Quantitative Assessment of Verticillium albo-atrum in Field Soils: Procedural and Substrate Improvements

O. C. Huisman and L. J. Ashworth, Jr.

Assistant Professor and Plant Pathologist, respectively, Department of Plant Pathology, University of California, Berkeley 94720.

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ABSTRACT

Improvements in a wet-sieving procedure for concentrating microsclerotia of *Verticillium albo-atrum* in residues of field soils, preparatory to culturing, increased assays from 18-24 to 36-40 per operator per day. Pectate (sodium polygalacturonate) was equal to cellophane as a principal carbon source. Advantages of a pectate substrate over sugarless Czapek's agar, overlaid with cellophane, are (i) ease of preparation, (ii) ease of removal of soil residues following incubation, and (iii) extended incubation periods are possible, thus offering the potential of visual counting of *Verticillium* colonies.

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Until recently, culture substrates limited successful quantitative assay (1) of *Verticillium albo-atrum* R. & B. (*V. dahliae* Kleb) in field soils, largely because of overgrowth by other soil microorganisms (5). The relatively low levels of the fungus in field soils requires the use of large soil samples, which intensifies this problem (2). The quantitative assay recently reported by Ashworth et al. (2) overcomes most of these problems. However, erosion of the cellophane film used in the substrate makes assay of some soils difficult. We report here some useful modifications of the earlier procedure (2) and introduce the use of pectate instead of cellophane as the principal carbon source. The use of pectate for isolation of *V. albo-atrum* was first reported by Zehsazian (7).

Triplicate 15-g samples of air-dry, naturally infested soils were used in all tests. Soil samples were suspended in 200 ml of water containing 1% Calgon (Calgon Corp., Pittsburgh, Pa.) and 0.01% Tergitol-NPX (Sigma Chemical Company, St. Louis, Mo.) and blended for 30 sec in a Waring Blendor. The soil suspension then was washed through 125- and 37-μm sieves (2). The residue retained by the 37-μm sieve was collected in 30-ml centrifuge tubes, centrifuged for 5 min at 1,600 g, and the excess water removed by aspiration. The final residue was spread over 15 agar plates which were incubated for 12 days at 26 C. Following incubation, the soil residue and most microorganisms were removed from the agar surface by washing under a gentle stream of water. The discrete colonies of *V. albo-atrum*, which developed below the agar surface, were counted with the aid of a dissecting microscope (2).

Pectate substrate was prepared as follows: pectate (sodium polygalacturonate), 0.5%, and agar, 1.5%, were slowly added with vigorous stirring to 900 ml of hot distilled water (approximately 95 C) and the resulting solution autoclaved at 121 C. For each liter of final medium, the following substances were prepared and added as follows: a solution consisting of 85 ml distilled water, 1.0 ml Tergitol-NPX (dissolved with stirring), 3.0

ml of 1 M guanidine HCl, 7.0 ml of 1 M KH_2PO_4, and 1.0 ml of micronutrients (5.0 mM $MnCl_2 \cdot 4H_2O$, 3.0 mM $ZnCl_2$, 0.3 mM H_3BO_4, 0.25 mM $CuCl_2 \cdot H_2O$, 0.1 mM $NaMoO_4 \cdot 2H_2O$, 0.1 mM $CoCl_2 \cdot 6H_2O$) was autoclaved separately. To the above autoclaved salts solution was added through a Millipore filter (0.22-μm pore size) a solution consisting of 0.5 ml biotin (10 mg/ml), 0.5 ml of 2 M $MgSO_4 \cdot 7H_2O$, 200 mg streptomycin sulfate, and 2.0 ml of 17 mM ferrous citrate. The final salts solution was added to the autoclaved pectate-agar solution just prior to the pouring of media plates. Guanidine (R. Sbragia, *unpublished*) and biotin (4) were used in the substrate to promote production of microsclerotia. Tergitol-NPX was used to restrict colony size (6). Although 1.0 ml/liter of the above is commonly used, 0.1 ml/liter was equally effective in restricting colony size.

Use of Calgon and Tergitol-NPX, along with 30 sec mixing in a Waring Blendor, dispersed clay particles and significantly reduced the time required to sieve samples. It was possible to process 36 to 40 samples per day per operator with the above procedure compared to 18 to 24 samples per day per operator with the procedure reported earlier (2). Hand rubbing of soil on sieves was unnecessary but gentle agitation with a rubber policeman speeded the operation.

A comparison of the pectate substrate with the sugarless Czapek's-cellophane substrate (2) showed that the two were equal as carbon sources for the isolation of *Verticillium albo-atrum*. In 24 direct comparisons performed during the past year, the pectate media gave slightly better recoveries than the cellophane media in only 58% of the trials. The substrates were also compared in germination tests of microsclerotia isolated individually from field soils. Microsclerotia were isolated from soil organic residues (3) and transferred to each substrate in each of two replicated (three times) experiments. In the first test, 51 and 53% of the microsclerotia produced colonies on, respectively, cellophane and pectate media, while 37 and 39% germination was observed in the second test.

The pectate substrate has three major advantages over the cellophane substrate. Less time is required to prepare finished culture plates for use. Timing of removal of soil residues from cellophane covered plates is critical (6 days) due to severe erosion of the film by cellulolytic microorganisms in some soils (2). This is because accurate counting of colonies is dependent upon seeing colonies which are principally located on the surface of the film. On the other hand, colonies are principally subsurface in the pectate substrate and no adverse effects were observed when pectate plates were incubated for 3 wk or more before removal of the soil residue. Lastly, pectate plates have the potential of being read directly, without use of a dissecting microscope, because extended incubation periods permitting colony development are possible.

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