

Serological Studies on Some Australian Isolates of *Verticillium* spp.

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Abstract

The production of antisera to three mycelial fractions of *V. dahliae* is described. Serological reactions with antigens from similar fractions of other fungi, including closely related species, were examined and specific sera, essential for immunofluorescent studies, were developed.

Gel-diffusion studies showed that *V. dahliae* shares extremely close serological affinities with *V. alboatrium* while *V. tricornutum* shares less, and *V. nigrescens* very little, antigenic resemblance to *V. dahliae*. Immunoelectrophoretic separation using γ -globulin from two anti-*V. dahliae* sera demonstrated the distinction between *V. dahliae* and *V. alboatrium*.

Detection of *V. dahliae* among other common root-inhabiting fungi was possible using immunofluorescence. Distinction between *V. dahliae* and *V. alboatrium* was also shown using cross-absorbed conjugate. The technique proved to be more sensitive than gel diffusion.

Introduction

The taxonomic distinction between species of *Verticillium* has long been the subject of controversy. This dispute has centred on the relationship between *Verticillium dahliae* Kleb. and *V. alboatrium* Reinke & Berth. Morphological and physiological studies have led some workers to separate these two species (Isaac 1949; Smith 1965). However, other researchers recognize only the one species, *V. alboatrium* (Wilhelm 1948; Brandt 1964).

Serological studies using gel diffusion and immunoelectrophoresis have been made to determine inter- and intraspecific similarities between a wide range of fungi. In studies on species of *Verticillium*, Wyllie and DeVay (1970), Schnathorst (1973) and Teranishi *et al.* (1973) used the gel-diffusion technique with antigens from conidial preparations. Serological heterogeneity and homologous cross-reactions were reported between species. Teranishi *et al.* (1973) showed that *V. alboatrium* and *V. dahliae* contain distinctly different antigenic compounds and that *V. tricornutum* Isaac has little antigenic relationship to either of these species. However, Schnathorst (1973) failed to demonstrate any serological distinction between *V. dahliae* and *V. alboatrium*.

Antigens from mycelial preparations may reveal more reliable serological information compared to conidial suspensions as conidial antigens have been shown to vary within individual species (Pelletier and Aube 1970). Holland and Choo (1970) found that preparations of whole-cell and cell-wall fractions of mycelia could be used as determinant antigens; however, the cell-wall fraction gave greater specificity.

This paper describes the production of antisera to three mycelial fractions of *V. dahliae* to investigate serological affinities between this fungus and other common root-inhabiting fungi, including closely related species. Specific antisera were then

used to develop fluorescent conjugates specific to mycelia of *V. dahliae* to enable rapid differentiation between *V. dahliae* and *V. alboatrum*, *V. tricornis* and *V. nigrescens* Pethybridge.

Materials and Methods

Fungal Cultures

Isolates of *V. dahliae* and *V. tricornis* were trapped from roots of common thornapple (*Datura stramonium* L.) and *V. nigrescens* from roots of fat hen (*Chenopodium album* L.) using the method of Evans and Gleeson (1973). Isolates of *Verticillium* spp. were collected from respective plant species growing in different localities in the Namoi Valley, N.S.W. *V. alboatrum* has not been recorded in New South Wales, so isolates from tomato (*Lycopersicon esculentum* Mill.) were obtained from South Australia. All cultures were maintained on Czapek-Dox slopes.

Preparation of Antigens

Cultures were grown on Czapek-Dox liquid medium in 750-ml Erlenmeyer flasks containing 270 ml of medium. Before dispensing, the culture solution was autoclaved for 15 min (121°C, 137.8 kPa) and filtered to remove precipitates. Each flask was then reautoclaved at the same temperature and pressure.

Flasks were inoculated with conidia washed from agar slopes with 2.5 ml of sterile water. Twenty isolates of each particular species were used to inoculate antigen-producing cultures. This was to offset any variation in cytoplasmic content which may occur within isolates of individual species. These were incubated as still cultures in continuous light for 7 days at 25°C. (Constant light reduced the tendency for *V. dahliae* and *V. tricornis* to form microsclerotia.)

Mycelia were harvested by filtering through sterile cheese-cloth in a contamination-free chamber, then washed with 5 litres of sterile phosphate-buffered saline, pH 7.2 (PBS: 0.14 M NaCl, 0.0025 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 0.0075 M Na_2HPO_4). Washed mycelia were diluted with 15 ml of chilled PBS, macerated for 15 min in a sterile Sorvall Omni-mixer (immersed in ice), and then divided into three equal portions, one of which was poured into 28-ml McCartney bottles and stored at -20°C as the whole-cell antigen. The remaining macerate was transferred to sterile centrifuge tubes and disintegrated by an MSE L.667 ultrasonic disintegrator (amplitudes 6-8) in two 15-min runs (1 h between each). The disrupted suspension was then centrifuged (12 000 g, 20 min, 4°C) to separate the cell-wall and soluble fractions. The grey lipid layer which formed on the top of the soluble antigen was carefully pipetted off, as was the soluble fraction which was stored as described previously. The cell fraction was washed in PBS, recentrifuged and collected by resuspending in 2.5 ml PBS. Protein concentrations of all antigens were measured by the method of Lowry *et al.* (1951).

Cell-wall and soluble antigens were also prepared from mycelia of *Pythium irregulare* Buisman, *Fusarium oxysporum* Schlecht. and *Cylindrocarpum destructans* (Zinns.) Scholten, all common root-inhabiting fungi.

Preparation of Antisera

Six albino rabbits (2-3 kg), two for each *V. dahliae* antigen, were bled and then injected. Initially, three intramuscular injections (1 ml antigen emulsified with 1 ml Freund's complete adjuvant) were administered to rabbits at fortnightly intervals. After 6 weeks, eight weekly intravenous injections (0.5 ml antigen without adjuvant) were given and a final bleed taken at the thirteenth week when as much blood as possible was collected from the marginal ear vein. Sera were filtered (0.45 µm cellulose acetate) and stored frozen (-20°C) in 5-ml aliquots.

Purification of γ -Globulin from Crude Sera

The method used was essentially that of Horejsi and Smetana (1956) using Rivanol precipitation for the removal of albumin and the α - and β -globulins.

Cross-absorption of γ -Globulin

Equal volumes of freshly prepared antigen and serum to be absorbed were mixed and incubated at 4°C for 24 h. The absorbed serum was centrifuged at 12 000 g and reincubated. This procedure was repeated until no further precipitate appeared.

Conjugation of Fluorochrome

The protein concentrations of the purified cell-wall and soluble γ -globulin to be conjugated were estimated by the method of Porter (1955). These globulins were conjugated with fluorescein isothiocyanate (FITC, Calbiochem, Los Angeles, California, U.S.A.) by the methods of Marshall *et al.* (1958) and Riggs *et al.* (1958), which resulted in chromatographically pure conjugate.

Immunodiffusion Plates

Diffusion plates, diam. 85 mm, were prepared using 20 ml of agar (consisting of 0.75% (w/v) Oxoid Ionagar No. 2 in 0.02 M phosphate buffer, pH 7.2, containing 0.14 M NaCl and 0.02% (w/v) sodium azide) per Petri dish. Wells, 6 mm in diameter and 4 mm apart, were cut in a symmetrical fashion. Equal volumes of fresh crude antigen (2.5 mg/ml protein) and antisera to be challenged were placed in appropriate wells. For titre tests, antibody dilutions ranging from 2 to 256 were used. Gel plates were incubated for 2–3 days at 25°C or 5–7 days at 4°C and observed by dark field illumination.

Immunoelectrophoresis

Apparatus used combined a Shardon type 2541 power supply with a Gelman electrophoresis chamber and immunoframes. Each immunoframe held three, 25 by 75 mm glass slides. Slides were washed in chromic acid, placed in frames and painted with a thin layer of adhesive agar (consisting of 0.1 g agar and 0.5 ml glycerine in 100 ml distilled water). This was allowed to dry before molten gel (consisting of 1% (w/v) Oxoid Ionagar No. 1 in barbital (Veronal) buffer, pH 8.6, with 0.02% (w/v) sodium azide) was slowly pipetted onto each frame. Two wells, 6 mm in diameter and 7 mm apart, were cut into each slide and filled with appropriate crude antigens. Nine slides were used for each test. Frames were loaded into the chamber and connected with the electrophoretic buffer (Tris–barbital sodium–barbital, pH 8.8, Gelman Instrument Co., Michigan) by soaked, lintless paper wicks.

Electrophoretic separation of cell-wall and soluble antigens of *V. dahliae* and *V. alboatrum* were performed independently at room temperature as follows: cell-wall antigens were separated by a current of 2 mA per slide for 1.5 h with a potential difference across the frame of 6.1 volts/cm. Soluble antigens received 1 mA per slide for 3 h with a potential difference of 3.48 volts/cm. During separation, wells were kept full with PBS to avoid tension points developing as the agar dried.

Immediately following electrophoresis, frames were removed and a central trough, 3 mm wide and 4 cm long, was cut out of the agar. This trough was filled with the globulin fraction of the appropriate antiserum and cross-reacted for 3 days in a humid chamber at 25°C before being washed in physiological saline. Immunograms were examined by dark field illumination.

Fluorescent Antibody Staining

Cell-wall and soluble FITC conjugates were used to label *V. dahliae* among hyaline mycelia of *V. alboatrum*, *V. tricorpus*, *V. nigrescens*, *P. irregulare*, *F. oxysporum* and *C. destructans*. Mycelia of all fungi were mixed together in watch glasses and stained by the direct method (Malajczuk *et al.* 1975) for 3 h before being washed with PBS for 1 h. Staining was done at 25°C under darkness to avoid deterioration of the fluorochrome. Mycelial strands were transferred to a microscope slide and mounted in carbonate-buffered glycerol (pH 8.7) consisting of one part of the carbonate buffer to nine parts of glycerol.

Examination of Slides

Immunofluorescent observations were made with a Leitz Ortholux II microscope using Narrow Band Blue (NBB) incident excitation from an HBO 200 W mercury vapour lamp (Osram). The filter system used consisted of a heat absorption filter (4 mm BG38) and blue excitation filters (2KP490 + 1 mm GG475), coupled with a dichroic mirror (TK510) and barrier filters (K510, K530) (Leitz Wetzlar, Germany).

Results

Immunodiffusion Analyses

Homologous and heterologous cross-reactions and absorption tests were carried out using both crude serum and purified γ -globulin. The specificity of anti-*V. dahliae*

whole-cell, cell-wall and soluble crude sera was checked against homologous fractions of other common root-inhabiting fungi (described previously). No cross-reactions were seen to occur, indicating a level of generic specificity in unpurified sera. Homologous reactions with diluted crude sera (Table 1) showed that antibodies against soluble antigen were produced in the highest concentration. More precipitin bands formed to whole-cell and soluble sera than to cell-wall serum.

Table 1. Number of precipitin bands formed on immunodiffusion between series diluted antisera and their homologous antigens

<i>V. dahliae</i> antiserum	Antiserum titre level							
	1	2	4	8	16	32	64	128
Whole-cell	9	6	6	4	4	2	1	—
Soluble	7	7	5	3	3	3	1	1
Cell-wall	5	5	5	3	1	1	1	—

Whole-cell serum produced numerous non-specific cross-reactions during initial specificity tests with antigen from individual *Verticillium* spp. This serum was eliminated from further serological studies.

The serological relationship between *V. dahliae* and other species was examined using both crude and purified sera. Results are expressed in Table 2. In homologous

Table 2. Number of precipitin bands formed in homologous and heterologous immunodiffusion reactions between anti-*V. dahliae* sera and soluble and cell-wall antigens of four *Verticillium* species

Anti- <i>V. dahliae</i> serum	Number of precipitin bands							
	Soluble antigen				Cell-wall antigen			
	<i>V.d.</i> ^A	<i>V.aa.</i> ^B	<i>V.tri.</i> ^C	<i>V.nig.</i> ^D	<i>V.d.</i>	<i>V.aa.</i>	<i>V.tri.</i>	<i>V.nig.</i>
Not cross-absorbed								
Crude serum								
<i>V.d.</i> (soluble)	7 ^E	4 ^E	4 ^E	2 ^E	2	1	1	0
<i>V.d.</i> (cell-wall)	3	2	0	0	6 ^E	4 ^E	3 ^E	0 ^E
Gamma-globulin								
<i>V.d.</i> (soluble)	2 ^E	2 ^E	1 ^E	1 ^E	1	1	0	0
<i>V.d.</i> (cell-wall)	1	1	0	0	3 ^E	2 ^E	1 ^E	0 ^E
Cross-absorbed (crude serum)								
Soluble								
with <i>V.tri.</i> soluble antigens	2 ^E	1 ^E	0 ^E	0 ^E				
with <i>V.aa.</i> soluble antigens	1 ^E	0 ^E	0 ^E	0 ^E				
Cell-wall								
with <i>V.tri.</i> cell-wall antigens					2 ^E	2 ^E	0 ^E	0 ^E
with <i>V.aa.</i> cell-wall antigens					0 ^E	0 ^E	0 ^E	0 ^E

^A*V.d.*, *V. dahliae*. ^B*V.aa.*, *V. alboarum*. ^C*V.tri.*, *V. tricornis*. ^D*V.nig.*, *V. nigrescens*.

^EHomologous fraction reaction.

fraction reactions involving cell-wall and soluble sera, fewer cross-reactions were observed when using cell-wall antigens of other species compared with reactions to soluble antigens (Fig. 1). This was the case when using both crude and purified globulin. Antibodies to cell-wall antigens were therefore regarded as being more specific to *V. dahliae* than antibodies to soluble antigens.

Heterologous fraction reactions with soluble and cell-wall antisera revealed the presence of common globulins within each antiserum. However, each antiserum displayed sufficient specific reaction for it to be identified using the gel-diffusion technique. Specific antisera, however, could only be demonstrated after cross-absorption of crude anti-*V. dahliae* soluble serum with soluble antigens of *V. alboatrum*. Cross-absorption with cell-wall antigens and serum did not produce a specific precipitin band. Absorption of γ -globulin resulted in no lines being formed.

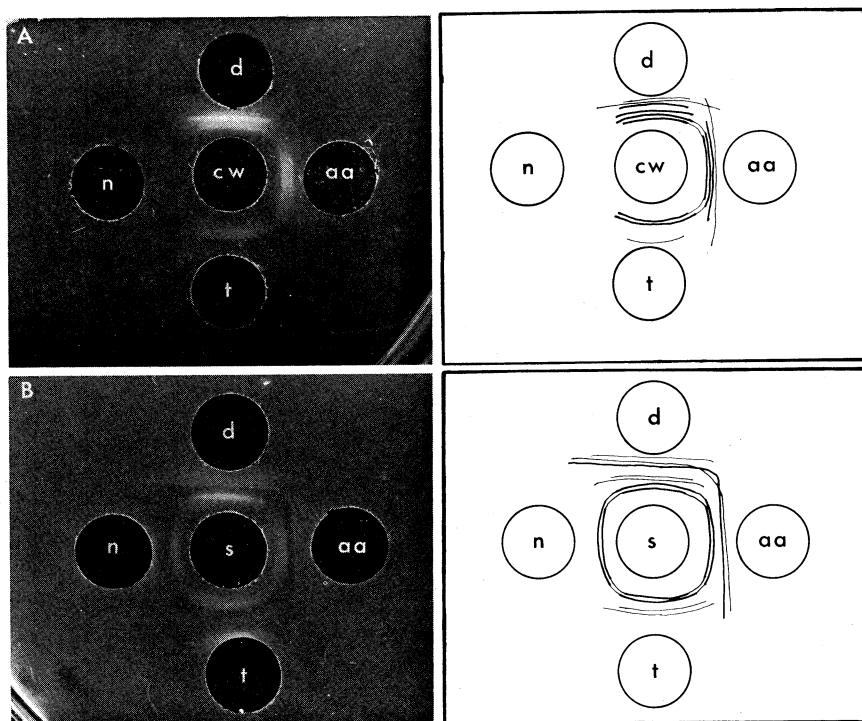


Fig. 1. Precipitin reactions in agar gel. (A) Cell-wall antigens of *V. dahliae* (d), *V. alboatrum* (aa), *V. tricorpus* (t) and *V. nigrescens* (n) challenged with crude anti-*V. dahliae* cell-wall serum (cw). (B) Soluble antigens of *V. dahliae* (d), *V. alboatrum* (aa), *V. tricorpus* (t) and *V. nigrescens* (n) challenged with crude anti-*V. dahliae* soluble serum (s).

Immunoelectrophoretic Analyses

Gel-diffusion reactions using soluble and cell-wall antigens showed that *V. dahliae* has extremely close serological affinities with *V. alboatrum*. In addition, antisera against cell-wall and soluble antigens share common globulins. This was particularly so when antisera consisted of γ -globulin.

Specific anti-*V. dahliae* γ -globulin is a prerequisite for immunofluorescent studies. It was therefore imperative to show that the γ -globulin fractions of cell-wall and soluble antisera are serologically unique and that these globulins react differently from antigens from *V. dahliae* and *V. alboatrum*. This was attempted without cross-absorption using thin-layer immunoelectrophoresis.

Differentiation was shown between antigens contained in soluble and cell-wall mycelial fractions of *V. dahliae* (Fig. 2A) and antigenic components of *V. dahliae* and

V. alboatrum (Figs 2B, 2C). A separate, distinct, antigen-antibody complex was consistently formed to *V. dahliae* when either cell-wall or soluble fraction reactions were employed.

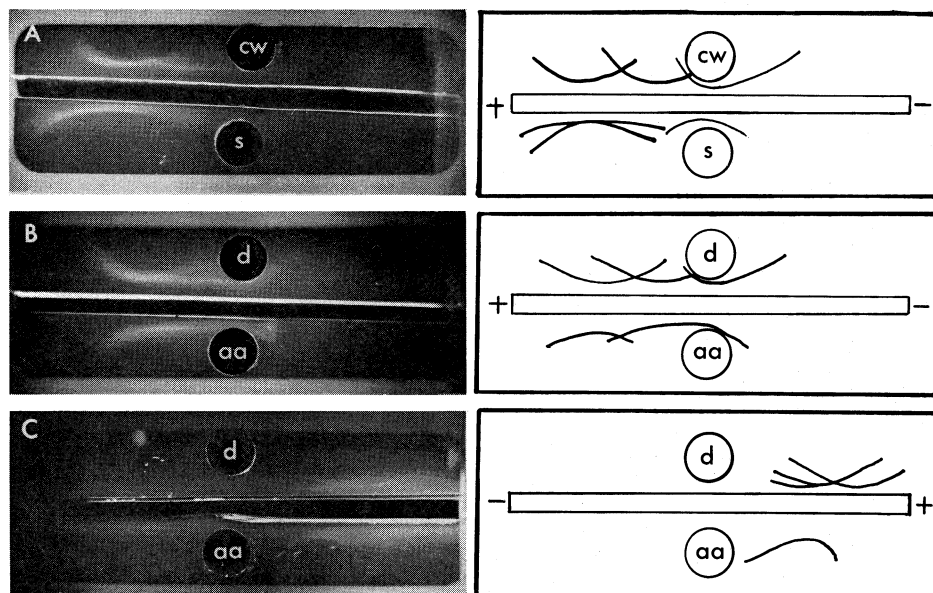


Fig. 2. Immunogram. (A) Cell-wall (cw) and soluble (s) antigens from *V. dahliae*, electrophoresed then challenged with anti-*V. dahliae* cell-wall γ -globulin. (B) Cell-wall and (C) soluble antigens of *V. dahliae* (d) and *V. alboatrum* (aa), electrophoresed and challenged with anti-*V. dahliae* homologous γ -globulin.

Fluorescent Identification of V. dahliae in Mixed Cultures

This technique was investigated as a further means of distinguishing mycelia of *V. dahliae* from hyaline mycelia of morphologically similar fungi including other species of *Verticillium*.

V. dahliae was easily recognized among mixed mycelial cultures of *V. tricorpus*, *V. nigrescens*, *P. irregulare*, *F. oxysporum* and *C. destructans* (Fig. 3). Both cell-wall and soluble conjugates appeared to have little specificity in staining different morphological structures. Both conjugates reacted strongly with conidia as indicated by the level of fluorescence. In addition, hyphal tips and cell walls reacted more strongly than hyphal cytoplasm when stained with either conjugate.

V. dahliae hyphae could not be distinguished from those of *V. alboatrum* using the conjugates in their existing form. Fluorescence from hyphae of these two fungi appeared similar both in colour and intensity. To distinguish between these species with either conjugate it was necessary to cross-absorb anti-*V. dahliae* sera with homologous antigen from *V. alboatrum* before staining. Fluorescent comparison of *V. dahliae* and *V. alboatrum* is seen in Fig. 4. The intensity difference could only be resolved using oil immersion since cross-absorption resulted in overall diminution in fluorescence. Cross-absorption of conjugated cell-wall immunoglobulin resulted in the greatest loss of fluorescent intensity. Although cross-absorption of γ -globulin resulted in no formation of specific precipitin bands in agar gel, the fluorescent

antibody technique was sufficiently sensitive to detect the small amount of remaining conjugated globulin which is specific to *V. dahliae*. Autofluorescence, which is enhanced by the use of oil immersion, accounts for a major proportion of the fluorescent image of *V. alboatrum* in Fig. 4B.

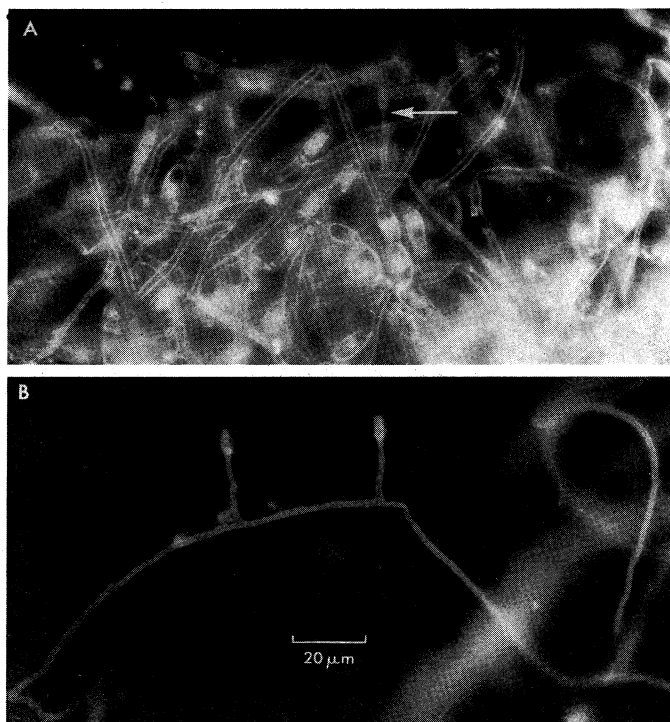


Fig. 3. Dark field and fluorescent photomicrographs. Hyphae of *V. dahliae*, *V. nigrescens*, *V. tricorpus*, *P. irregulare*, *F. oxysporum* and *C. destructans* stained with FITC cell-wall conjugate by the direct method and viewed under (A) dark field and (B) NBB incident light through the Leitz Ortholux II fluorescence microscope. Only hyphae of *V. dahliae* indicated by arrow in (A) were seen to fluoresce in (B). Plus-X film (Kodak Pty Ltd); exposure time (A) 11 s, (B) 2 min.

Discussion

Results presented here support the observations of Holland and Choo (1970) that the increased number of precipitin bands shown by whole-cell antigens over cell-wall antigens indicates the existence of antigens in the soluble fraction of fungal cells not found in the cell wall. This was verified by gel-diffusion and immunoelectrophoretic studies where antisera to soluble and cell-wall fractions were shown to be distinct.

Teranisihi *et al.* (1973), using antisera against conidial antigens of both *V. dahliae* and *V. alboatrum*, reported that *V. tricorpus* had no serological resemblance to either of these two species. In the present study, both antigen fractions from *V. tricorpus* cross-reacted with anti-*V. dahliae* sera. Therefore, it would seem that antiserum produced to conidial antigens is more specific than that produced against mycelial preparations.

Cross-reactions also occurred between *V. nigrescens* and anti-*V. dahliae* serum; however, this was confined to antigens from the soluble fraction only. It would appear that *V. dahliae* has more serological affinities with *V. tricornis* than with *V. nigrescens*.

Conclusions drawn from gel-diffusion analyses are in complete agreement with Isaac (1967), who suggested that taxonomically *V. dahliae* is much closer to *V. alboatrum* than it is to *V. tricornis* or *V. nigrescens*. Furthermore, this work and that of Schnathorst (1973) complement each other as antisera to both mycelial and conidial antigens have produced almost identical results in gel-diffusion assays. Both studies have shown that *V. dahliae* shares extremely close serological affinities with *V. alboatrum*, while *V. tricornis* shares less, and *V. nigrescens* very little, antigenic resemblance with *V. dahliae*. However, Schnathorst (1973) failed to demonstrate any serological distinction between *V. dahliae* and *V. alboatrum*.

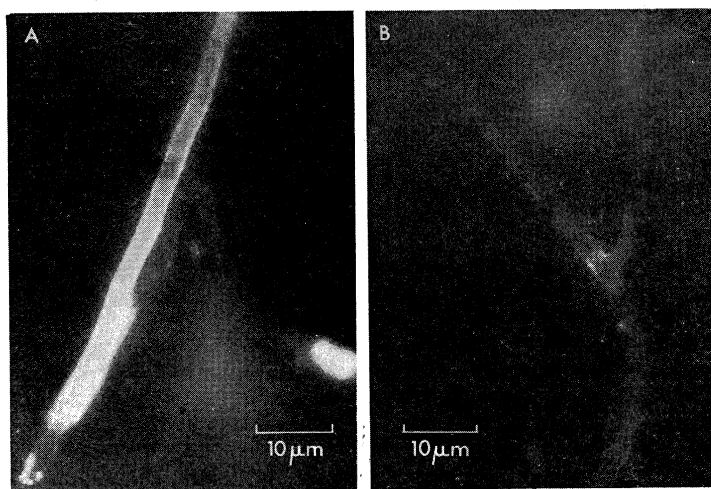


Fig. 4. Fluorescent photomicrographs. (A) *V. dahliae* and (B) *V. alboatrum* stained by the direct method with soluble conjugate which has been cross-absorbed with *V. alboatrum* soluble antigens. Plus-X film (Kodak Pty Ltd); exposure time (A) 5 min, (B) 7 min.

The distinction between *V. dahliae* and *V. alboatrum* has been clearly shown on an antigenic basis using both immunoelectrophoresis and gel diffusion. In the case of immunoelectrophoresis however, cross-absorption of antisera was not necessary. This work lends support to the thesis that *V. dahliae* and *V. alboatrum* remain as separate species and that these are the valid respective names of the microsclerotial and dark mycelial forms of *Verticillium*.

The fluorescent antibody technique has previously been used with varying degrees of success to distinguish between morphologically similar fungi. For example, Choo and Holland (1970) and Price (1973) found the technique to be less reliable than conventional serological methods. However, Al-Doory and Gordon (1963), Kumar and Patton (1964) and Amos and Burrell (1967) reported that this technique provided an excellent means for identifying morphologically similar fungi belonging to different genera or species.

To obtain reliable fluorescent distinction between hyphae of one species and morphologically similar hyphae of another species, it may be desirable to conjugate

globulins produced against the soluble fraction of sexual or asexual spores. These globulins have a number of advantages since they can be produced at a much higher concentration as shown by Schnathorst (1973) and appear to be more specific for differentiating between species as shown by Teranishi *et al.* (1973). Furthermore, the work reported here has shown that labelled globulin from soluble mycelial fractions react with both vegetative and reproductive structures. However, species differentiation by immunofluorescence may involve the comparison of fluorescent image brightness. Such a comparison is difficult to quantify (Nairn 1969).

In work reported here, *V. dahliae* mycelia could be distinguished from that of *V. alboatrum* after cross-absorption. The use of oil immersion and incident light illumination overcame the problem of fluorescence diminution following conjugate cross-absorption. Hence, the technique has taxonomic merit for rapid differentiation between species of *Verticillium*.

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References

- Al-Doory, Y., and Gordon, M. A. (1963). Application of fluorescent antibody procedures to the study of pathogenic dematiaceous fungi. *J. Bacteriol.* **86**, 332–8.
- Amos, R. E., and Burrell, R. G. (1967). Serological differentiation in *Ceratocystis*. *Phytopathology* **57**, 32–4.
- Brandt, W. H. (1964). Morphogenesis in *Verticillium*: effect of light and ultra-violet radiation on microsclerotia and melanin. *Can. J. Bot.* **42**, 1017–23.
- Choo, Y. S., and Holland, A. A. (1970). Direct and indirect fluorescent antibody staining of *Ophiobolus graminis* Sacc. in culture and in the rhizosphere of cereal plants. *Antonie van Leeuwenhoek; J. Microbiol. Serol.* **36**, 549–54.
- Evans, G., and Gleeson, A. C. (1973). Observations on the origin and nature of *Verticillium dahliae* colonizing plant roots. *Aust. J. Biol. Sci.* **26**, 151–61.
- Holland, A. A., and Choo, Y. S. (1970). Immunoelectrophoretic characteristics of *Ophiobolus graminis* Sacc. as an aid in classification and determination. *Antonie van Leeuwenhoek; J. Microbiol. Serol.* **36**, 541–8.
- Horejsi, J., and Smetana, R. (1956). The isolation of gamma-globulin from blood serum by rivanol. *Acta Med. Scand.* **155**, 65–70.
- Isaac, I. (1949). A comparative study of pathogenic isolates of *Verticillium*. *Trans. Br. Mycol. Soc.* **32**, 137–57.
- Isaac, I. (1967). Speciation in *Verticillium*. *Annu. Rev. Phytopathol.* **5**, 201–22.
- Kumar, D., and Patton, R. F. (1964). Fluorescent antibody technique for detection of *Polyporus tomentosus*. (Abstr.) *Phytopathology* **54**, 898.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–75.
- Malajczuk, N., McComb, A. J., and Parker, C. A. (1975). An immunofluorescence technique for detecting *Phytophthora cinnamomi* Rands. *Aust. J. Bot.* **23**, 289–309.
- Marshall, J. D., Eveland, W. C., and Smith, W. C. (1958). Superiority of fluorescein isothiocyanate (Riggs) for fluorescent antibody technic with a modification of its application. *Proc. Soc. Exp. Biol. Med.* **98**, 898–900.
- Nairn, R. C. (Ed.) (1969). 'Fluorescent Protein Tracing.' 3rd edn. p. 69. (E. and S. Livingstone Ltd: Edinburgh and London.)
- Pelletier, G., and Aube, C. (1970). Conidial size and contents in *Verticillium* as affected by environmental factors. *Can. J. Microbiol.* **16**, 231–6.
- Porter, R. R. (1955). The fractionation of rabbit γ -globulins by partition chromatography. *J. Biochem. (Tokyo)* **59**, 401–7.

- Price, T. V. (1973). Serological identification of *Eutypa armeniacae*. *Aust. J. Biol. Sci.* **26**, 389-94.
- Riggs, J. L., Seinwald, R. J., Burckhalter, J. H., Downs, C. M., and Metcalf, J. J. (1958). Isothiocyanate compounds as fluorescent labelling agents for immune serum. *Am. J. Pathol.* **34**, 1081-97.
- Schnathorst, W. C. (1973). Verticillium wilt of cotton. In proceedings of a work conference, National Cotton Pathology Research Laboratory, College Station, Texas, August 1971. ARS-S-19, (1973). pp. 1-19.
- Smith, H. C. (1965). The morphology of *Verticillium alboatrum*, *V. dahliae* and *V. tricorpus*. *N.Z. J. Agric. Res.* **8**, 450-78.
- Teranishi, J., Figueiredo, M. B., Cardoso, Rosa Maria G., and Namekata, T. (1973). The value of serological techniques for the differentiation between *Verticillium alboatrum* Reink and Berth. and *V. dahliae* Kleb. *Arg. Inst. Biol., S. Paulo* **40**(1), 45-51.
- Wilhelm, S. (1948). The effect of temperature on the taxonomic characters of *Verticillium alboatrum* (Abstr.) *Phytopathology* **38**, 919.
- Wyllie, T. D., and DeVay, J. E. (1970). Immunological comparison of isolates of *Verticillium alboatrum* and *V. nigrescens*, pathogenic to cotton. *Phytopathology* **60**, 1682-6.

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