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Production and specificity of polyclonal antibodies against soluble proteins from the arbuscular mycorrhizal fungus *Glomus intraradices*

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Abstract Rabbit polyclonal antibodies were produced against a soluble protein fraction from a vesicle and spore mixture of the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices*. The protocol for isolation of vesicles and spores from plant roots was optimized to minimize debris contamination. Protein extract purification and preparation for immunization was adapted to increase protein content and immunogenicity. Active antisera were produced starting from the second boost immunization. Antibodies obtained were specific for surface antigens of AMF and revealed different patterns of soluble protein antigens in *G. intraradices*, *G. constrictum* and an unidentified *Glomus* species.

Keywords Arbuscular mycorrhizal fungi · Polyclonal antibodies · Immunoelectrophoresis · Biodiversity

Introduction

Reliable identification and evaluation of genetic diversity amongst arbuscular mycorrhizal fungi (AMF) is a significant problem for modern mycology. The contemporary systematics of AMF is based mainly on morphological features of asexual spores and sporocarps (Trappe 1982; Walker 1992), which may vary slightly in different species as well as be affected by the physiological status of the fungus or by environmental conditions (Morton 1988). Methods such as isozyme analysis, RAPD, PCR-RFLP, DNA cloning and screening allow to estimate genetic diversity more accurately and to differentiate between AMF taxa (Van Tuinen et al. 1994).

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Immunochemical methods based upon highly selective binding of antibodies (Ab) to the corresponding antigens have been applied for the identification and systematics of bacteria and fungi (Dewey et al. 1991; Breuil et al. 1992; De Ruiter et al. 1993). Polyclonal antibodies (pAb) against AMF extraradical mycelium and spore material (soluble fractions and wall epitopes) have been used for AMF identification (Sanders et al. 1992), ecological studies of AMF (Adwell and Hall 1986; Friese and Allen 1991) and research into AMF taxonomy (Adwell et al. 1985). However, low titers of the antisera obtained and a wide range of Ab cross-reactivities have hindered the application of immunochemical approaches for reliable identification and definition of AMF taxonomic criteria. An increase in pAb specificity was achieved by using a purified precipitate of the soluble protein fraction from spores of *Gigaspora rosea* Nicolson and Schenck (BEG 9) as antigen (Cordier et al. 1994). The Ab obtained were specific for *Gigaspora* and could differentiate between species of this genus. The monoclonal Ab (mAb) technology (Köhler and Milstein 1975) permits to direct antibodies against single epitopes and can, therefore, increase specificity. Production of mAb against a crushed spore suspension of AMF resulted in species-specific mAb discriminating between isolates of *Glomus occultum* Walker (Wright et al. 1987), species-specific mAb for *G. etunicatum* Becker and Gerdemann and mAb cross-reacting with two *Glomus* species out of six tested (Hahn et al. 1993). Genus-specific mAb directed against hyphae from *G. monosporum* Gerdemann and Trappe have been reported; pAb against the same antigen showed cross-reactions with several species of non-mycorrhizal fungi (Göbel et al. 1995). In this paper, we report the production of pAb against soluble proteins from *G. intraradices* and application of these Ab in investigations of AMF antigen diversity by immunoelectrophoresis.

Materials and methods

Fungal material

Six different isolates of AMF were used in this research. Strain 7 of *G. intraradices* Schenck and Smith (S7) was isolated, identified and tested for efficiency in our laboratory (Muromtsev et al. 1985). *G. constrictum* Trappe (S44) was obtained from the Institute of Semi-Arid Agriculture, India. *G. fasciculatum* (Thaxter sensu Gerdemann) Gerdemann and Trappe (S38) was provided by Dr. Lee (New Zealand). *G. mosseae* Gerdemann and Trappe (S2) was received from Rothamsted Experimental Station. *Glomus* sp. (S162) and *G. albidum* Walker and Rhodes (S105) were provided by Dr. Cudlin (Institute of Microbiology, Czech Academy of Sciences). All isolates were grown in a sand/soil mixture (1:3 v/v) with *Plectranthus australis* R. Br. as host plant. Non-mycorrhizal fungi were obtained from the collection of the All-Russian Research Institute for Agricultural Microbiology and grown on Czapek-Dox agar (Singleton et al. 1992).

Isolation of vesicles and spores from plant roots

Mycorrhizal roots of *P. australis* were lightly macerated (0.25 M $CaCO_3$, 3% glucose) for 4 h and crushed in a disintegrator to release vesicles and spores. The suspension obtained was filtered through 200- and 40-µm-mesh nylon sieves to remove large root segments and small wall fragments, respectively. The suspension was then diluted with distilled water to an OD_{620} of 0.3 and centrifuged in a sucrose density gradient with 60%, 50%, 40% and 10% layers at 300 *g* for 12 min. The 10% sucrose fraction containing a mixture of vesicles and spores was collected and filtered through 200- and 90-µm-mesh sieves to remove the rest of the root cells. The purified mixture of vesicles and spores was collected on a 40-µm-mesh sieve and stored frozen at –20°C.

Extraction of soluble proteins and preparation of antigen for immunization

The vesicle and spore mixture (600–700 mg) was suspended in 3 ml of cold distilled water and homogenized in a glass homogenizer for 5–7 min, depending on the AMF isolate. Light microscopy was used to check the degree of disintegration. The homogenate was diluted to 5–6 ml with washes from the homogenizer and extracted for 16 h at 4°C. Mertiolate was added before extraction at a dilution of 1:10,000 to minimize exogenous contamination of the homogenate. The extract was centrifuged at 4°C for 60 min at 12,000 *g*. The supernatant was transferred into dialysis tubes, dialyzed against distilled water for 20 h and lyophilized.

Immunization and antibody production

A rabbit (Chinchilla) was immunized subcutaneously with 2–5 mg of protein extract of *G. intraradices* dissolved in 0.5 ml of distilled water and emulsified with an equal volume of Freund's complete adjuvant. The immunization protocol included 4 injections and 5 boost immunizations of one injection each (Table 1). A sample of pre-immune serum was taken from the marginal ear vein before the first injection. The test bleedings (3 ml of blood) were carried out on the 50th day after starting immunization and 7th–8th day after each boost immunization. The serum was tested for Ab activity and titer by immunodiffusion in a 2-mm-thick layer of 1% agarose. Consecutive dilutions of the protein extract from *G. intraradices* (4.4, 2.2, 1.1, 0.55 and 0.275 mg/ml) in aliquots of 20 µl each were applied in corresponding antigen wells placed alongside the antiserum well (2×64 mm) containing 250 µl of serum. Antiserum activity was revealed by a precipitate between antigen and antiserum wells on the 2nd day of incubation at room temperature. The antiserum titer was evaluated by the minimal antigen concentration corresponding to the precipitate. When sufficient antiserum activity was observed, 50 ml of blood was taken 1–2 days after the test bleeding. Immunoglobulins were isolated from antiserum and purified by $(NH_4)_2SO_4$ precipitation and chromatography on DEAE-Sephadex A 50 (Harboe and Ingild 1983). Purified immunoglobulins were concentrated to one-third of the original volume by dialysis against 0.1 M NaCl, 15 mM NaN_3, 20% (w/v) PEG 20,000 at 4°C for 8 h, portioned and lyophilized.

Table 1 Immunization protocol and serum activity (+ precipitation of antigen by serum antibodies, – lack of precipitation, *n.t.* not tested, S_1–S_4 samples of antibodies isolated from the corresponding sera)

Injection		Day	Antigen (mg)	Serum activity	Antibody sample
Immunization	1	0	2.16	n.t.	
	2	14	2.00	n.t.	
	3	28	2.97	n.t.	
	4	42	4.82	–	
Boost immunization	I	77	3.28	–	
	II	112	3.60	+	S_1
	III	248	4.00	+	S_2
	IV	285	1.68	+	S_3
	V	346	3.02	+	S_4

Table 2 The cross-reactivities of polyclonal antibodies (pAb) with surface antigens of endomycorrhizal and non-mycorrhizal fungi tested by immunofluorescence (– no signal, + signal)

	Species	Structures	pAb Reaction
Mycorrhizal fungi	*Glomus intraradices*	Vesicles, hyphae	+
	Glomus constrictum	Vesicles, hyphae	+
	Glomus mosseae	Vesicles, hyphae	+
	Glomus fasciculatum	Vesicles, hyphae	+
	Glomus albidum	Vesicles, hyphae	+
	Glomus sp.	Vesicles, hyphae	+
Non-mycorrhizal fungi	*Sclerotinia* sp.	Hyphae	–
	Trichoderma sp.	Hyphae	–
	Gliocladium sp.	Hyphae	–
	Paecilomyces sp.	Hyphae	–
	Penicillium sp.	Hyphae	–
	Pythium sp.	Hyphae	–
	Mucor sp.	Hyphae	–
	Wardomyces sp.	Hyphae	–

Analysis of Ab specificity

Heat-fixed smears on slides of 5-day cultures of non-mycorrhizal fungi (Table 2) or heat-fixed vesicles on slides isolated from AMF-colonized roots as described above were incubated with 25 µg/ml bovine serum albumin conjugated to rhodamin for 15 min at room temperature for saturation of aspecific antigenic sites. The fungal material was then incubated successively with the Ab obtained and with anti-rabbit specific Ab conjugated to sodium fluorescein isothiocyanate for 20 min at room temperature. All the incubations were carried out in a humid chamber. Three washing steps with phosphate-buffered saline (50 mM Na_2HPO_4, 5 mM NaH_2PO_4, 0.85% NaCl, pH 7.6) followed each incubation. To test the specificity of pAb to surface antigens of AMF mycelium, vesicles were isolated from roots as described above, placed on nitrocellulose membrane filters, put into nylon mesh and germinated in soil close to the roots of *P. australis.* After 10 days, filters were taken out, washed, air dried and incubated in a humid

chamber with dye solution [0.2% gentian violet, 34% (v/v) ethanol] for 15 min at room temperature to eliminate autofluorescence. After four washing steps using distilled water, filters were air dried, put on slides and stained as described above. A luminescent microscope (LUMAM R–3) was used for evaluation of the fluorescence intensity.

Immunoelectrophoresis

Separation of soluble protein antigens of AMF and precipitation by the pAb produced was carried out by crossed immunoelectrophoresis in a 1.5-mm layer of 1% agarose and veronal-acetate buffer (pH 8.6, μ 0.02) according to Weeke (1977b). Ten microliters of protein extract dissolved in the buffer (25–30 mg/ml) was applied to each well. The potential gradients of the first- and the second-dimension electrophoresis were 4 V/cm² and 1 V/cm², respectively. Second-dimension gels contained 10 μl per cm² of purified and concentrated immunoglobulins. Immunoprecipitates were stained with Coomassie brilliant blue R-250 according to Weeke (1977a).

Results

Antigen production

The protocol described for isolation of vesicles decreased the amount of debris in the final pellet to 5–10%. The main fraction of host root material and destroyed AMF structures appeared in bottom layers of the gradient. The 10% sucrose fraction contained a mixture of vesicles and spores as well as a small amount of root cells, which was removed by the subsequent filtration. In 1 day it was possible to obtain 40–200 mg dry weight of purified vesicles and spores, depending upon mycorrhization. Pure spores and vesicles retained viability and structure and could be used for inoculation and protein extraction. Successful protein extraction required 600–700 mg of purified AMF structures. The dry weight of the obtained protein extract was 4–6 mg, equivalent to 0.7–1% of the original vesicle biomass.

Production of pAb and cross-reactivities

Precipitation of protein extract of *G. intraradices* by antiserum was observed in the immunodiffusion test with the second boost immunization (Table 1). The antiserum titer was approximately equal to 0.55 mg/ml of antigen. No precipitation was observed in immunodiffusion tests with the pre-immune serum. A sample of antiserum was collected after each boost immunization, starting with the second. Because of the low titer, it was necessary to isolate pAb from all samples and to concentrate it for the succeeding manipulations. Four samples (S_1–S_4) of pAb corresponding to four consecutive boost immunizations were obtained.

The specificity of pAb to the surface antigens of AMF and soil-borne non-mycorrhizal fungi was tested by immunofluorescence (Fig. 1; Table 2). The Ab cross-reacted with both vesicle and mycelium surface antigens of six AMF species. No cross-reaction was observed with eight isolates of non-mycorrhizal fungi.

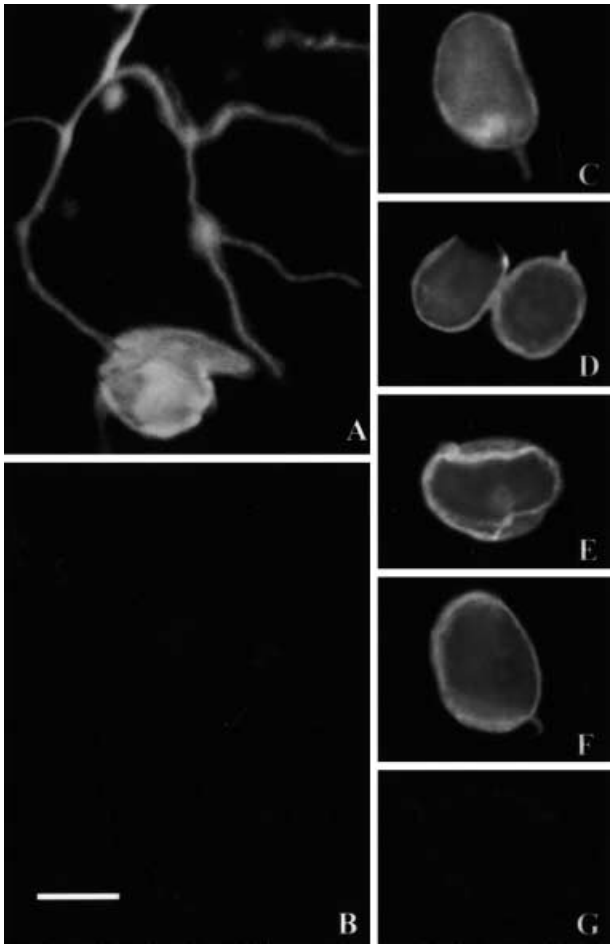

Fig. 1A–F Immunofluorescent labeling of AMF and non-mycorrhizal fungi using obtained antibodies. **A** Vesicle of *Glomus intraradices* germinated on a nitrocellulose membrane filter in soil; **B** hyphae of *Mucor* sp.; **C–F** vesicles of *G. intraradices* (**C**), *G. constrictum* (**D**), *G. mosseae* (**E**), *Glomus* sp. (**F**); **G** *G. intraradices* vesicle, primary antibodies omitted, control; *bar* 50 μm

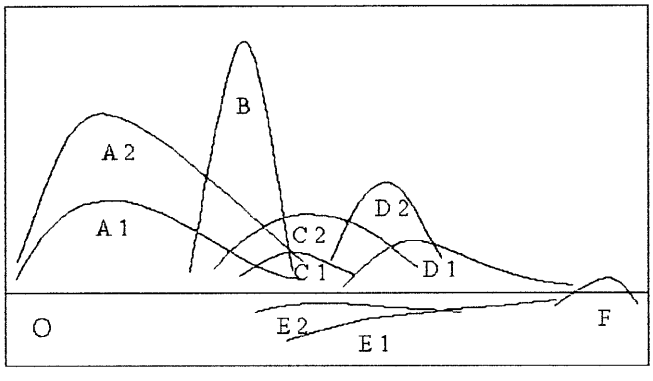

Fig. 2 Pattern of all the revealed antigens (summary scheme) (*A–F* detected antigens)

Analysis of antigens

Immunoelectrophoresis of soluble protein extracts of *G. intraradices* revealed at least 10 antigens precipitated by the pAb obtained (Fig. 2). Each precipitate corre-

sponds to at least one antigen. The antigens detected were mostly major, except for antigen B.

Comparison of the antigen patterns recognized by four samples of pAb (S_1–S_4) showed an increasing number of antigens: antigen B was detected by S_3 and S_4 but not by S_1 and S_2, i.e. pAb activity increased (Fig. 3A–D; Table 3).

Table 3 Activity of antibody (Ab) samples (S_1–S_4) obtained by four consecutive boost immunizations [+ precipitation of *G. intraradices* antigens by produced antibodies, *(+)* ambiguous reaction, – no precipitation]

Antigen (precipitate)	Activity of Ab samples			
	S_1	S_2	S_3	S_4
A1	+	+	+	+
A2	(+)	(+)	+	+
B	–	–	+	+
C1	+	+	+	+
C2	+	+	+	+
D1	+	+	+	+
D2	+	+	+	+
E1	+	+	(+)	(+)
E2	+	+	+	+
F	+	+	+	+

Detection of antigens in protein extracts of *G. intraradices*, *G. constrictum* and *Glomus* sp. by the pAb produced showed different antigen patterns for the three species (Fig. 3E–G). Five antigenic determinants were shared by all three species, seven antigenic components were shared by *G. intraradices* and *Glomus* sp. and three components (B, A2 and C2) were only detected in *G. intraradices* (Table 4). Application of both S_1 and S_4 showed no antigen B in either the *G. constrictum* or *Glomus* sp. protein extracts.

Discussion

The analysis of data on molecular and morphological characters is essential for establishing identification and taxonomic criteria for AMF. Analysis of antigen polymorphism by immunoelectrophoresis using pAb has been shown to be valuable in this respect. Polyclonal antibodies raised against whole hyphae (Göbel et al. 1995) as well as cell wall preparations from spores and germinating hyphae (Kough et al. 1983; Wilson et al. 1983; Friese and Allen 1991) showed a wide specificity from cross-reactions with non-mycorrhizal fungi to genus specificity. The use of purified soluble spore proteins as

Table 4 Pattern of soluble protein antigens recognized by pAb in three species of AMF

AMF species	Recognized antigens	Antigens recognized in *G. intraradices* only
Glomus intraradices	A1, A2, B, C1, C2, D1, D2, E1, E2, F	B, A2, C2
Glomus constrictum	A1, D1, E1, E2, F	
Glomus sp.	A1, C1, D1, D2, E1, E2, F	

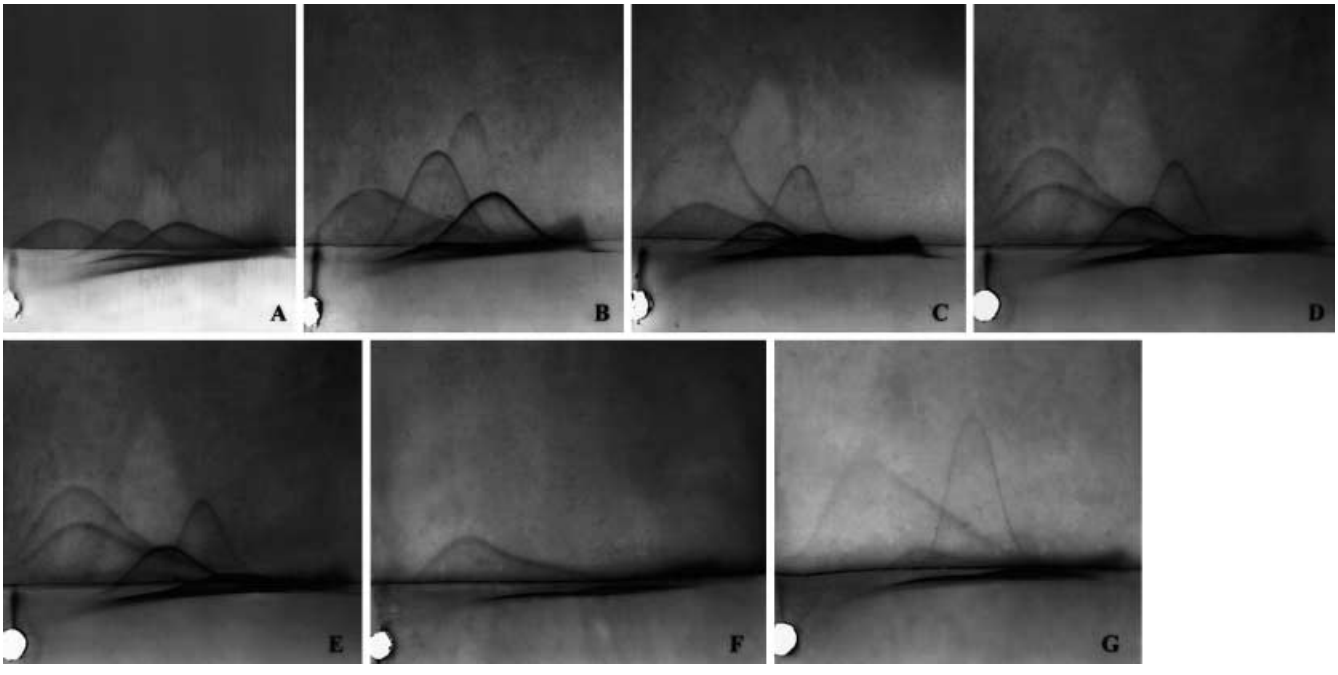

Fig. 3 A–D Immunoelectrophoretograms of *Glomus intraradices* soluble antigens revealed by four antibody samples: **A** S_1, **B** S_2, **C** S_3, **D** S_4. **E–G** Immunoelectrophoretograms of antigens revealed by the S_4 antibodies in a soluble protein extract of *G. intraradices* (**E**), *G. constrictum* (**F**) and *Glomus* sp. (**G**)

an immunogen increased the specificity of pAb produced and allowed *Gigaspora* species to be distinguished (Cordier et al. 1994).

In this present work, we developed a protocol to increase antigen yield for immunization by dialysis and lyophilization of the protein extract of *G. intraradices* spores and vesicles. The pAb produced against soluble proteins from *G. intraradices* showed cross-reactions with surface antigens of five other *Glomus* species, probably due to the presence of shared antigens. In crossed immunoelectrophoresis of soluble protein extracts, seven shared antigens were detected among three species of *Glomus* and three antigens of *G. intraradices* were found which were not detectable in *G. constrictum* or *Glomus* sp. Estimation of the specificity of these three antigens requires further analysis of several genera and species of AMF. Should these antigens prove to be specific, they could be isolated from the total protein extract and used to produce more specific antibodies.

In conclusion, crossed immunoelectrophoresis with pAb revealed different antigen patterns in three *Glomus* species. Such an analysis offers a novel approach to the study of AMF diversity.

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References

Adwell FEB, Hall IR (1986) Monitoring spread of *Glomus mosseae* through soil infested with *Acaulospora laevis* using serological and morphological techniques. Trans Br Mycol Soc 87:131–134

Adwell FEB, Hall IR, Smith JMB (1985) Enzyme-linked immunosorbent assay as an aid to taxonomy of the Endogonaceae. Trans Br Mycol Soc 84:399–402

Breuil C, Luck BT, Rossignol L, Little J, Echeverri CJ, Banerjee S, Brown DL (1992) Monoclonal antibodies to *Gliocladium roseum*, a potential biological control fungus of sap-staining fungi in wood. J Gen Microbiol 138:2311–2319

Cordier C, Gianinazzi-Pearson V, Gianinazzi S (1994) Immunodétection de champignons endomycorhiziens à arbuscules in planta. Acta Bot Gallica 141:465–468

De Ruiter JA, van Bruggen-van der Lugt AW, Bos W, Notermans SHW, Rombouts FM, Hofstra H (1993) The production and partial characterization of a monoclonal IgG antibody specific for moulds belonging to the order Mucorales. J Gen Microbiol 139:1557–1564

Dewey M, Evans D, Coleman J, Priestley R, Hull R, Horsley D, Hawes C (1991) Antibodies in plant science. Acta Bot Neerl 40:1–27

Friese CF, Allen MF (1991) Tracking the fates of exotic and local VA mycorrhizal fungi: methods and patterns. Agric Ecosyst Environ 34:87–96

Göbel C, Hahn A, Hock B (1995) Production of polyclonal and monoclonal antibodies against hyphae from arbuscular mycorrhizal fungi. Crit Rev Biotechnol 15:293–304

Hahn A, Bonfante P, Horn K, Pausch F, Hock B (1993) Production of monoclonal antibodies against surface antigens of spores from arbuscular mycorrhizal fungi by an improved immunization and screening procedure. Mycorrhiza 4:69–78

Harboe NMG, Ingild A (1983) Immunization, isolation of immunoglobulins and antibody titre determination. Scand J Immunol 17:345–351

Köhler G, Milstein C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495–497

Kough J, Malajczuk N, Linderman RG (1983) Use of indirect immunofluorescent technique to study the vesicular-arbuscular fungus *Glomus epigaeum* and other *Glomus* species. New Phytol 94:57–62

Morton JB (1988) Taxonomy of VA mycorrhizal fungi: classification, nomenclature, and identification. Mycotaxon 32:267–324

Muromtsev GS, Marshunova GN, Jacoby LM (1985) Strain 7 of endomycorrhizal fungus *Glomus* sp. increasing yield of agricultural plants (in Russian). Patent certificate No. 1145503; bulletin No. 34 of USSR State Committee of Inventions and Discoveries

Sanders IR, Ravolanirina F, Gianinazzi-Pearson V, Gianinazzi S, Lemoine MC (1992) Detection of specific antigens in the vesicular-arbuscular mycorrhizal fungi *Gigaspora margarita* and *Acaulospora laevis* using polyclonal antibodies to soluble spore fractions. Mycol Res 96:477–480

Singleton LL, Mihail JD, Rush CM (eds) (1992) Methods for research on soil-borne phytopathogenic fungi. APS Press, St. Paul, Minn

Trappe JM (1982) Synoptic keys to the genera and species of zygomycetous mycorrhizal fungi. Phytopathology 72:1102–1108

Van Tuinen D, Dulieu H, Zézé A, Gianinazzi-Pearson V (1994) Biodiversity and characterization of arbuscular mycorrhizal fungi at the molecular level. In: Gianinazzi S, Schüepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhäuser, Basel, pp 13–23

Walker C (1992) Systematics and taxonomy of the Glomales: a possible way forward. Agronomie 12:887–897

Weeke B (1977a) General remarks on principles, equipment, reagents and procedures. In: Axelsen NH, Krøll J, Weeke B (eds) A manual of quantitative immunoelectrophoresis. Methods and applications. Mir, Moscow, pp 11–42

Weeke B (1977b) Crossed immunoelectrophoresis. In: Axelsen NH, Krøll J, Weeke B (eds) A manual for quantitative immunoelectrophoresis. Methods and applications. Mir, Moscow, pp 58–74

Wilson IM, Trinick MJ, Parker CA (1983) The identification of vesicular-arbuscular mycorrhizal fungi using immunofluorescence. Soil Biol Biochem 15:439–445

Wright SF, Morton JB, Sworobuk JE (1987) Identification of a vesicular-arbuscular mycorrhizal fungus by using monoclonal antibodies in an enzyme-linked immunosorbent assay. Appl Environ Microbiol 53:2222–2225