SEROLOGICAL IDENTIFICATION OF *EUTYPA ARMENIACAE*

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Abstract

The production of antiserum specific to *E. armeniacae* is described. *E. armeniacae* was identified by immunodiffusion tests; cross-reactions did not occur with isolates of *Eutypella virescens*, *Eutypella parasitica*, *Valseutypella tristicha*, *Valsa pini*, *Valsa eugeniae*, *Valsa ambiens*, *Leucostoma cincta*, *Leucostoma* sp., or *Cytospora* sp. The antiserum was used to identify isolates from diseased apricot trees from Switzerland and South Africa.

I. INTRODUCTION

Eutypa armeniacae Hansf. & Carter, the causal organism of apricot "gummosis" or "dieback", does not produce perithecia in culture (Carter 1957) and is therefore difficult to identify. Isolates from diseased apricot wood have previously been identified in culture solely on their morphological similarity to isolates of *E. armeniacae* derived from ascospores. Isolates of the pathogen, however, can show sufficient variation in their morphology for them to be confused with the vegetative states of other Ascomycetes which may colonize dead vascular tissue below pruning wounds of apricot trees. This has caused diagnostic problems especially in relation to epidemiological studies of the disease (Carter and Moller 1967). Francki and Carter (1970) prepared antisera to *E. armeniacae* ascospores and mycelium and attempted to identify the fungus more precisely by immunodiffusion and immunofluorescence techniques. They found that ascospores and mycelium differed antigenically from each other. Cross-reactions occurred when anti-ascospore serum was tested against ascospores of *Cryptovalsa ampelina* (Nits.) Fuckel, but the antimycelial serum did not show a similar response against mycelium of *C. ampelina*. However, the antisera were not tested against other species of fungi.

This paper reports further work on the preparation of an antiserum specific to *E. armeniacae* and its use in identifying the fungus.

II. MATERIALS AND METHODS

Ascospores of *E. armeniacae* were collected as previously described (Francki and Carter 1970) and plated on sterile Czapek–Dox agar containing 0·05% Difco yeast extract (CDY). Disks, 1 cm in diameter, were cut from the edge of a growing colony with a sterile cork-borer and transferred to 250-ml Erlenmeyer flasks containing 50 ml liquid CDY medium (pH 5·6) and 0·01% streptomycin. Inoculated flasks were placed on a rotary shaker (100 r.p.m.) and incubated at 25°C.

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Mycelial antigen was prepared by harvesting the mycelium obtained from four inoculated flasks after incubation for 5 days on a double layer of sterile muslin in a Buchner funnel. The fungal mats were washed three times with sterile distilled water, formalinized by suspending in 0·5% formaldehyde in phosphate-buffered saline (PBS, 8·5 g NaCl, 1·07 g anhydrous Na_2HPO_4, 0·39 g $NaH_2PO_4.2H_2O$, distilled water 1 litre pH 7·1), centrifuged at 3000 r.p.m. for 10 min at 4°C, re-suspended in PBS, and homogenized in a sterile blender (Servall Omni-Mix) for 2 min at 120 V. The turbidity of the suspension was adjusted to 42% transmission at 550 nm (10·6 mg/ml dry wt.) in an EEL colorimeter and 2-ml aliquots were then dispensed in sterile tubes and stored at −18°C.

Preparation of Antisera

A rabbit was bled before being injected subcutaneously with 0·5 ml of mycelial antigen emulsified with an equal volume of Freund's complete adjuvant. Three further 1-ml injections of emulsified antigen were given at weekly intervals followed by a final injection 2 weeks later. The rabbit was bled from the ear vein 5 days after the last injection and again after a further 2 weeks. A second series of injections was given a year later. The rabbit was injected at weekly intervals with 1·0, 1·0, and 0·7 ml of emulsified antigen, bled the fourth week, injected with 0·4 ml the fifth week, followed by a final bleeding 1 week later.

Blood samples were allowed to clot and then centrifuged at 3000 r.p.m. for 10 min at 4°C. The serum was extracted and stored at −18°C with thiomersalate (1 : 10,000).

Immunodiffusion tests were carried out as described by Francki and Carter (1970). Soluble fungal antigen was prepared as follows: Cultures of the test organisms were grown in liquid CDY medium on a rotary shaker for 1 week, harvested on muslin, washed twice with distilled water, suspended in 20 ml sterile PBS, and homogenized in a Servall Omni–Mix for 2 min at 120 V. The homogenized suspension was ground in a glass tissue homogenizer for 5 min at 4°C, and disintegrated for 10 min in a MSE 50 ultrasonic disintegrator. The ultrasonically dispersed suspension was centrifuged at 3000 r.p.m. for 10 min to remove the hyphal cells, and the supernatant then centrifuged at 15,000 *g* for 30 min to remove cell walls. Protein concentration was measured by the method of Lowry *et al.* (1951) and adjusted to a concentration of 2·0 mg protein per millilitre.

Immunofluorescence tests were carried out by the indirect method. Mycelial smears were made on clean glass slides with a drop of Haupt's adhesive. They were allowed to dry, fixed in acetone for 10 min at 4°C, irrigated with rabbit antiserum, and incubated in a moist chamber for 30 min. The smears were then washed with PBS for 10 min, irrigated with sheep anti-rabbit immunoglobulin conjugated with fluorescein isothiocyanate, and incubated in a moist chamber for 30 min. The smears were again washed with PBS for 10 min, mounted in phosphate-buffered glycerol (PBS 10%, glycerol 90%, pH 7·2) and examined under ultraviolet illumination with a Leitz Ortholux microscope fitted with primary filters BG12 (5 + 1 mm) and BG38 (4 mm) and secondary filter K530.

III. Results

Immunodiffusion tests of antiserum with *E. armeniacae* antigen revealed the presence of antibodies; a titre of 1/16 was obtained after the first series of injections and a titre of 1/64 after the second series. Unidentified fungal isolates from field experiments were tested for specificity against the antiserum from the first series. Those with close morphological similarity to *E. armeniacae* when grown on CDY gave a positive reaction in gel-diffusion plates. Cultures with less morphological similarity to *E. armeniacae* on agar media were also tested. These were even less similar morphologically when grown in liquid media, often producing a few rounded balls of hyphae instead of the abundant mycelium produced by *E. armeniacae*. These isolates, without exception, gave a negative reaction in gel-diffusion plates. Although some degree of specificity was indicated by these results it was necessary to test the antiserum against a wider variety of known isolates of related species of fungi.

A year later, 11 named isolates were obtained from culture collections in America, England, and Europe. Nine isolates, from apricot trees showing dieback symptoms, were obtained from Switzerland (Carter and Bolay 1972) and South Africa for identification. All these fungi were tested against the antiserum from the

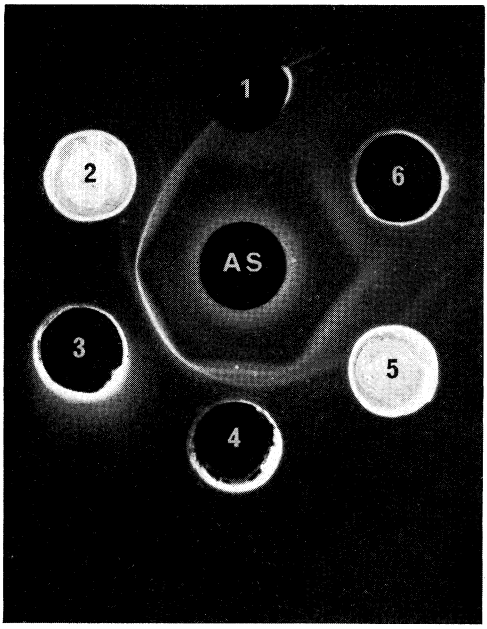

Fig. 1.—Serological reactions in a gel-diffusion test between *E. armeniacae* anti-mycelial serum (AS) and cell wall-free antigens from mycelia of: (1) B887; (2) IMI 165987; (3) B885; (4) IMI 165988; (5) B886; (6) B888. See Table 1 for origins of mycelia.

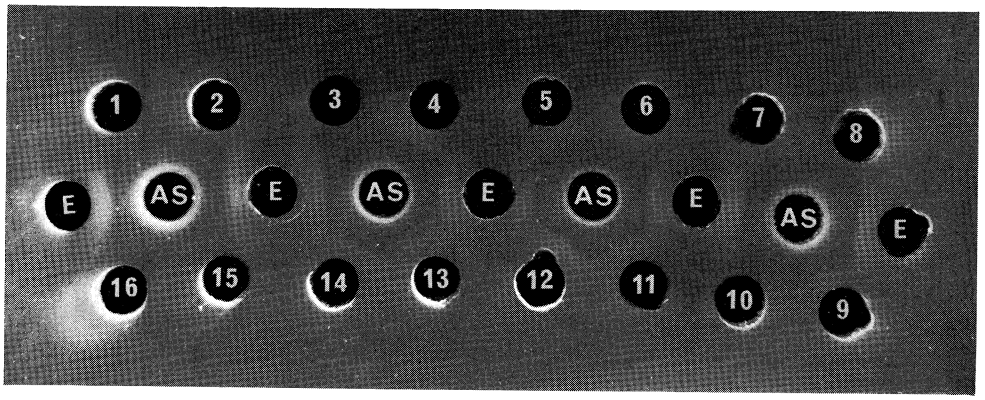

Fig. 2.—Serological reactions in a gel-diffusion test between *E. armeniacae* anti-mycelial serum (AS) and cell wall-free antigens from mycelia of (E) *E. armeniacae* IMI 165987: (1) IMI 148025; (2) CBS205·36; (3) blank, (4) B888; (5) SA79; (6) B887; (7) IMI 165988; (8) M746; (9) mycelial isolate 1; (10) M704; (11) B886; (12) B885; (13) SA80; (14) SA106; (15) CBS210·39; (16) SA87. See Table 1 for origins of mycelia.

second series. In order to produce sufficient mycelium those that grew poorly on CDY medium were grown on malt extract medium (malt extract 2%, peptone 0·1%, dextrose 2%) in still culture.

The results (Table 1) show that the antimycelial serum was specific to *E. armeniacae*. Isolates identical with *E. armeniacae* produced precipitation bands which joined each other (Fig.1) whereas closely related fungi gave none (Fig. 2).

TABLE 1

ISOLATES TESTED BY GEL-DIFFUSION ASSAY AGAINST *E. ARMENIACAE* ANTI-MYCELIAL SERUM

Isolate No.*	Organism	Host	Origin	Immuno-diffusion reaction (±)
IMI 165987	*Eutypa armeniacae* Hansf. & Carter	*Prunus armeniaca*	*South Australia*	+
IMI 165988	*E. armeniacae*	*Prunus armeniaca*	California	+
CBS210·39	*Eutypella parasitica* Davidson & Lorentz	—	Netherlands	−
CBS205·36	*Eutypella virescens* Wehmeyer	—		−
CBS465·59	*Valseutypella tristicha* (de Not) Hohnel	—		−
IMI44946	*Valsa eugeniae* Nutman & Roberts	*Eugenia* sp.	Zanzibar	−
IMI80102	*Valsa pini* (Albertini & Schweintz) Fries	*Pinus sylvestris*	Norway	−
M768	*Valsa ambiens* (Persoon ex Fr.) Fries	*Pyrus malus*	Switzerland	−
M662	*Valsa* sp.	*Pyrus malus*		−
4109	*Leucostoma cincta* (Fr.) v. Hohn.	*Prunus armeniaca*		−
M746	*Leucostoma* sp.	*Prunus armeniaca*		−
M704	*Leucostoma* sp.	*Prunus spinosa*		−
29	*Cytospora* sp.	—	Illinois	−
IMI 148025	*Fusarium lateritium* Nees	*Prunus armeniaca*	South Australia	−
SA79	Mycelial isolates	*Prunus armeniaca*	South Africa	+
SA80				+
SA87				−
SA89				−
SA106				+
B885			Switzerland	+
B886				+
B887				+
B888				+
1			South Australia	+
2				+
3				−
4				−
5				−
6				−
7		*Prunus persica*		+
8		*Prunus domestica*		−
9		*Prunus amygdalus*		+

*IMI, Commonwealth Mycological Institute, Kew; CBS, Centralbureau voor Schimmelcultures, Baarn, Netherlands; M, Professor H. Kern, Zurich; B, Dr. A. Bolay, Nyon, Switzerland; SA, Dr. Erasmus, Stellenbosch, South Africa.

The fluorescent antibody staining technique was also applied to the test organisms. Unstained preparations showed no fluorescence but stained preparations treated with normal serum and antiserum gave variable results. Generally the fluorescent antibody technique was less reliable than the gel diffusion assay.

IV. Discussion

Serological tests using gel diffusion assay have been used to determine relationships between a wide range of species and strains of fungi such as *Fusarium* (Tempel 1957; Madhosingh 1964*a*), *Fomes* (Madhosingh 1964*b*; Gooding 1966), *Cronartium* (Gooding and Powers 1965), *Pythium* and *Phytophthora* (Morton and Dukes 1967; Gill and Powell 1969), *Rhizoctonia* and *Ceratobasidium* (Manning *et al.* 1967) and *Ascochyta* (Madhosingh and Wallen 1968). Unfortunately, isolates of other *Eutypa* species were unobtainable; however, the present study indicates that antiserum to *E. armeniacae* mycelium is highly specific and could be used to identify the fungus by gel-diffusion assay. The technique was also successfully used to identify unknown isolates from diseased apricots and other stone fruits which were suspected of being *E. armeniacae*.

A major problem in the use of serology for the identification of fungi has been lack of specificity. This has been partly owing to the different antigenic components of the mycelium. Francki and Carter (1970) suggested that cell walls may be more immunogenic and that the use of purified fungal cell walls may lead to a more specific response. Holland and Choo (1970) used immunoelectrophoresis to examine the precipitation bands obtained when whole cells and cell walls of *Ophiobolus graminis* Sacc. were used as the antigen. They obtained a greater number of bands with antiserum to whole-cell antigen, but found the antiserum to cell walls to be more specific. Gooding (1966) found that crude homogenates of *Fomes annosus* (Fr.) Karst gave rise to multiple precipitation bands whereas purified macromolecular antigens gave a single precipitation band. In the present study, a single precipitation band was obtained when cell wall-free extract was used as the antigen in gel-diffusion tests.

The present study has yielded a reliable method for identifying a pathogen which, although widely distributed in temperate regions of the world, has hitherto been generally overlooked, and recognized only by those familiar with work on apricot dieback at this Institute.

V. Acknowledgments

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