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USE OF THE INDIRECT IMMUNOFLUORESCENT TECHNIQUE TO STUDY THE VESICULAR-ARBUSCULAR FUNGUS *GLOMUS EPIGAEUM* AND OTHER *GLOMUS* SPECIES

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SUMMARY

An antiserum for spores and hyphae of *Glomus epigaeum* was developed employing extracted chlamydospore walls as an antigen. The reaction with fungal material was visualized by an indirect immunofluorescent technique. The staining was most intense and uniform on hyphae from root preparations and on the inner spore walls and hyphal attachments from spore preparations. The staining reaction was strongest for *G. epigaeum*, but *G. mosseae* and *G. deserticola* also reacted. The staining reaction appeared to be specific for the genus *Glomus*, as endomycorrhizal species of *Gigaspora*, *Acaulospora* and other soil and root-inhabiting fungi tested did not react.

INTRODUCTION

The study of the soil phase of vesicular–arbuscular mycorrhizae (VAM) has been hampered by the difficulty of tracing and distinguishing their hyphae from those of the other soil fungi. The fluorescent antibody (FA) technique has proved useful in examining other fungi including those inhabiting soil and roots (Burrell *et al.*, 1966; Sen and Holland, 1970; Schmidt *et al.*, 1974; Malajczuk, McComb and Parker, 1975; MacDonald and Duniway, 1979; Fitzell, Evans and Fahy, 1980; Fitzell, Fahy and Evans, 1980; Frankland *et al.*, 1981). The use of the FA technique in VAM research has been impeded by the laborious methods involved in obtaining sufficient quantities of clean fungal material for use as an antigen. This difficulty is compounded by the present inability to culture VAM fungi in the absence of a living host plant.

The VAM fungus *Glomus epigaeum* produces large, relatively clean sporocarps on the soil surface of plant cultures (Daniels and Trappe, 1979). Surface contaminants can be removed by rinsing the spores, yielding a sufficient quantity of spore wall material to proceed with an immunological preparation. This study was designed to determine (1) if chlamydospores would serve as the antigen to produce serum that would react with spores and hyphae of the fungus and (2) if

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the antiserum would be specific for *G. epigaeum* alone or if other VAM or non-VAM fungi would react.

MATERIALS AND METHODS

Preparation of fungal antigen

Mature sporocarps of *G. epigaeum* were collected from the soil surface of asparagus (*Asparagus officinalis* L.) pot cultures. The average weight of fresh sporocarps was 30 mg. This weight represents approximately 6000 spores. Twenty to 30 sporocarps were collected for each extraction. The chlamydospores were separated by hand dissection from the sporocarps in sterile distilled water (SDW) and sonicated until a suspension of individual chlamydospores was obtained. Care was taken to avoid heating during sonication. The spores were then washed in SDW, agitated, and centrifuged at 500 r min^{-1} for 2 min. This procedure was repeated 10 times with SDW and twice with sterile phosphate buffered saline (PBS) (0·005 M $K_2HPO_4 . 3H_2O$, 0·005 M KH_2PO_4, 0·15 % w/v NaCl). The spores were withdrawn in a minimum of PBS, and an aliquot plated on potato dextrose agar (PDA) to check for sterility. The remaining spores were frozen, lyophilized, and extracted by the method Ayers *et al.* (1976). Using this procedure, 0·1 g of fresh sporocarpic material yielded 35 to 50 mg of lyophilized, extracted material. The cell wall preparation was stored at −10 °C no more than 5 days before being suspended in Ringer's physiological saline for injection into rabbits.

Immunization and determination of titre

One millilitre of extracted wall suspension (50 mg ml^{-1}) was intravenously injected into the marginal vein of the ear in each of two rabbits. The injections were repeated on days 2 and 3, then the rabbits were rested for 11 days. On days 14 and 28 the injection sequence was repeated as before. On days 35 and 42 samples of approximately 5 ml were withdrawn from the marginal vein of the ear for determination of titre. The injection sequence was repeated on day 59 and the rabbits were bled on day 76. The blood was allowed to stand overnight to coagulate, after which the red blood cells were spun down. The serum was collected and subsequently used for the antibody titre assay.

The antibody titre was determined by a modification of the agglutination technique for mycelial wall preparations described by Malajczuk *et al.* (1975). Sonicated chlamydospores suspended in PBS were ground with a mortar and pestle, then left for 2 h to allow the larger fragments of cell walls to settle out. The remaining suspension was removed and used as an antigen in an agglutination test. The reaction with rabbit serum was carried out at 40 °C in a covered water bath for 12 h. The agglutination reaction was assessed by examination under a dissecting microscope. A positive reaction resulted in a diffuse cloud of agglutinated particles; a negative reaction was a clear suspension. Based on these results, all further serum collections were diluted with PBS (1:25) and stored in 10 ml aliquots at −10 °C until needed.

Specificity tests with spores, hyphae and roots

Chlamydospores of *G. epigaeum* were collected from sporocarps as described above, but were not extracted. Spores of other VAM species were obtained by sieving and decanting (Gerdemann and Nicolson, 1963) from pot cultures of asparagus (*G. mosseae*, *G. deserticola*, *Gigaspora gigantea*, *Acaulospora trappei*) or

subclover (*Gigaspora margarita* and *A. spinosa*). The other fungi tested were from cultures growing on agar plates (*Phytophthora cinnamoni*, *Verticillium dahliae*, *Fusarium oxysporum* f. sp. *pini*) or in liquid culture (*Pisolithus tinctorius* and *Cenococcum geophilum*).

The fungal preparations to be examined were placed in a 25 mm membrane filter apparatus (Millipore Inc) equipped with a 0·45 μm nucleopore filter. They were washed with 500 ml sterile PBS after which 0·5 ml rabbit serum (diluted 1 : 25 with PBS) was introduced. This combination was allowed to react in a covered water bath at 40 °C for 1 h. Control fungal preparations with PBS but without rabbit serum or with non-immune rabbit serum were kept in the same chamber. The samples were then rinsed with 500 ml PBS, and 0·5 ml of 1 : 50 fluorescein-labelled goat anti-rabbit serum (Antibodies Inc, Davis, CA) was added to all samples including the controls. These were then returned to the water bath to react for 1 h after which the samples were rinsed with 500 ml of PBS, and stored in moist plastic seal bags in the dark at 4 °C for up to 2 days before examination.

Mycorrhizal root preparations of *G. epigaeum*, *G. mosseae*, *G. deserticola*, *Gigaspora gigantea*, and *Acaulospora trappei* from asparagus pot cultures were rinsed in 1·0 M NaOH for 5 h before staining to decrease non-specific staining of rhizoplane debris (Mayersbach, 1969). After the NaOH rinse, the root preparations were rinsed with 500 ml of PBS, treated with immune rabbit serum or control solutions and incubated by the procedures described above. Root preparations were quenched for tissue autofluorescence with either 0·01 % aqueous Evans blue or a 10 % cold water extract of bark of Douglas fir (Bohlool, pers. comm.). All preparations were examined under incandescent and u.v. illumination on a Zeiss standard microscope. The u.v. light source was a halogen lamp filtered to pass 450 to 490 nm wavelength light through the subject and allowing no wavelength shorter than 510 to 520 nm to pass through the eyepiece. Slides were taken with Kodak 160 ASA tungsten film processed to 320 ASA. Exposure times under u.v. illumination were from 30 to 60 sec.

Results and Discussion

An antiserum to extracted chlamydospore walls of *G. epigaeum* reacted most intensely with crushed chlamydospores of *G. epigaeum* [Fig. 1(*a*)]. The fluorescein label was not uniformly distributed on the outer spore surface of *G. epigaeum* and was more intense on the inner walls and hyphae [Fig. 1(*a*), (*e*)–(*h*)]. Roots colonized by *G. epigaeum* alone and together with *G. mosseae* or *G. deserticola* also stained, but the label occurred on the spores and hyphae of all three fungi [Fig. 1(*a*)–(*d*)]. However, spore or root preparations of VAM species in other genera tested did not react. Hyphae of non-VAM aseptate fungi present on the root surface remained unlabelled [Fig. 1(*e*), (*f*)]. The mycelial preparations of ectomycorrhizal and pathogenic fungi also did not label. There was autofluorescence present in the spore walls of some VAM species under u.v. illumination: *Gigaspora margarita* fluoresced a dull ochre colour and some immature *G. epigaeum* spores an intense orange colour. Both these reactions were easily distinguished from the apple-green colour of the fluorescein label. The spotty nature of the stain on the outer spore wall of *G. epigaeum* could have resulted from the presence of pigmentation in the wall obscuring the antigenic sites (Sen and Holland, 1970; Fitzell *et al.*, 1980). However, the spores and hyphae of *G. deserticola* are pigmented but reacted with the label [Fig. 1(*c*), (*d*)].

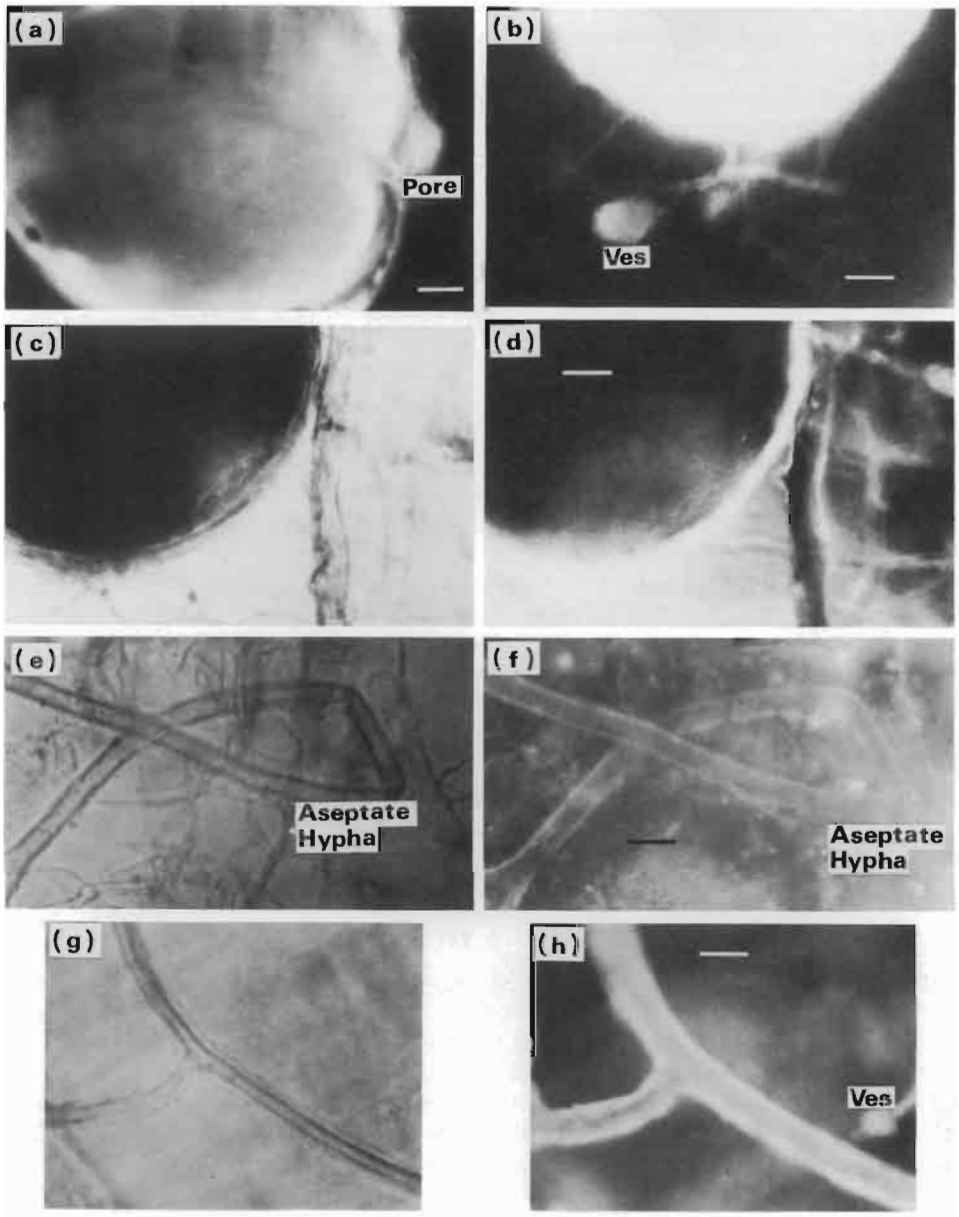

Fig. 1. Photomicrographs of fluorescein-labelled VAM fungi viewed with u.v. illumination except (c), (e) and (g) which were viewed with incandescent illumination. (a) Crushed chlamydospore of *G. epigaeum*, arrow indicates location of occluded pore. Note intensity of staining on inner wall of spore. Bar = 10 μm. (b) Mature chlamydospore of *G. mosseae* with external vesicle (ves). Bar = 10 μm. (c), (d) Chlamydospore and pigmented hyphae of *G. deserticola*. Labelled hyphae presumably of *G. epigaeum* in background. Bar = 10 μm. (e), (f) *G. epigaeum* hyphae on asparagus root with unlabelled aseptate fungal hypha. (g), (h) *G. mosseae* hypha and vesicle (ves) above an asparagus root. Bar = 10 μm.

A problem existing in many FA studies of fungal material is the cross-reaction of the antiserum to different strains of the same or closely related species. Some researchers have been able to distinguish between species in the same genus using serum developed with sexual or asexual spores as antigens (Amos and Burrell, 1967). However, this was not the case in the present study as cross-reaction occurred between all of the *Glomus* species tested. Titres for cross-reaction were not determined because of the difficulty of obtaining sufficient quantities of the cross-reacting species. One means of eliminating cross-reaction is by the absorption of the cross-reacting globulins (Amos & Burrell, 1967; Sen and Holland, 1970; Fitzell *et al.*, 1980; Frankland *et al.*, 1981), but this procedure was not attempted due to the low antibody titre of the serum collected.

The titre of specific antibodies in the blood serum never exceeded 1:50 as determined by the agglutination tests. The serum that was collected was therefore diluted 1:25 for use in these tests. Although this low titre was not an impediment in the present study, further globulin purification, removal of cross-reacting fractions, or direct labelling of the globulins with fluorescein isothiocyanate would require a higher titre. The extraction procedure is probably destructive to much of the antigenic material in spore walls. Ayers *et al.* (1976) found that their cell wall preparations of *Phytophthora megasperma* var. *sojae* contained only 10% protein. Since protein moieties are highly antigenic, a low protein content in extracted chlamydospores could result in reduced antigen activity in the preparations and hence a low titre. Since the chlamydospores were not contaminated after sonication and washing, a washed and crushed wall preparation could be employed as an antigen perhaps more successfully than the extracted preparations.

The FA technique should be useful in tracing the soil phase of VAM fungi and spores, especially when used in conjunction with a concentration technique based on soil sieving (Malajczuk, Bowen and Greenhalgh, 1978). The FA technique could also be used to study the development of hyphae along the root surface. Since the technique can distinguish between genera of VAM fungi, external colonization of roots by mixed inoculum, and differences in hyphal proliferation could be assessed. All this could be done with less disruption of the rhizosphere than presently occurs when using the clearing and staining technique of Phillips and Hayman (1970).

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