizal network is structurally and functionally able to capture high quantities of phosphate from the soil (Smith et al. 2003).

A reverse flow of sugars occurs from host plants to fungal symbionts. The amount of C, obtained from the host plant and transformed by the fungal symbiont into trehalose and other polyols may reach 20 % of total photosynthate, depending on different plant-fungus combinations (Jakobsen and Rosendahl 1990).

Since AMF have a wide host range, mycorrhizal networks can simultaneously colonise diverse root systems, interconnecting plants belonging to the same and different species, genera and families (Eason et al. 1991; Lerat et al. 2002; Giovannetti et al. 2004). Thus, common mycorrhizal networks (CMNs) represent the physical structures through which carbon, mineral nutrients and water can move from plant to plant (Johansen and Jensen 1996; Egerton-Warburton et al. 2007; Simard et al., Chap. 5, this Vol.), allowing plants to share ecosystem resources which may modify and/or facilitate plant coexistence.

The occurrence of AMF mediated interplant C transfer, requiring a net flux of C from the fungal symbiont to the host, was reported in some mycoheterotrophic plants (Bidartondo et al. 2002), while C allocation from one green plant to another through AMF mycelial networks is much more controversial. Early findings suggesting C transfer between plants through AMF hyphae (Hirrel and Gerdemann 1979; Francis and Read 1984; Grime et al. 1987; Martins 1993) were followed by other reports that showed the occurrence of interplant C flow, but pointed out that transferred C remained in fungal root tissues without moving into the shoots (Watkins et al. 1996; Graves et al. 1997; Fitter et al. 1998). Some findings supported the view of an exchange of C between plants, at least in particular conditions (Lerat et al. 2002; Carey et al. 2004), while others, utilising *in vitro* mycorrhizal root organ cultures or plants, further confirmed that C originating from a donor plant was retained in fungal cells (Pfeffer et al. 2004; Voets et al. 2008; Lekberg et al. 2010).

It has been suggested that N and P can move from a plant to another through mycorrhizal networks (Whittingham and Read 1982; Haystead et al. 1988). Studies on N fixing plants utilizing ^{15}N showed that AMF mediated N transfer may occur (Frey and Schüepp 1992; Martins and Cruz 1998), although also indirect pathways may be significant (Ikram et al. 1994; Rogers et al. 2001). However, a laboratory experiment that utilized two plant compartments linked only by AMF hyphae and separated by an air gap, confirmed N transfer together with transfer of analogs of P and K (Meding and Zasoski 2008). Direct interplant P transfer through hyphal connections, suggested by early field and laboratory studies (Chiariello et al. 1982; Francis et al. 1986), was not confirmed by other experiments, suggesting that the observed flow could result from the release of P from donor roots into the soil or to the mobilization of nutrients from a dying donor plant (Newman and Ritz 1986; Newman and Eason 1989, 1993; Johansen and Jensen 1996). An elegant experimentation using

^{32}P as a tracer confirmed belowground P transfer from donor to receiver plants mediated by interconnected mycorrhizal networks (Mikkelsen et al. 2008). Recently, a differential allocation of P and N to plant hosts either belonging to diverse species or with high/low C source strength was demonstrated for CMNs formed by different AMF isolates (Fellbaum et al. 2014; Walder et al. 2015).

Mycorrhizal networks interconnecting different plants may function as plant-plant underground communication ways, allowing signals transfer among plants and activating defence pathways before pathogen attacks. For example, tomato plants connected by *Funneliformis mosseae* (formally *Glomus mosseae*) CMNs showed increased expression of defence-related genes and higher levels of disease resistance enzymes in healthy “receiver” plants after inoculation of ‘donor’ plants with the pathogen *Alternaria solani* (Song et al. 2010). In addition, aphid-free *Vicia faba* connected to aphid-infested plants via a CMN showed aphid repellence and aphid enemy attraction due to systemic changes in the production of volatiles (Babikova et al. 2013). CMNs are also able to widen the action of allelochemicals in natural environments and to affect interactions within plant communities (see Jakobsen and Hammer, Chap. 4, this Vol.), as plant-derived allelopathic substances and herbicides supplied to mycorrhizal plants may be transferred to the target plant, leading to their accumulation at levels that cannot be reached by soil diffusion (Barto et al. 2011; Achatz et al. 2013). In addition, CMNs may allow water flux between interconnected plants, facilitating plant survival during drought (Egerton-Warburton et al. 2007).

Further studies aimed at detecting and quantifying mineral nutrient and carbon transfer in the fungal network could improve our understanding of its functional significance and of the role played by AMF in the distribution of resources in plant communities. Moreover, since mycorrhizal networks may also represent a channeling system for a wide exchange of information molecules between plants, they appear to play a fundamental role in the dynamics and evolution of the complex network of interactions regulating ecosystem functioning.

In this chapter we will review the developments which contributed to give a picture of mycorrhizal networks as one of previously unimagined dynamism. We will discuss the structure of AMF networks, the cellular events leading to anastomosis formation, and the phenomenon of self/nonself recognition and nonself incompatibility between hyphae belonging to the same and to genetically different AMF.

2.2 Structure of Mycorrhizal Networks

The structure, viability, extent and interconnectedness of AMF mycelium are of critical importance for the establishment and maintenance of the flow of nutrients from soil to plant roots and were investigated by many authors. Using a destructive approach, the extent of AMF networks was estimated to range from 2.7 to 20.5 m/g of soil, depending on the fungal species (Giovannetti and Avio 2002; Mikkelsen et al. 2008).

Some non destructive observations of AMF extraradical mycelium (ERM), carried out by using root observation chambers (Friesse and Allen 1991) and in vitro dual systems (Bago et al. 1998a), provided qualitative information on its architecture and development before and after symbiosis establishment. A non-destructive in vivo bi-dimensional experimental system (sandwich system), allowed the visualization and quantification of the whole intact AMF network produced by the AM fungus *F. mosseae* living in symbiosis with three different plant species: *Allium porrum*, *Thymus vulgaris* and *Prunus cerasifera* (Fig. 2.1). After 7 days' growth the length of ERM spreading out from root-based hyphae into the surrounding environment ranged from 5169 mm in *T. vulgaris* to 7471 mm in *A. porrum*, corresponding to 10 and 40 mm mm⁻¹ root length, respectively. The mean growth rate was 738–1067 mm d⁻¹, depending on the host plant (Giovannetti et al. 2001). By contrast, in a tri-dimensional soil system lower values were detected, ranging from 3.1 to 3.8 mm d⁻¹ for *F. mosseae* and *F. caledonius* ERM spreading from *Trifolium subterraneum* mycorrhizal roots (Mikkelsen et al. 2008).

Besides ERM extent, fungal biomass is important for the role played by mycorrhizal networks in the transfer of C from atmosphere to soil (Bago et al. 2000; Treseder and Allen 2000), since they can supposedly sequester large quantities of organic C in their walls in the form of recalcitrant compounds, such as chitin and

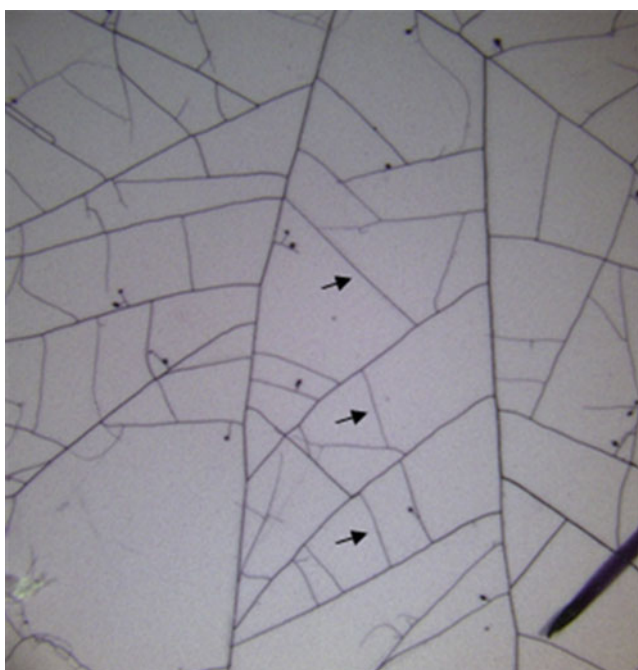


Fig. 2.1 Visualisation of intact extraradical mycelium of *Funneliformis mosseae* spreading from colonised roots of *Allium porrum* showing the network structure realised by means of frequent anastomoses interconnecting nearby hyphae (arrows)

chitosan (Gooday 1990). Specific fungal weights, assessed using different AMF species and experimental systems, ranged from 3.85 to 7.84 $\mu\text{g}/\text{m}$ of mycelium (Bethlenfalvay and Ames 1987; Frey et al. 1994; Miller et al. 1995; Fortuna et al. 2012). The extraradical network of the AM fungus *F. mosseae* IMA 1, visualised by means of a bi-dimensional experimental system, appeared highly branched ($0.86\text{--}0.97$ branches mm^{-1}), while its viability, determined by the localization of formazan salts depositions (SDH activity), was 100 %, after 7 days' growth (Fig. 2.2) (Giovannetti et al. 2001).

The interconnectedness of mycorrhizal networks is the result of fusions (anastomoses) between contacting hyphae. The number of anastomoses in extraradical mycelium ranged between 0.75 per 100 cm of hypha in *Gigaspora margarita* to 0.51 per mm of hypha in *F. mosseae*, whereas fusion frequencies ranged between 0 in *Ambispora leptoticha*, *Gigaspora albida*, *Gigaspora gigantea* and *Dentiscutata heterogama*, to 64 % in *F. mosseae* (Table 2.1). Fusion frequencies showed the highest values in in vivo systems, both in bi-dimensional (up to 64 %) and in tri-dimensional models (up to 37.4 %) (Table 2.1). In some isolates of the AMF species *Rhizoglyphus clarus*, anastomosis frequencies recorded in extraradical (symbiotic) mycelium were different from those observed in hyphae originating from germinating spores (asymbiotic) mycelium (Purin and Morton 2013), whereas slight differences were reported for *F. mosseae* (Giovannetti et al. 1999, 2004). In an in vitro root organ culture system, characterised by high soluble nutrient levels, a low number of anastomoses was recorded, with a maximum of 17 fusions per meter of *Rhizoglyphus proliferus* hyphae, although 100 % of such fusions were between different hyphae in *Rhizoglyphus intraradices*, *R. proliferus* and *Glomus hoi* (de la Providencia et al. 2005; Voets et al. 2006). Networks formed by Gigasporaceae showed no fusions in vivo (Purin and Morton 2011), whereas a low number of anastomoses was produced in root-organ cultures: interestingly, about 95 % of



Fig. 2.2 Visualisation of succinate dehydrogenase activity (SDH) evidenced by formazan salt depositions, showing complete fusions of hyphal walls and the establishment of protoplasmic continuity in anastomosing extraradical hyphae of *Funneliformis mosseae*

Table 2.1 Summary of the different patterns of anastomosis formation across different lineages of arbuscular mycorrhizal fungi during both the symbiotic and asymbiotic stage (a spore germinating without a plant host connection)

Within isolate	Asymbiotic mycelium				Symbiotic mycelium				References	
	PF	PrFI	PFI	NI	PF	PrFI	PFI	NI		
<i>Acaulospora scrobiculata</i> A38	42–72.9	0	0	27.1–58					Barreto de Novais et al. (2013)	
<i>Acaulospora spinosa</i> 95-UFLA	14–32.8	0	0	67.2–86					Barreto de Novais et al. (2013)	
<i>Ambispora leptoticha</i> CR312					0	10.7	-	89.3	Purin and Morton (2011)	
<i>Claroideoglossum (Glomus) etunicatum</i> SCT101A	63.8–75.4	0	0	24.6–36.2					Barreto de Novais et al. (2013)	
<i>Funneliformis (Glomus) caledonius</i> BEG 20	34–55	-	-	-					Giovannetti et al. (1999)	
<i>Funneliformis (Glomus) mosseae</i>										
IMA1	40–60.4	0	0	39.6	44–64	0–8.9	0	36–56	Giovannetti et al. (1999, 2003, 2004), Sbrana et al. (2011)	
IN101C	75.8	0	0	24.2					Giovannetti et al. (2003)	
BEG25	76.7	0	0	23.3					Giovannetti et al. (2003)	
AZ225C	85.1	0	0	14.9					Giovannetti et al. (2003)	
BEG69	72.0	0	0	28					Giovannetti et al. (2003)	
<i>Glomus formosanum</i> A20	79.8–91.4	0	0	8.6–20.2					Barreto de Novais et al. (2013)	
<i>Glomus hoi</i>					5.7/100 cm (100 % bh)				de la Providencia et al. (2005)	
<i>Paraglomus occultum</i> WY112A					0	0	-	100	Purin and Morton (2011)	
<i>Rhizoglossum (Glomus) proliferus</i>					6.6/100 cm (100 % bh)				de la Providencia et al. (2005)	
<i>Rhizoglossum (Glomus) proliferus</i> MUCL 41827					0.172/cm				Voets et al. (2006)	
<i>Rhizoglossum (Glomus) clarus</i>										
MUCL46238	64.2	-	-	-					Cárdenas-Flores et al. (2011)	

(continued)

Table 2.1 (continued)

Within isolate	Asymbiotic mycelium				Symbiotic mycelium				References
	PF	PrFI	PFI	NI	PF	PrFI	PFI	NI	
351-UFLA	47.9	0	0	52.1					Barreto de Novais et al. (2013)
WV101					6.7	32.1	-	61.2	Purin and Morton (2013)
1UnC#7	6.3–8	25*	0*		15.6–37.4	5*	0*		Purin and Morton (2013)
6AmA#2	6.6–19.7				10.3–24.5				Purin and Morton (2013)
CRwest#8	17.4–37.5				11.7–32.4				Purin and Morton (2013)
WV123A#6	13.4–30.3				2.4–5.4				Purin and Morton (2013)
WV123A#7	11.4–18.1				4.7–11.1				Purin and Morton (2013)
WV310#5	20.6–35.7				6.8–17.8				Purin and Morton (2013)
<i>Rhizoglonus (Glomus) intraradices</i>									
LPA 8 (BEG 141)	59	–	–	–					Giovannetti et al. (1999)
IMA 5	69–90	–	–	–					Giovannetti et al. (1999)
MUCL43204					5.4/100 cm (100 % bh)				de la Providencia et al. (2005)
ON201B					13.9	7.5	-	78.6	Purin and Morton (2011)
<i>Rhizoglonus (Glomus) irregularis</i>									
DAOM197198	60	0	0	40					de la Providencia et al. (2013)
DAOM240415	38	0	0	62					de la Providencia et al. (2013)
DAOM234328	55	0	0	45					de la Providencia et al. (2013)
Lineages of MUCL43194	41–78	0	0	-					Cárdenas-Flores et al. (2010)
MUCL41833	89	0	0	-					Cárdenas-Flores et al. (2010)

(continued)

Table 2.1 (continued)

Within isolate	Asymbiotic mycelium					Symbiotic mycelium					References
	PF	PrFI	PFI	NI		PF	PrFI	PFI	NI		
MUCL43194						7.6/100 cm (100 % bh)					de la Providencia et al. (2005)
MUCL43194						0.086/cm					Voets et al. (2006)
A4	48.1	0	0	51.9							Croll et al. (2009)
C2	51	0	0	49							Croll et al. (2009)
C3	47.5	0	0	52.5							Croll et al. (2009)
D1	45.8	0	0	54.2							Croll et al. (2009)
B3	49.4	0	0	50.6							Croll et al. (2009)
<i>Rhizoglyphus (Glomus) manihotis</i> A83	9.3–24.3	0	0	75.7–90.7							Barreto de Novais et al. (2013)
<i>Dentiscutata (Scutellospora)</i> <i>heterogama</i> SN722						0	13.2	-	86.8		Purin and Morton (2011)
<i>Dentiscutata (Scutellospora)</i> <i>reticulata</i>											
EMBRAPA CNPAB1						0.02/cm (94.7 % wh)					Voets et al. (2006)
EMBRAPA CNPAB11						0.79/100 cm (5.2 % bh, 94.8 % wh)					de la Providencia et al. (2005)
EMBRAPA CNPAB11						<1					de Souza and Declercq (2003)
<i>Gigaspora albida</i> URM-FMA 01	0	0	0	100							Barreto de Novais et al. (2013)
<i>Gigaspora gigantea</i> MN414D						0	15.4	-	84.6		Purin and Morton (2011)
<i>Gigaspora margarita</i> BEG 34						0.75/100 cm					de la Providencia et al. (2005)

(continued)

Table 2.1 (continued)

Within isolate	Asymbiotic mycelium				Symbiotic mycelium				References
	PF	PrFI	PFI	NI	PF	PrFI	PFI	NI	
					(9.8 % bh, 90.2 % wh)				
<i>Gigaspora margarita</i> BEG 34					0.009/cm (95.5 % wh)				Voets et al. (2006)
<i>Gigaspora rosea</i>									
BEG 9	0	–	–	–					Giovannetti et al. (1999)
A35	0	0	0	100					Barreto de Novais et al. (2013)
BEG 9					1.2/100 cm (4.2 % bh, 95.8 % wh)				de la Providencia et al. (2005)
<i>Racocetra (Scutellospora)</i> <i>castanea</i> BEG 1	0	–	–	–					Giovannetti et al. (1999)
<i>Scutellospora calospora</i> A80	0	0	0	100					Barreto de Novais et al. (2013)
Between isolates	Asymbiotic mycelium				Symbiotic mycelium				References
	PF	PrFI	PFI	NI	PF	PrFI	PFI	NI	
<i>Funneliformis (Glomus) mosseae</i>	IMA1 × BEG25	0	51	0	49				Giovannetti et al. (2003)
	AZ225C × IN101C	0	49	0	51				
	AZ225C × BEG25	0	46	0	54				
	IMA1 × AZ225C	0	43	0	57				
	AZ225C × BEG69	0	43	0	57				
	IN101C × BEG25	0	36	0	64				
	IMA1 × IN101C	0	33	0	67				
	SY710 × IN101C	0	32	0	68				

(continued)

Table 2.1 (continued)

Between isolates		Asymbiotic mycelium				Symbiotic mycelium	References
		PF	PFI	PFI	NI		
<i>Rhizoglyphus (Glomus) irregularis</i>	DAOM240415 × 234328	1.2	1.3	1.3	96		de la Providencia et al. (2013)
	DAOM197198 × 240415	0	2.3	0.7	97		
	DAOM197198 × 234328	0	0	2	98		
	A4 × C2	4.6	1.9	13.9	79.6		Croll et al. (2009)
	A4 × C3	10.3	4.1	11.3	74.2		
	A4 × D1	0	7.3	13.4	79.3		
	A4 × B3	1.9	8.5	8.5	81.1		
	C2 × C3	5.4	0	19.4	77.4		
	C2 × D1	1.9	8.5	20.8	70.8		
	C2 × B3	1.9	6.6	12.3	79.2		
	C3 × D1	1.1	4.2	15.8	82.1		
	C3 × B3	1	5.8	10.7	82.5		
	D1 × B3	4.6	3.4	9.2	87.4		
<i>Rhizoglyphus (Glomus) clarus</i>	6AmA#2 3 1UnC#7	0				0	Purin and Morton (2013)
	6AmA#2 3 CRwest#8	0				0	
	1UnC#7 3 CRwest#8	0				0	
	1UnC#5 3 1UnC#7	5.8				0	
	1UnC#7 3 WV310#5	0				0	
	1UnC#7 3 WV123A#6	0				0	
	1UnC#7 3 WV123A#7	0				0	
	WV310#5 3 WV123A#6	0.9				0	
	WV123A#7 3 WV123A#6	1.6				0	
	WV123A#7 3 WV310#5	2.2				0	

(continued)

Table 2.1 (continued)

Between isolates	Asymbiotic mycelium				Symbiotic mycelium		References
	PF	PtFI	PFI	NI	PF	NI	
Between lineages							
<i>Rhizoglyphus (Glomus) irregularis</i>	MUCL 43194	41–77	0	0	–		Cárdenas-Flores et al. (2010)
Between life cycle phases				PF	PtFI	PFI	NI
<i>Funneliformis (Glomus) mosseae</i> IMA1	Asymbiotic × symbiotic hyphae	4.9–23.9		5.2–17.6	1.2–14.9	46.6–88.6	Sbrana et al. (2011)

*Indicate mean data for all the isolates.
Data are given as frequencies (percent) of perfect fusions (PF), pre-fusion incompatibility (PrFI), post-fusion incompatibility (PFI), no interactions (NI) or as number of perfect fusions per hyphal length. In the latter case, frequencies of fusions within the same hypha (wh) and between different hyphae (bh) are reported.

fusions detected in these two genera occurred within the same hypha (de la Providencia et al. 2005; Voets et al. 2006).

It is interesting to note that, using data obtained from studies of ERM development in soil and hyphal fusion frequencies in tri-dimensional agar or soil systems, even the apparently low anastomosis rate results in the production of 100–410 interhyphal connections per gram of soil (Giovannetti and Avio 2002; Voets et al. 2006; Mikkelsen et al. 2008).

Other studies, carried out on mycorrhizal networks produced by geographically different isolates of the globally distributed AMF species *F. mosseae* and *R. intraradices* (two isolates for each species) living in symbiosis with *Medicago sativa*, revealed that the structure of the network significantly differed among AMF isolates, since the hyphal length ranged from 4 to 21 mm per mm of root length and the number of anastomoses per hyphal contact varied between 30 and 67; Avio et al. 2006). Such a high interconnectedness was shown to play an important role in the translocation and flow of nutrients from soil to host plants, affecting plant growth and nutrition (Avio et al. 2006).

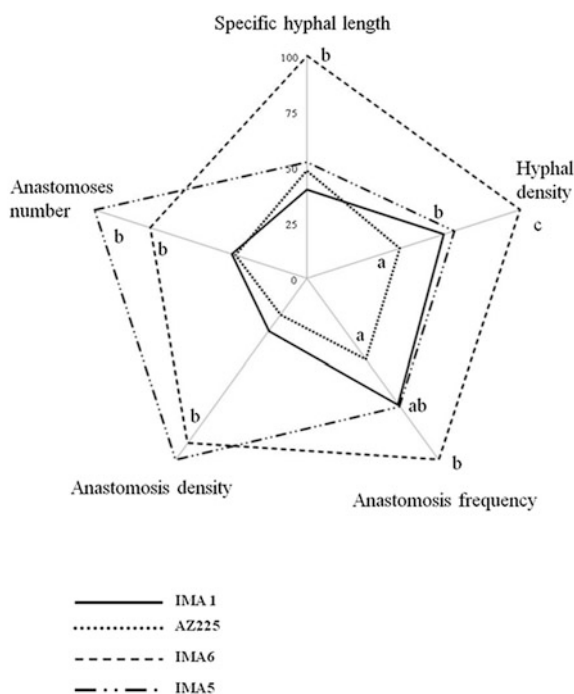


Fig. 2.3 Radar chart showing mycorrhizal network variables characterizing different isolates of *Funnelformis mosseae*, IMA1 and AZ225, and *Rhizoglossus intraradices*, IMA5 and IMA6, expressed as percentages of the highest obtained value. Fungal variables are measured as: specific hyphal length, mm mm⁻¹ colonised root length; hyphal density, mm mm⁻²; anastomosis number mm⁻¹ hyphal length; anastomosis density mm⁻²; anastomosis frequency, percentage of hyphal contacts leading to hyphal fusions

Although the sandwich system is bi-dimensional and may affect hyphal anastomosis and growth rate, its use extended the knowledge of the mechanisms underlying plant interconnectedness, revealing an unexpected and remarkable outcome: the root systems of plants belonging to different species, genera and families and colonised by the same fungal symbiont could be interconnected by means of linkages between contiguous mycorrhizal networks (Giovannetti et al. 2004). The extraradical hyphae spreading from *Allium porrum* root system were able to establish connections with those originating from *Daucus carota*, *Gossypium hirsutum*, *Lactuca sativa*, *Solanum melongena*, colonized by the same strain of the AM symbiont *F. mosseae* (Fig. 2.4). The percentages of hyphal contacts leading to anastomosis between extraradical networks originating from different plant species, ranging from 44 % in the pairing *A. porrum*-*S. melongena* to 49 % in *A. porrum*-*G. hirsutum*, were significantly lower than those detected between hyphae belonging to the same plant, which ranged from 46 % in *D. carota* to 64 % in *L. sativa* and showed also a host plant effect.

According to such data, connections between different plants are not exclusively established through hyphae spreading from mycorrhizal roots and colonising contiguous host plants (Newman 1988; Graves et al. 1997; Van der Heijden et al.



Fig. 2.4 Hyphal connections established between extraradical mycorrhizal networks originating from *Allium porrum* (left) and *Solanum melongena* (right) colonised by the same *Funneliformis mosseae* isolate IMA1

1998), but also by means of fusions between mycorrhizal networks originating from different plants, which could potentially create indefinitely large numbers of linkages through which nutrients can be transported over long distances. As the visualisation of such linkages in soil is not possible, because every kind of sampling would destroy the structure of the fungal network, an indirect approach confirmed the occurrence of anastomosis between contiguous ERM in a soil experimental system (Mikkelsen et al. 2008).

In our laboratory we recently demonstrated the ability of hyphae originating from individual germinated spores to fuse and incorporate into hyphae of the mycorrhizal network produced by plants colonised by the same fungal strain, and to establish vital connections with nuclei flowing in anastomosis bridges (Sbrana et al. 2011; Table 2.1). This phenomenon represents an important mechanism evolved by AMF to increase their chances of survival. Indeed, although AMF are obligate biotrophs, their spores can germinate in the absence of host-derived chemical signals, giving rise to coenocytic colonies where an active bidirectional flow of nuclei, mitochondria, fat droplets, vacuoles and organelles is easily detectable (Bago et al. 1998b; Logi et al. 1998) and whose extent may range from 18 to 50 mm (Bécard and Piché 1989; Gianinazzi-Pearson et al. 1989; Logi et al. 1998). Such asymbiotic hyphae, being unable to establish a symbiosis, rapidly undergo a programmed growth arrest accompanied by protoplasm withdrawal and resource reallocation towards mother spores (Mosse 1959; Hepper 1983; Bécard and Piché 1989; Logi et al. 1998). This energy-saving behaviour, though important to allow long-term maintenance of spore viability and host-infection ability (Beilby and Kidby 1980; Koske 1981; Tommerup 1984; Logi et al. 1998), could have represented an evolutionary selective disadvantage. The ability of fungal germings to plug into pre-existing extraradical mycelium may increase their probability of survival, allowing them to gain access to plant-derived carbon circulating in the network before asymbiotic growth arrest.

2.3 Cytology of Anastomosis Formation

The word anastomosis derives from Greek and originally referred to an opening or junction through a mouth as of one body of water with another. In human anatomy, it commonly refers to a connection that is created between tubular structures, such as blood vessels, involving the concept of fluid flow. In mycology, anastomoses (vegetative hyphal fusions), first described in 1933 (Buller 1933), occur between hyphae of Ascomycota and Basidiomycota (Gregory 1984; Ainsworth and Rayner 1986; Leslie 1993). Anastomoses were formerly believed to be lacking or rare in Zygomycetes (Gregory 1984; Carlile 1995) but some authors mentioned their occurrence without giving any quantitative data on their frequency or the cytological events involved (Godfrey 1957; Mosse 1959; Tommerup 1988; Giovannetti et al. 1993).

Anastomoses between living hyphae of AMF were first studied and monitored in asymbiotic mycelium originating from individually germinated spores (Giovannetti et al. 1999). By using a combination of time-lapse and video-enhanced light microscopy, image analysis, and epifluorescence microscopy the dynamics of anastomosis formation was monitored, cytoplasmic flow and nuclear exchange were visualised, and the occurrence and frequency of anastomosis between hyphae of germings belonging to the same and to different isolates, species and genera were assessed. Anastomoses formed in hyphae belonging to the same germling or to different germings of the same strain were characterized by cellular compatibility, consisting in complete fusion of hyphal walls, cytoplasmic flow and migration of organelles and nuclei through hyphal bridges (Table 2.1). The histochemical localization of formazan salts in hyphal fusions, evidencing SDH-succinate dehydrogenase activity, allowed the detection of successful anastomoses, characterised by viable hyphal connections and protoplasmic continuity (Giovannetti et al. 1999).

The morphological types of hyphal fusions were mainly tip-to-side, and rare tip-to-tip fusions were observed. During pre-contact interactions, approaching hyphal tips were actively attracted towards the nearby hyphae and showed growth orientation, while the approached hyphae initiated new hyphal tips, suggesting the existence of an interhyphal remote signalling. When a tip contacted a trunk hypha, it stopped growing and in some cases appeared swollen, but more often the walls fused without any apparent tip swelling, while a protoplasmic flow was established through the fusion pore. The cascade of cellular and biochemical events, including cell wall degradation and synthesis, leading to the formation of a hyphal bridge connecting the two previously separated hyphae remains to be unravelled. Further investigations should be performed to answer the question as to whether the complex process of anastomosis formation starts with a physiological switch making hyphae growing nearby fusion-competent as a result of remote chemical signals controlling pre-fusion events, similarly to what happens during the sexual phase of other fungi (Bistis 1981; Snetselaar et al. 1996).

The complete formation of hyphal fusions in living hyphae of AMF was accomplished in 35 min, after hyphal contact in *F. mosseae* and *F. caledonius* (Giovannetti et al. 1999), and in 4 h after a hyphal tip showed directed growth towards another hypha in *R. irregularis* (Sbrana, unpublished results). In hyphal fusions, the intense protoplasmic flow subsequent to anastomosis was visualised by the bidirectional movement of particles—vacuoles, mitochondria, nuclei, and fat droplets—migrating at the speed of $1.8\text{--}0.26\ \mu\text{m s}^{-1}$ in *F. caledonius*, *F. mosseae* and *R. irregularis* (Giovannetti et al. 1999; Sbrana, unpublished results). Nuclear occurrence in hyphal bridges, evidenced by DAPI staining and epifluorescence microscopy was detected between hyphae belonging to the same germling and to different germings of the same AMF isolate, in *F. caledonius*, *R. intraradices*, *F. mosseae*, showing the complete compatibility and interconnectedness of the mycelia.

Nuclear migration in AMF hyphal fusion bridges was confirmed by the visualisation—by immunofluorescence microscopy—of nuclei closely associated to cytoplasmic microtubules, which are believed to mediate nuclear division and

migration processes in fungi (Morris and Enos 1992; Åstrom et al. 1994). In fungi three types of cytoplasmic microtubule (cMT)-dependent nuclear movements have been observed using live cell imaging: short-range oscillations (up to 4.5 $\mu\text{m}/\text{min}$), rotations (up to 180° in 30 s), and long-range nuclear bypassing (up to 9 $\mu\text{m}/\text{min}$) (Lang et al. 2010). In *Ashbya gossypii* long-range nuclear movements were regulated by cytoplasmic microtubule cytoskeleton emanating from each nucleus and by dynein, and nuclear pulling was due to cytoplasmic microtubule cytoskeleton cortical sliding (Grava et al. 2011).

The migration and intermingling of nuclei in hyphal bridges indicate that anastomoses in AMF play a fundamental role not only in the establishment of “mycelial interconnectedness”, allowing intrahyphal communication and homeostasis, as proposed by Rayner (1996), but also in the information flow leading to a physiological and genetic integration among vegetatively compatible individual germings. Cytological observations of *C. etunicatum* mycelium showed that nuclear mobility contributed to mix different lineages of nuclei within the coenocytic hyphae, and that the occurrence of asynchronous nuclear replication allowed changes in relative rates of such nuclear lineages. Moreover, a selective elimination of compromised nuclei, through a programmed death process, was observed during spore development, suggesting that also a nuclear-level selection operates in Glomeromycota (Jany and Pawlowska 2010).

Anastomosis frequency ranged from 35 to 69 % between contacting hyphae of the same germling and from 6 to 90 % between hyphae of different germings of the same strain (Table 2.1) in different experimental systems. However, no information is available on the factors controlling anastomosis frequency, involving either the extracellular environment or the intrahyphal microenvironment, possibly differentiating hyphae into fusion-competent regions, as observed in other fungal species (Hickey et al. 2002).

No hyphal fusions over 220 and 460 contacts were detected in *Gigaspora rosea* and *Racocetra castanea* mycelium, revealing an additional character differentiating the family Glomeraceae from the Gigasporaceae (Giovannetti et al. 1999). Such a difference was confirmed by in vitro experiments carried out using RiT-DNA transformed carrot roots, which reported very low values of anastomosis formation between different hyphae in AMF species belonging to Gigasporaceae (de Souza and Declerck 2003; de la Providencia et al. 2005; Table 2.1). Interestingly, the fusions observed were likened to a healing process (Gerdemann 1955; de Souza and Declerck 2003), which could be functional to the restriction of damages as a result of ageing, lytic events or physical lesions. In some species, short-length hyphal sections were able to undergo septa formation rapidly to shelter from the external environment and new hyphal tips growing from detached sections formed anastomoses among them. A differential behaviour was observed between *Dentiscutata reticulata*, where only a recovery of hyphal integrity was achieved, and *R. clarus* where the healing mechanism led to hyphal recovery and to a new growth into the surrounding medium (de la Providencia et al. 2007). Differences in hyphal fusion regulating mechanisms between these two species, mostly still unknown, could

explain such different behaviour, supporting the view that Glomeraceae and Gigasporaceae have developed different survival strategies.

Anastomosis between vegetative hyphae may represent the first step of the parasexual cycle, allowing the formation of a heterokaryotic coenocytic mycelium where distinct nuclear genotypes are maintained for an indefinite/definite period of time (Pontecorvo 1956). However, no evidence of parasexual hybridization by means of hyphal fusions has so far been reported in AMF, as described in other fungi (Schardl et al. 1994). Though, in *R. intraradices* high-mobility domains containing transcriptional factors, with significant similarity to genes controlling mating type in *Phycomyces blakesleeana* (Idnurm et al. 2008) and transcripts encoding for the meiotic recombination machinery, as well as meiosis-specific proteins (Tisserant et al. 2012), were detected. Moreover, 51 genes showing homology to those required for the proper completion of meiosis in *Saccharomyces cerevisiae* were identified in *Glomus* spp. (Halary et al. 2011), indicating the possibility of sexual reproduction in AMF. Indeed, findings consistent with recombination were reported for different AMF species, suggesting the occurrence of gene exchange, which could be realised by means of intermingling of nuclei during anastomosis (Vandenkoornhuyse et al. 2001; Croll et al. 2009; den Bakker et al. 2010; de la Providencia et al., 2013; Beaudet et al. 2015; Boon et al. 2015; Weichert and Fleißner 2015). Further research is needed to understand how fusions between genetically different lineages may alter the genetic structure and the reproductive success of AMF populations.

2.4 Vegetative Compatibility and Incompatibility in Anastomosing Hyphae

Studies on fungal somatic fusions revealed beneficial outcomes of frequent self-anastomoses, which increase the absorbing surface and the foraging ability of hyphal colonies (Aanen et al. 2008; Richard et al. 2012). Although enhanced mycelial fitness has been reported also after nonself fusions, their frequency is low, as vegetative compatibility is under the control of het or vic (heterokaryon or vegetative incompatibility genes) genes (Glass and Kuldau 1992; Leslie 1993; Glass and Kaneko 2003). The occurrence of incompatible het/vic alleles in fusing hyphae triggers incompatible responses, including programmed cellular compartmentalization and death (Glass and Dementhon 2006). Such incompatibility systems have probably evolved to limit mycelial damages resulting from genetic conflicts, due to DNA exchange, and from the transfer of pathogenic elements (viruses, deleterious mitochondria and plasmids) (Biella et al. 2002; Malik and Vilgalys 1999).

In AMF, experiments carried out on hyphae of germings belonging to different genera and species, and to geographic isolates of the same species, revealed their ability to discriminate self from nonself. Hyphae belonging to different species or

genera do not form anastomoses and, during interspecific and intergeneric interactions, do not show any contact interference. For example, no hyphal fusions were detected on a total of 90, 140, 232 and 98 hyphal contacts between hyphal germings of *F. mosseae* and *F. caledonius*, *F. mosseae* and *Gigaspora rosea*, *F. caledonius* and *G. rosea*, *G. rosea* and *R. castanea*, respectively.

No hyphal compatibility between germings belonging to geographically different isolates of *F. mosseae* was observed, even if pre-contact tropism, directional growth and branching of approaching hyphae occurred. In the interaction between *F. mosseae* isolates IN101, BEG25 and AZ225C (Giovannetti et al. 2003), approaching hyphae showed directed growth, branching and initiation of tips contacting the receiving hyphae, which were able to sense the presence of approaching hyphae and produced new lateral tips growing towards them. Interestingly, either prior to or after physical contact between hyphae, clear pre-fusion incompatible responses (rejection responses), were evidenced, such as apical swellings, wall thickening and cell wall depositions in the contacting hypha, followed by protoplasm withdrawal from hyphal tips, vacuolization and septa formation (Fig. 2.5).

The different ranges of events leading to the development of hyphal bridges and to the formation of anastomoses suggested the existence of a highly regulated system of self-recognition, leading to compatibility between hyphae belonging to the same network and between germings and mycelia originated from spores produced by the same isolate. Such events are mirrored by nonself discrimination mechanisms, leading to nonself incompatibility between hyphae of AMF belonging to different genera, species, and geographic isolates of the same species. Though,

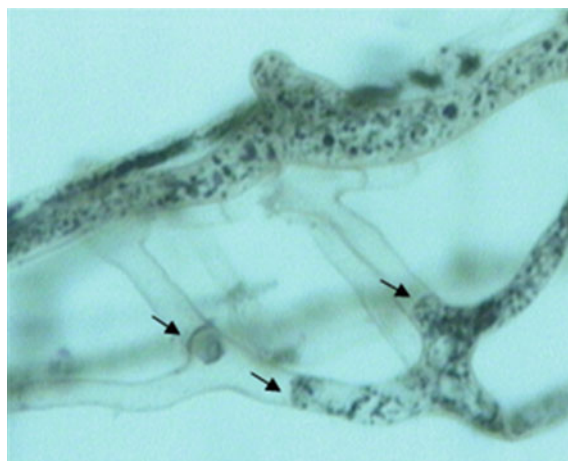


Fig. 2.5 Pre-fusion incompatible interactions between hyphae belonging to two geographically different isolates of the AMF species *Funnelformis mosseae*, IMA1 and AZ225, after SDH staining. Note the retraction septa developed by an approaching hypha after protoplasm withdrawal (arrows)

the specific chemical signals triggering interhyphal attraction and regulating vegetative compatibility/incompatibility, and leading to self recognition and nonself discrimination, remain poorly understood.

Post-fusion incompatible interactions, showing protoplasm withdrawal and cross wall formation in fused hyphae, were demonstrated in germinating spores and vegetative hyphae of Ascomycota, where incompatibility results from heterodimers of het or vic proteins (Glass et al. 2000; Saupe 2000; Glass et al. 2004). In AMF, nonself vegetative fusions (Fig. 2.6) were detected between genetically different single spore isolates (clonal lineages) of *R. irregularis*, which established vital connections, thereby creating the possibility for genetic exchange (Table 2.1; Fig. 2.7). Molecular analyses of the progeny of the mycelium derived from such nonself vegetative fusions evidenced the transmission of specific genetic markers, showing that genetic exchange had indeed occurred, despite the low anastomosis frequencies (Croll et al. 2009). Recent findings confirmed the occurrence of nonself anastomoses in *R. clarus* and the possibility of genetic exchange and heteroplasmy as a result of either perfect fusions or post-fusion incompatible interactions in *Rhizoglossus* isolates (Purin and Morton 2011, 2013; de la Providencia et al. 2013; Beaudet et al. 2013; Lin et al. 2014).

In conclusion, AMF hyphae are capable of recognition and fusion, thus producing large mycorrhizal networks where important nutritional, genetic and information flows are active. Such property is crucial for the survival of AMF populations, because it can directly affect their fitness, viability and reproductive success. The visualisation of AMF networks and of their structure unravelled a high level of interconnectedness, fundamental for facilitating the interchange of mineral nutrients, water and sugars flowing from soil to plants and from plants to soil.



Fig. 2.6 Post-fusion incompatible interactions between hyphae belonging to two genetically different isolates of *Rhizoglossus irregularis*. Note the retraction septum and protoplasm withdrawal developed after fusion (arrow)

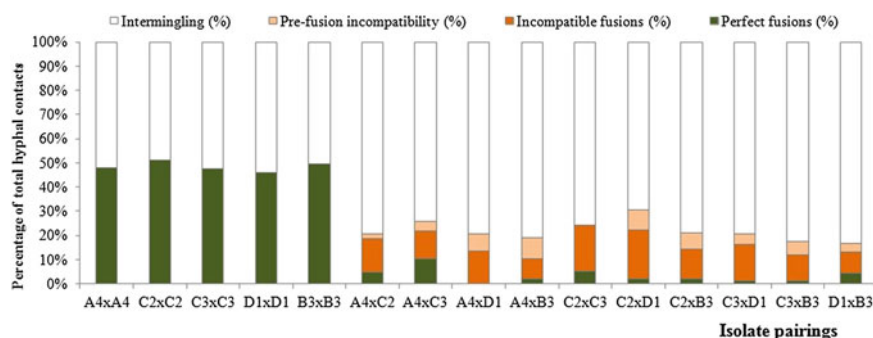


Fig. 2.7 Frequencies of the different types of interaction between hyphae of the same and genetically different lineages of *Rhizoglyphus irregularis* (isolates A4, B3, C2, C3 and D1 originated from different nearby fields; isolates C2 and C3 originated from the same field. Modified from Croll et al. 2009)

In addition, the ability of self recognition and nonself discrimination of AMF hyphae suggests that the mycorrhizal network is also a site of information flow. The capacity of extraradical hyphae of fusing by means of anastomosis, interconnecting many different plants in the community, confirms that mycorrhizal networks can contribute to the formation of indefinitely large potential functional guilds (see Simard et al., Chap. 5, this Vol.), playing a key role in the complex web of interactions that regulates the functioning of natural and agricultural ecosystems.

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