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Immunochemical detection of arbuscular mycorrhizae

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Abstract. The difficult systematics of the arbuscular mycorrhiza (AM) forming-fungi, belonging to the Glomales, can be facilitated with immunochemical methods. Polyclonal antibodies, however, are seldom able to reach below the generic level. Monoclonal antibodies (mAb) have been produced which can differentiate AM fungal spores on the species and strain/isolate level. Together with an improved enrichment procedure for spores and hyphae of AM fungi, isolation and identification of the extraradical structures is possible with these mAb, lowering the risk of unspecific binding. Quantification of the data is discussed as the next step.
Key words. Immunochemistry; arbuscular mycorrhizal fungi; *Glomus*; surface antigens; monoclonal antibodies.

Introduction

The arbuscular mycorrhizal (AM) fungi belong to the order Glomales and currently include six genera. *Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis and Scutellospora* together comprise around 150 species described to date[20]. The largest part of the soil hyphae occurring in northern temperate latitudes stem from mycorrhizae[14]. Especially in vegetation systems dominated by herbaceous plants, where phosphate is the major growth-limiting nutrient, AM fungi mobilize phosphate and make it available for their host plants[16]. In addition, short-circuiting of nutrient cycles by AM fungi connecting different plants can prevent nutrient losses from ecosystems[14]. With the recognition of these processes, AM fungi are gaining growing attention in reclamation attempts and agricultural applications[2].
However, different species of AM fungi differ in their ability to colonize host plants. Stringent taxonomic classification of the employed isolate is therefore required for all experiments dealing with the impact of AM on ecosystems. A drawback in the systematics of AM fungi is their inability to grow axenically, i.e. without their host plants. It is time-consuming and requires expert knowledge to isolate and identify single spores before multiplying these isolates for later inoculation. It is virtually impossible to distinguish different AM fungi by the morphological appearance of their hyphae. We are confident that these difficulties can be overcome using immunochemical methods.

Immunochemistry

Immunochemical methods are based on the selective binding properties of antibodies. An antibody (Ab) is an immunoglobulin molecule, a glycoprotein produced in response to an antigenic stimulus by vertebrate plasma cells derived from B-lymphocytes. For directed Ab production this stimulus is an injection of a high-molecular-mass foreign substance (antigen) into laboratory animals (e.g. rabbit, mouse, goat, sheep or horse). The basic structure of an immunoglobulin is a pair of heavy and light identical polypeptide chains held together by disulfide bonds[17]. Each chain has one hypervariable complementary determining region (cdr), which is part of an antibody binding site or paratope formed by the amino-terminal ends of a light and a heavy chain. One paratope is capable of recognizing and binding one epitope or antigenic determinant of an antigen. Although there are five distinct classes of antibodies in most higher mammals, immunochemical techniques usually rely upon immunoglobulin of the G class (IgG) as the major immunoglobulin. IgG has two paratopes.
An Ab recognizes only a restricted part of the antigen, the antigenic determinant or epitope. Every antigen has a particular set of epitopes, some of which are shared with other antigens (generic or public epitopes) and some that are not (type-specific or private epitopes). In most instances, an organism will produce more than one Ab per epitope. These differ in their affinity for the epitope.

Isolation of antigen

For the production of immunochemical probes, large amounts of a defined antigen are needed. This is especially true when mAb-producing cell cultures are to be screened. For a large-scale production of pure AM spore material, the aeroponic method[8] is usually the method of choice. However, we met with difficulties in using it, presumably due to the different climatic conditions in Germany, compared to the southern United States. We therefore had to devise a strategy to isolate antigenic material from a complex substrate in quantitative amounts.
Host plants are grown in sterilized sand substrate together with surface-sterilized fungal inoculum. The

system is kept in translucent plastic bags to exclude the possibility of cross-contamination. Mycorrhized substrate can be harvested after 4 to 5 months. The isolation starts with a wet sieving step. The fraction containing the fungal material is then sonified and separated by flotation in water. The supernatant contains spores and the sediment contains hyphal matter in the substrate matrix; this is subsequently separated in an upstream flotation process. Two centrifugation steps purify the fractions enough for them to be used directly in immunoassays or for immunizations[7]. Figure 1 gives an example of the purity that can be attained with this method. Spores and hyphae of the AM fungus *Glomus etunicatum* are depicted after the extraction process.

With this method it was possible to produce sufficient amounts of antigen from a variety of different AM fungi. Table 1 gives an overview of the isolates that have been successfully purified in our laboratory by this method, along with the amount of spores that could be derived from greenhouse cultures, with sand as a substrate. The amount of mycelium obtained was not quantified in these experiments. The diversity of the fungi demonstrates that this method can be applied to most species of *Glomus*. The limits of the method are reached with fungi producing sporocarps, such as *G. fasciculatum*; these cannot be extracted by this method.

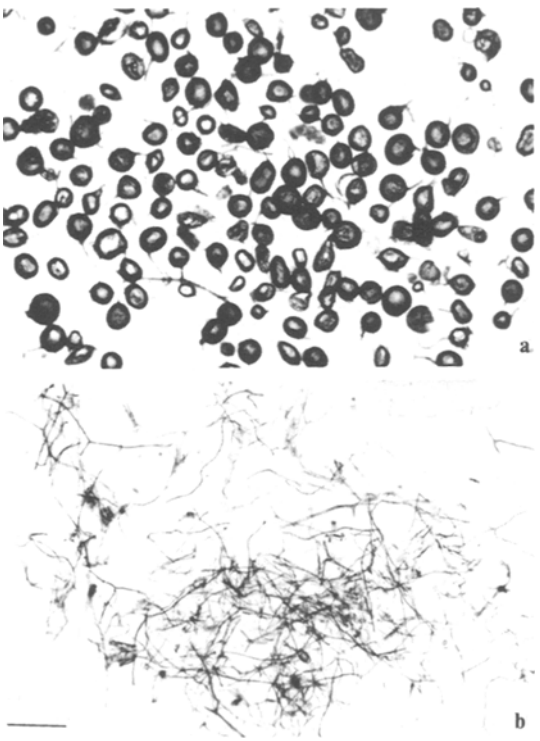

Figure 1. Spores (*a*) and hyphae (*b*) of *Glomus etunicatum* (Whs) extracted from sand substrate by the described method. Scale bar = 200 µm.

Table 1. Isolates of fungi extracted with the described method.

Fungal isolate	Number of spores per kg dry weight of sand substrate
Glomus albidum	1000
G. clarum	220
G. etunicatum	26,000
G. fasciculatum	0
G. hoi	1500
G. manihotis	260
G. mosseae	40,000

Production of polyclonal antibodies

For the production of polyclonal antibodies (pAb) against spores of AM fungi, rabbits were immunized four times at weekly intervals with 500 surface-sterilized, undestroyed spores of an isolate designated *Glomus* S328 ('*G. globisporum*'). Blood was taken two weeks after the fourth immunization and again four weeks after a fifth immunization. An indirect immunofluorescent staining procedure with an anti-rabbit IgG antibody coupled to FITC was employed to label spores of various species of *Glomus*.

In immunolabelling experiments, an unspecific fluorescent signal may result from the adsorption of the antibody molecules, often through the constant Fc fraction, to the antigen. In the anti-AM antisera, this signal could be suppressed by blocking with unspecific IgG from goat. This is demonstrated by unspecific murine IgG, acting as a control primary Ab. In contrast to the anti-AM antiserum in figure 2a, no fluorescent signal is seen in figure 2b. However, the antiserum showed cross-reactions with the tested *Glomus* species and other soil

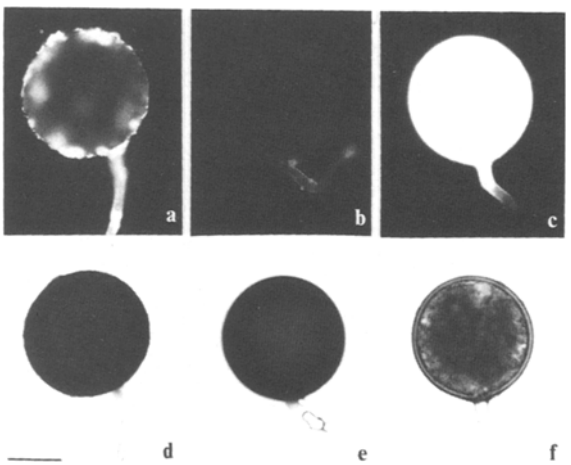

Figure 2. Indirect immunolabelling of spores of two isolates of *Glomus* with a polyclonal antiserum from rabbit and anti-rabbit IgG secondary antibody conjugated to the fluorescent stain FITC. *a* Anti-*Glomus* S328 antiserum as primary Ab with isolate S328. *b* Unspecific IgG from rabbit as control with isolate S328. *c* Anti-*Glomus* S328 antiserum as primary Ab with *G. etunicatum*; *d* to *f* Corresponding bright field micrographs. Scale bar = 50 µm.

fungi. Cross-reactions result from epitopes shared by different antigens or from insufficient specificity of the Ab binding sites. These cannot be suppressed by blocking steps; this is demonstrated in figure 2c, where the pAb raised against *G.* isolate S328 also yielded a fluorescent signal with the cytoplasma of *G. etunicatum* spores.

pAb used for taxonomic work in AM research have seldom been able to permit identification below generic level. This has been reviewed[1].

Production of monoclonal antibodies against AM antigens

In contrast to the heterogenous population of antibodies in polyclonal antisera, the clonal nature of the immune response guarantees that every B-cell produces just one type of Ab with a defined specificity and affinity. This results in the possibility of higher specificity of monoclonal antibodies (mAb). mAb are produced by isolation of B-cells and subsequent immortalization by fusion with non Ab-producing myeloma cells[9]. The resulting 'hybridomas' inherit from their myeloma the ability to grow as in-vitro cell cultures, and from their parent B-cell the ability to produce Ab. Subsequent cloning of the population and cultivation of single cell cultures yields mAb-producing cell populations.

Wright et al.[21] were the first to show that mAb can be raised against AM fungi. They produced mAb against soluble antigens from spores of *G. occultum*, which had a high specificity for their homologous antigen and gave significantly weaker signals with 29 other specific mycorrhizal and 5 non-mycorrhizal fungal isolates when tested in an ELISA. These mAb were even able to differentiate their antigen from isolates of the same species from different locations[13]. The perspectives and applications of this technique for AM research have been reviewed by Perotto et al.[15].

We followed a different strategy, producing mAb against surface antigens of the insoluble spore walls of AM fungi. Due to their size, undestroyed spores cannot be used for intraperitoneal immunizations; we resorted to a different immunization pathway.

In this method, the peritoneum of the laboratory animal (for mAb work, mice are usually employed) is opened in a small surgical operation and the antigen is injected directly into the spleen, the location of B-cell differentiation[12]. Table 2 compares the fusion rates and fusion yield of cells from mice immunized by the intrasplenic (i.s.) pathway with AM spores, in comparison to the conventional intraperitoneal (i.p.) method using crushed spores.

The i.s. immunization is the only way to introduce particulate matter as antigen into the organism of the laboratory animal. In comparison to the i.p. pathway, the amount of spores needed for an immune response is about ten times lower, the immunization protocol is shorter and fewer immunizations have to be executed before an immune response occurs[19].

For the selection of those hybridoma cells producing the desired mAb, an indirect immunohistochemical protocol was employed which comprised the following steps[6]: Spores of the AM fungi were embedded in resin, cut semi-thin (0.5 µm) and fixed to glass slides. The cell culture supernatants containing the mAb were incu-

Figure 3. Indirect immunogold-silver staining (IGSS) of AM spore walls of *G. etunicatum*.
a A positive cell culture supernatant; *b* ms-IgG as control. Scale bar = 20 µm.

Table 2. Fusion rates and fusion yields compared for different immunization pathways; data from 3 i.p. and 5 i.s. derived fusions.

Fusion parameter	Intraperitoneal immunization	Intrasplenic immunization
Rate (hybridomas per spleen cell)	3×10^{-8}	3.3×10^{-8}
Yield (anti-AM Ab hybridomas per spleen cell)	5×10^{-9}	5×10^{-9}
Number of stable mAb cell lines derived	5	8

bated with the samples. The bound mAb were detected by a secondary anti-murine IgG-Ab conjugated to biotin, which in turn was labelled by streptavidin colloidally bound to 5 nm gold particles. These particles were finally visualized as a black precipitate by silver reduction. Figure 3a shows a spore wall of *G. etunicatum*, labelled by supernatant from cell culture A5B1; this hybridoma cell line was derived from a mouse immunized with *G. etunicatum*. Figure 3b demonstrates the absence of unspecific binding using IgG from a normal mouse as a primary Ab control obtained from a subsequent semi-thin section of the same spore. The interior of the spores was preserved in only very few instances. This is attributed to the tough and elastic spore walls[10,11].

Figure 4 summarizes the steps to produce mAb against AM spores. Spleen cells from the immunized mouse are fused with myeloma cells to produce hybridomas, which can be kept in cell cultures. A series of screening and cultivation steps leads to the production of the needed amounts of mAb.

Finally, immunofluorescent labelling was used for probing the mAb with native spores to detect cross-reactions. Due to the low fluorescence signal, which is obtained with mAb as compared to pAb, the streptavidin-biotin enhancer step was retained and phycoerythrin B, a fluorescent dye with a high quantum yield[18], was used for labelling (c.f. Fig. 1). Figure 5 shows the resulting signals for the mAb A5B1 and H6A12 challenged with

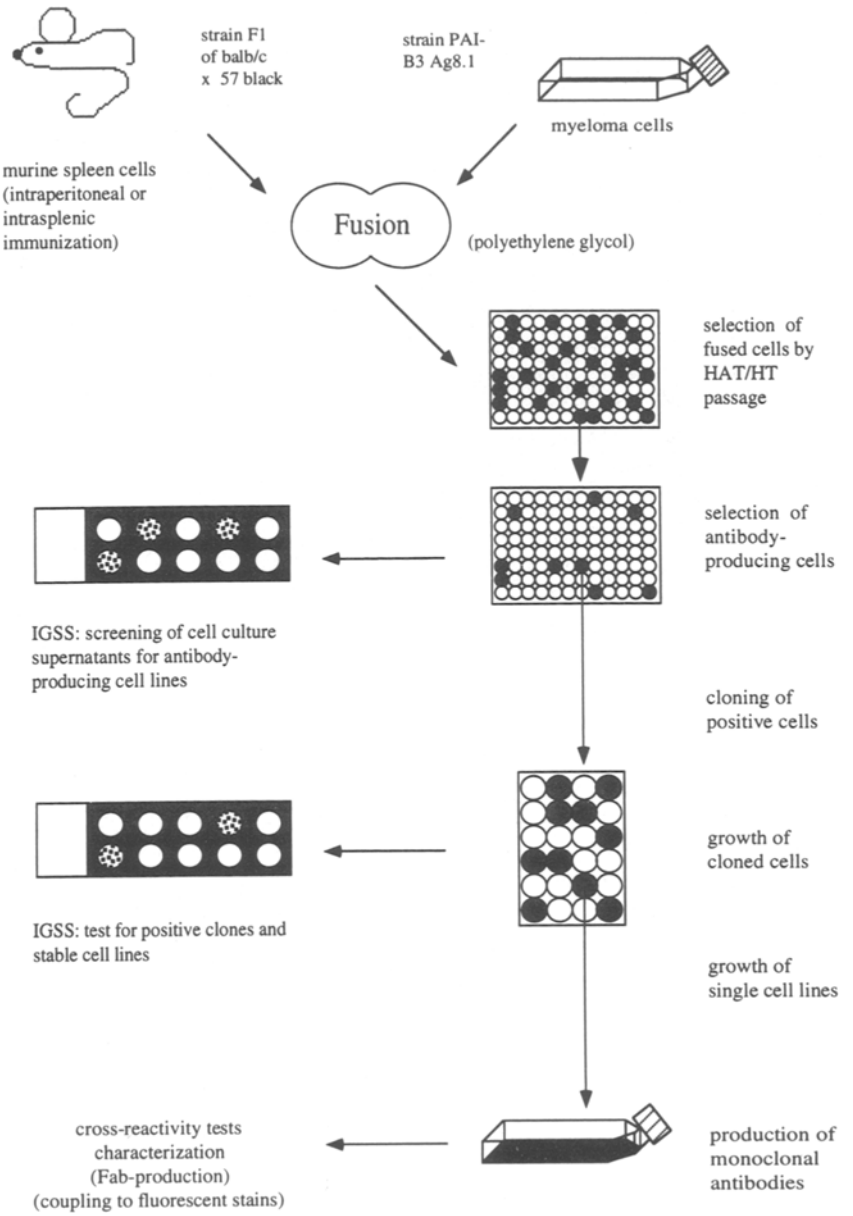

Figure 4. Screening of hybridoma cell culture supernatants for antibody production against insoluble cell wall antigens on AM spores using immunogold-silver staining (IGSS).

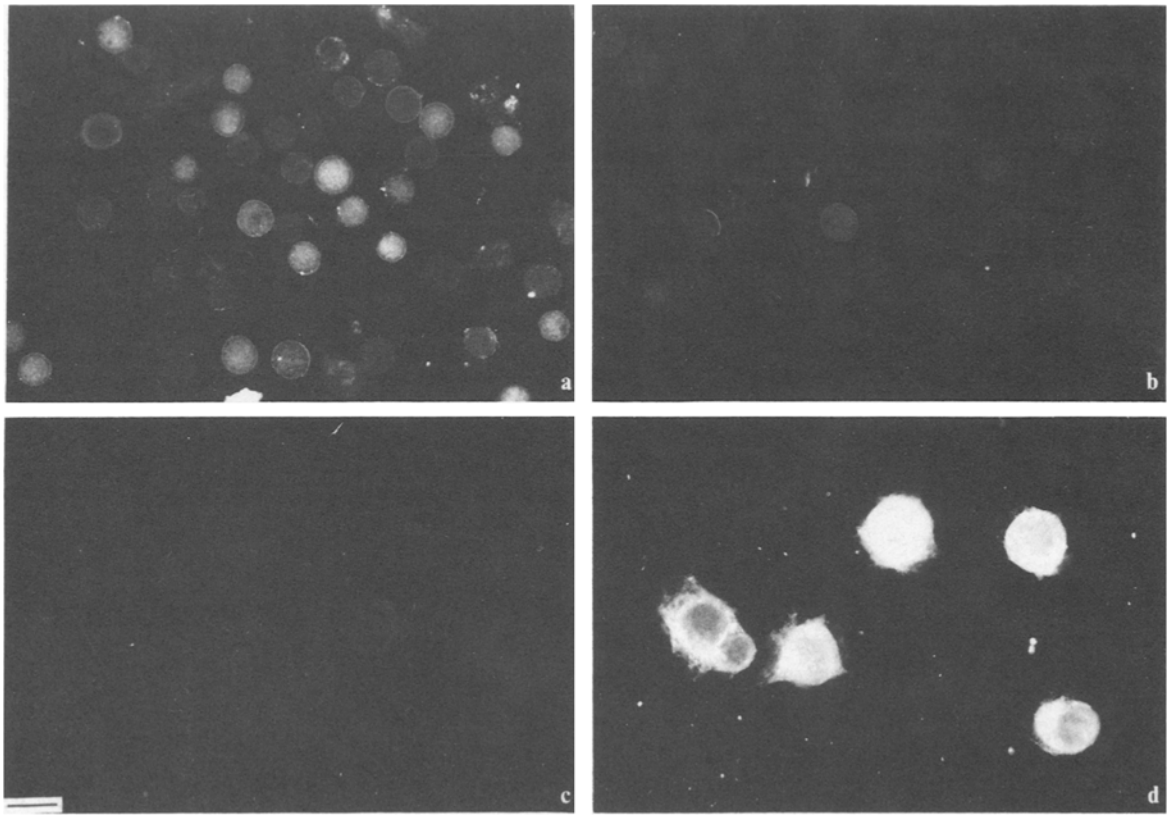

Figure 5. Indirect immunofluorescent labelling of *Glomus* spores with mAb A5B1 (*a*, *c*) and H6A12 (*b*, *d*) as primary Ab combined with a streptavidin-biotin enhancer step. *a*, *b G. etunicatum*; *c*, *d G. mosseae*. Scale bar = 200 µm.

G. etunicatum and *G. mosseae*. Only the homologous antigen is labelled.

A total of 13 mAb against a variety of isolates of *Glomus* have been produced to date, four of which have been characterized in more detail. Of these, two show species specificity. The mAb A5B1 is specific for spores of *Glomus etunicatum*, the mAb H6A12 for *G. mosseae*. A further mAb, H8F7, reacts with all AM isolates tested because it cross-reacts with *Bacillus cereus* var. *mycoides*, a bacterial strain present on all AM isolates tested so far. The fourth mAb shows cross-reactivity with a variety of AM species; owing to the uncertain origin of a number of isolates, the possibility that mixed cultures were used for immunization and screening cannot be excluded.

Applications of immunochemical methods in the quantification of AM antigens

In most field experiments, indigenous fungi, mycorrhizal and other, will be present in the samples. The competition of introduced fungi with indigenous microorganisms is a process which must be taken into account in field studies. Friese and Allen[5] have shown in an experiment with polyclonal Abs coupled to a fluorescent stain that inoculated AM fungi can survive in the

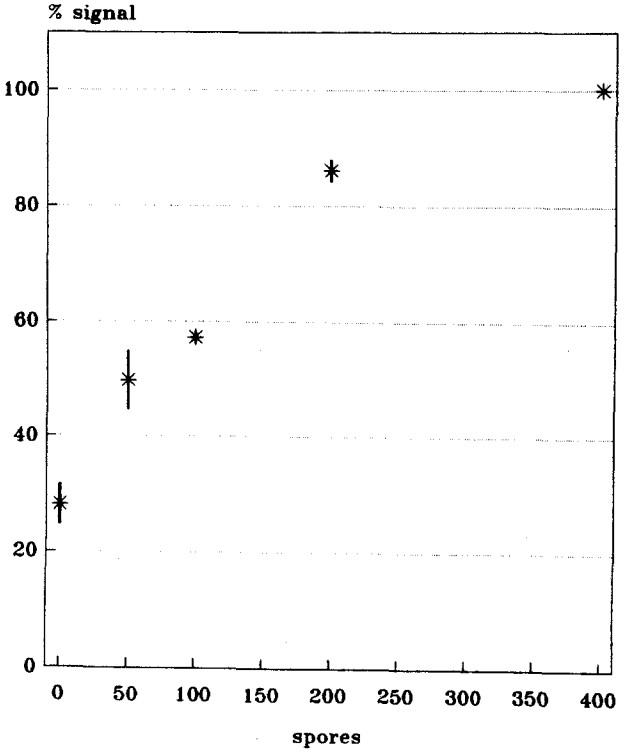

Figure 6. Spores of AM isolate *G. etunicatum* (strain Whs) assayed by chemiluminescence immunoassay.

field for up to two years, even in competition with indigenous AM fungi. However, the vertical placement of the inoculum can significantly affect this potential for survival. This documents the need for reliable quantitative assays of AM fungal matter. First experiments have yielded promising results. Figure 6 shows a calibration curve with the mAb A5B1 detecting undestroyed spores of its homologous antigen *G. etunicatum* in a chemiluminescence assay. The close correlation between absorption and spore count is visible between 0 and 200 spores. In this context, however, the amount of mycelium in the substrate is more relevant for measuring the infectivity of a substrate, as the spores are resting states, not necessarily liable to infect an introduced host plant. Work is in progress to raise mAb against hyphae of AM fungi.

Conclusions

The combination of intrasplenic immunization and IGSS as a screening method proved to be useful for the production of mAb against particulate antigens such as the spores of AM fungi. Although the production of mAb involves considerable effort, mAb can resolve systematic differences in AM spores at a level below the generic one. In addition, mAb can be produced in virtually unlimited amounts. Immunochemical methods are easier to follow and to interpret than the traditional morphological methods. A general use of these methods for the identification of AM fungi requires the availability of a greater number of mAb. A reference panel of mAb[4] could be used by workers in this field, guaranteeing a consolidation of data. An advantage of immunochemical methods compared to other highly specific methods such as DNA analysis[22] is seen in the possibility that the same propagules that were tested with the antibodies can subsequently be introduced as a viable inoculum. Fluorescence-activated cell sorters could separate and quantify spores directly out of mixed populations of AM fungi.

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