THE SEROLOGICAL PROPERTIES OF *EUTYPA ARMENIACAE* MYCELIUM AND ASCOSPORES*

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In epidemiological studies of the apricot vascular pathogen *Eutypa armeniacae* Hansf. & Carter in this laboratory (Moller and Carter 1965; Carter and Moller 1967) some diagnostic problems have been encountered. The pathogen is sufficiently variable in culture to be confused with other Ascomycetes, such as species of *Valsa* and *Cryptovalsa* which may colonize dead vascular tissue below pruning wounds which have been invaded by *E. armeniacae*. It is also difficult to differentiate *E. armeniacae* ascospores from those of related Ascomycetes caught in spore traps during wet weather.

The preparation of antisera to antigens of *E. armeniacae* ascospores and mycelium and their possible use in identification of the fungus are the subjects of this communication.

Materials and Methods

Perithecial stromata of *E. armeniacae* were soaked in water for 1 hr and attached to the lid of a closed container for several hours to collect the liberated ascospores. The ascospores were then suspended in a small quantity of distilled water and the concentration estimated by haemacytometer. A quantity of the suspension containing $2–5 \times 10^7$ ascospores was then emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into several sites on the flank of a rabbit. Two further injections containing similar quantities of spores were administered 1 and 5 weeks after the initial injection. The rabbit was bled through an ear vein 1 week after the final injection and several more times during the month following.

E. armeniacae was cultured on Czapek–Dox agar and after 2–3 weeks of growth the hyphae were scraped off the agar, suspended in 0·14M NaCl, and ground in a glass tissue-homogenizer. Microscopic examination of the suspension showed the presence of small pieces of hyphae. This material was emulsified with an equal volume of adjuvant and used to immunize a rabbit as described above for the ascospore antigen.

Serological tests with mycelial antigens were carried out by the double-diffusion precipitation technique (Crowle 1961) using 0·75% agar in 0·02M phosphate buffer, pH 7·2, containing 0·14M NaCl and 0·02% sodium azide. Agglutination tests using ascospores as antigens were carried out on microscope slides. Immunofluorescence tests with both mycelium and ascospores were carried out by the indirect method (Weller and Coons 1954). Smears of the antigens were prepared by drying suspensions on microscope slides which were then irrigated with rabbit antiserum for 15 min. The smears were washed thoroughly with 0·14M NaCl and irrigated with fluorescein isocyanate-labelled sheep anti-rabbit globulin serum for 15 min and again washed with 0·14M NaCl. The smears were mounted in glycerol and examined in a Leitz Ortholux microscope equipped with both phase-contrast and ultraviolet illumination.

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Results

The presence of antibodies in sera from rabbits immunized with *E. armeniacae* ascospores and mycelium was demonstrated by agglutination and gel-diffusion tests respectively. Agglutination tests with ascospores were avoided in further work because of the tendency of the spores to agglutinate in normal sera after prolonged incubation.

When *E. armeniacae* ascospores were treated with homologous rabbit antiserum they fluoresced strongly on staining with fluorescein-labelled sheep anti-rabbit-serum. However, when rabbit anti-mycelium serum was used, the spores failed to fluoresce, indicating the absence of common antigens between the mycelium and ascospores of this fungus. Similarly, using the antiserum prepared against *E. armeniacae* mycelium, fluorescence was observed with mycelium as antigen but not with ascospores. The latter tests were not as convincing as the former owing to some non-specific fluorescence. The reaction appeared to be largely with the intracellular antigens of the hyphae as most of the fluorescence was associated with the material extruded from broken hyphae; whereas there was no apparent difference in the fluorescence of the hyphal walls treated with either homologous, anti-ascospore, or normal rabbit sera.

The non-specific fluorescence of *E. armeniacae* mycelium, and the absence of fluorescence with ascospores, is illustrated in Figures 1–3. Ascospores, germinated overnight in distilled water at room temperature, were treated with normal, anti-mycelium, and anti-ascospore sera. The ascospores fluoresced only after treatment with the homologous antiserum (Fig. 3) whereas the mycelium fluoresced non-specifically when treated with all three sera.

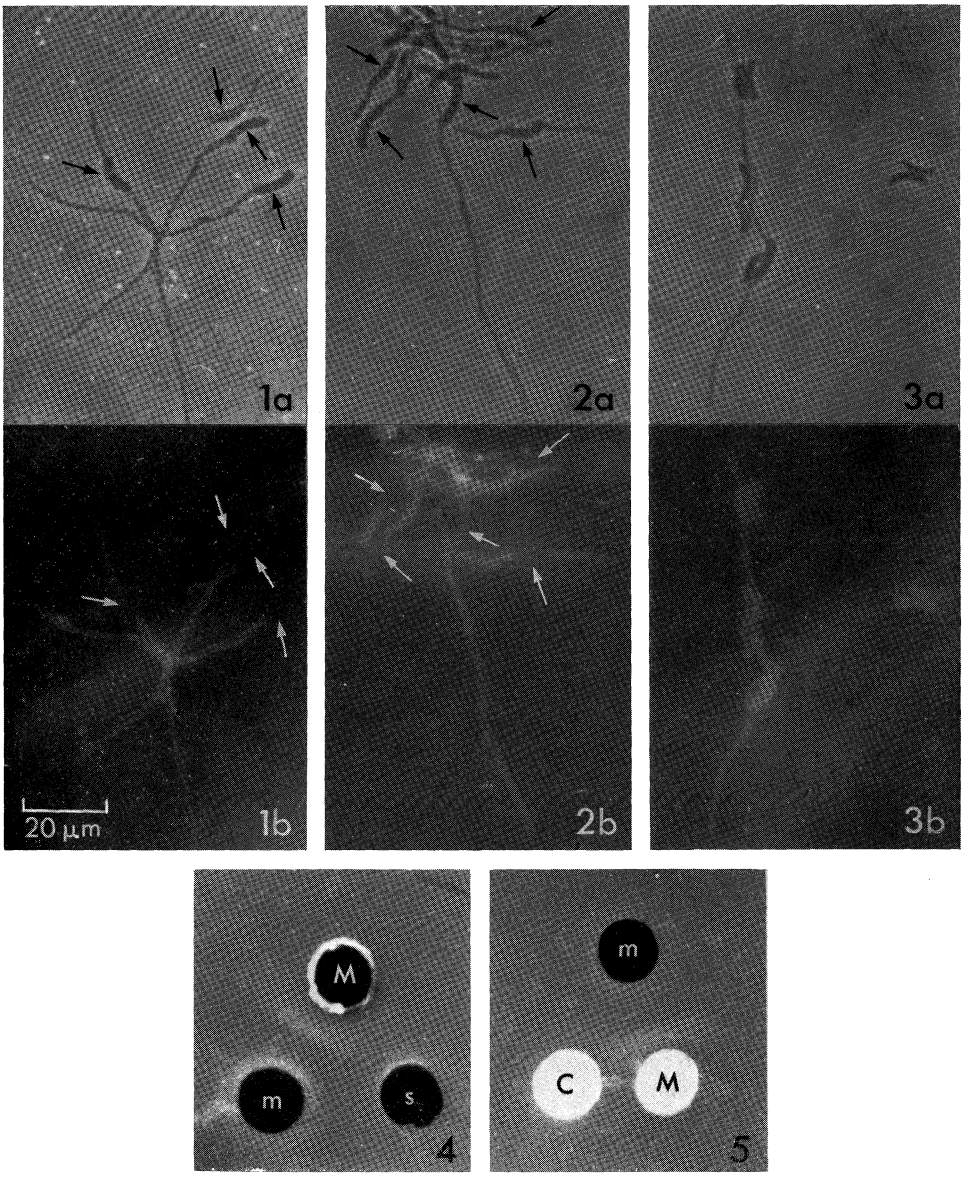
Gel-diffusion tests with mycelial antigen showed a positive reaction with homologous antiserum but no reaction could be detected with anti-ascospore serum (Fig. 4). Specificity was thus confirmed for the *soluble* mycelial antigens and the fluorescence observed when *E. armeniacae* mycelium was treated with the three sera is attributed to a non-specific reaction of the cell walls.

Strong fluorescence of ascospores of *Cryptovalsa ampelina* (Nits.) Fuckel was observed when tested with antiserum prepared against *E. armeniacae* ascospores, indicating antigenic relationship between ascospores of these two species. However, both immunofluorescence and gel-diffusion tests (Fig. 5) failed to demonstrate any antigenic relationship between the mycelia of these two fungi.

Discussion

Serological techniques for the detection and identification of fungi, recently reviewed by Preece and Cooper (1969), have not been widely used but have obvious potential. From our preliminary experiments with *E. armeniacae* two points of interest emerge:

(1) Ascospore and mycelial antigens of this fungus appear to differ; however, our experiments do not exclude the possibility that the two structures may have some antigens in common because the antiserum to ascospores probably contained antibodies only to spore-wall antigens. Results of the immunofluorescence experiments indicate that the anti-mycelium serum contained antibodies to intracellular antigens, but the presence of antibodies to



Figs. 1–3.—Photomicrographs of germinating *E. armeniacae* spores stained with fluorescein-labelled antibody by the indirect method (see text) and examined with phase-contrast (*a*) and ultraviolet (*b*) optics. 1, Stained with normal rabbit serum. 2, Stained with anti-mycelium rabbit serum. 3, Stained with anti-ascospore rabbit serum. Arrows in Figures 1(*a*), 1(*b*), 2(*a*), and 2(*b*) point to the ascospores.

Fig. 4.—Serological reaction in a gel-diffusion test between *E. armeniacae* mycelium extract (*M*) and homologous antiserum (*m*) and antiserum prepared against *E. armeniacae* ascospores (*s*).

Fig. 5.—Serological reaction in a gel-diffusion test between mycelial extracts of *E. armeniacae* (*M*) and *C. ampelina* (*C*) and antiserum prepared against *E. armeniacae* mycelium extract (*m*).

cell-wall antigens was difficult to assess owing to non-specific fluorescence of cell walls, a problem also encountered with other fungi (Preece and Cooper 1969). If intracellular antigens (mainly proteins) are more immunogenic than cell-wall antigens (mainly polysaccharides) there may be immunogenic competition within the animal. To ensure a better response to antigens, it may be necessary to use purified cell walls as the cell-wall immunogen.

(2) It appears that the mycelia of *E. armeniacae* and *C. ampelina* are antigenically distinct, making serological tests a convenient method for distinguishing the two species, the gel-diffusion technique being particularly suitable. However, ascospores of these two species appear to be antigenically similar, and although cross-absorbed sera may be of use in identification, serological methods are unlikely to be of practical value. Amos and Burrell (1967) also found antigens common to the spores of two or more species of *Ceratocystis* when whole spores were used as the immunogen.

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