

Diagnosis of Mal Secco Disease in Lemon by Enzyme-Linked Immunosorbent Assay

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Contribution 179-E, 1978 Series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

This research was supported by Grants 488 and 1716 from the U.S.-Israel Binational Science Foundation (BSF), Jerusalem, Israel.

Accepted for publication 13 November 1978.

ABSTRACT

NACHMIAS, A., M. BAR-JOSEPH, Z. SOLEL, and I. BARASH. 1979. Diagnosis of mal secco disease in lemon by enzyme-linked immunosorbent assay. *Phytopathology* 69: 559-561.

An antiserum was prepared against an acetone precipitate of a culture fluid of *Phoma tracheiphila*. The antiserum reacted positively by either the double diffusion technique or the double sandwich enzyme-linked immunosorbent assay (ELISA). The latter test was extremely sensitive, and yielded a positive reaction up to an antigen dilution of 10^{-7} . The molecular weights of the antigens as determined by fractionation on Sepharose 6B

were $0.5-1.5 \times 10^6$. The ELISA values ($A_{405 \text{ nm}}$) obtained with infected lemon tissues were considerably higher than those obtained with healthy tissue, wounded leaves, and chlorotic leaves (nonpathogenic cause) or with leaves colonized by saprophytic fungi. Field surveys of lemon trees indicated that ELISA values of extracts from mal secco-infected organs were higher than 0.42, whereas extracts from noninfected organs were lower.

Mal secco, a disease caused by *Phoma tracheiphila* (Petri) Kantshaveli and Gikachvili, is a vascular disease of lemons and other citrus that occurs throughout the Mediterranean and Black Sea area (9). After penetration via wounds, the pathogen develops systemically in the xylem elements and causes veinal chlorosis, wilt, and shedding of leaves, followed by dieback of branches. Diagnosis of the disease in individual trees is crucial for effective sanitation, currently the major control measure. The symptoms alone are not entirely specific and diagnosis must be confirmed by isolation of the pathogen. Isolation and identification of *P. tracheiphila* requires skill, experience, and a minimum of several days. A pink-orange pigment associated with mal secco in lemon stems is considered to be a reliable diagnostic symptom. However, this develops only at an advanced stage of the disease, after the xylem vessels are well colonized by mycelium.

Plant diseases have been diagnosed by serological methods. Recently the microplate enzyme-linked immunosorbent assay (ELISA) has proved to be a reliable and specific serological test for detecting plant viruses (2,10) and spiroplasmas (3) and it has been used for diagnosing fungal infections, such as aspergillosis and

candidiasis, in human patients (4). The present study describes the diagnosis of mal secco disease in lemon by ELISA and considers its suitability for large scale screening of the disease.

MATERIALS AND METHODS

Antigen preparation. A pathogenic isolate of *P. tracheiphila* was cultured in a defined liquid medium under conditions described previously (7). The medium was modified by adding $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and NiSO_4 (each at 2 mg/L). After 30 days 1,000 ml of the culture fluid was filtered to remove mycelium, and concentrated to 20 ml by flash evaporation at 45 C. The preparation was treated with 60 ml of acetone at -18 C and allowed to precipitate overnight. The precipitate was pelleted by centrifugation at 10,000 g for 15 min and resuspended in 5 ml of distilled water.

Antiserum preparation. The acetone precipitate (0.5 mg) was dissolved in 1.5 ml of 0.5 M phosphate saline buffer (pH 7.2) and emulsified by sonication in a Bronson B-12 sonifier for 2.5 min with 1.5 ml of Freund's complete adjuvant. The antigen was injected subcutaneously into a New Zealand white rabbit. Two booster injections of the same emulsion were made after 3 and 6 wk and bleedings were started 2 mo after the primary injection.

Column chromatography. Antigen solution (3 ml) was centrifuged 15 min at 35,000 g and the supernatant was applied to a

Sepharose 6B column (3 × 60 cm). Elution was with distilled water (flow rate of 25 ml/hr) at 4 C. Fractions (3 ml) were collected and analyzed for protein content (6) and serological reaction as described below.

Serological detection by ELISA. The ELISA procedure was conducted according to Clark and Adams (2). The γ -globulin fraction obtained from the antiserum was partially purified by column chromatography on DE 23 diethylaminoethyl cellulose (Whatman). The γ -globulin/enzyme conjugates were prepared by mixing 0.3 ml (1.7 mg) alkaline phosphatase (Sigma P-4502) with 0.7 ml of purified γ -globulin ($A_{280\text{ nm}} = 1.4$). The solution was dialyzed against 1 L of phosphate-buffered saline (PBS) which contained 0.025 M phosphate buffer, pH 7.2, and 0.075 M NaCl. Dialysis was followed by addition of 0.03 ml of 2% glutaraldehyde. After 4 hr of conjugation at room temperature the unbound glutaraldehyde was removed by dialysis against PBS.

The ELISA tests were conducted in microtiter plates (Dynatech M29 ARE [Greiner & Söhne, Nürtingen, W.G.] or IS-MRC [Linbro Scientific Co., Hamden, CT]). The plates were coated with 7 $\mu\text{g/ml}$, partly purified γ -globulin, incubated for 2–3 hr at 33–35 C, and washed three times with PBS containing 0.5 ml/L of Tween-20 (polyoxyethylene sorbitan monolaurate) (PBS Tween). Test samples (200 μl) were added to the wells and the plates were incubated for either 2–3 hr at 33–35 C, or overnight at 6 C. After removing the test samples, the plate was washed with PBS-Tween and 200 μl of enzyme antibody conjugate (6 $\mu\text{l/ml}$) in PBS-Tween containing 2% polyvinyl pyrrolidone (PVP-40) were added.

After an incubation period of 2–3 hr at 33–35 C the excess conjugate was removed by washing with PBS-Tween. The enzymatic reaction was initiated by addition of *p*-nitrophenyl phosphate substrate (200 μl , 0.12 mg) in diethanolamine buffer (pH 7.2) and incubation at room temperature for 0.5–1.0 hr. The reaction was terminated by the addition of 50 μl of 3 M NaOH (3) to each well. Enzymatic activity (hereafter called the "ELISA value") was expressed as $A_{405\text{ nm}}$ which was measured in a Varian Techtron 635 spectrophotometer.

Sample preparation for ELISA. Samples originating from either acetone precipitates of culture fluids or effluents of Sepharose 6B column chromatography were dissolved in PBS-Tween containing 2% PVP-40 (PBS-Tween/PVP-40). Samples obtained from leaves, bark, or wood were prepared as follows: The plant sample was sectioned into pieces of about 0.5 × 0.5-cm chips and about 0.5–1.0 g was homogenized in 5–7 ml of PBS-Tween/PVP-40 with an Ultra-Turrax homogenizer equipped with a T-18 generator (Janke & Kunkel KG, West Germany), using ice-cooled 22 × 100 mm, U-bottom tubes (1). The homogenates were either tested immediately or stored at –18 C for several days.

RESULTS

Detection of *P. tracheiphila* antigens in culture filtrates. In agar double diffusion tests (8) the antiserum which was collected after 8 wk reached a titer of 1:32 whereas the antigens could be detected in a dilution of 1:60. The partially purified γ -globulin from this serum was used for determination of ELISA values throughout the present study. A high dilution end point of the antigens in the acetone precipitate of culture filtrates was measured by ELISA. Values of 0.05, 0.2, 0.6, and 1.5 were observed with dilutions of 10^8 , 10^7 , 10^6 , and 10^5 , respectively. Culture filtrates of several fungi namely *Phytophthora citrophthora*, *Stemphylium botryosum*, *Alternaria* sp., and *Fusarium* sp. at a dilution of 10^3 gave low ELISA values of 0.14, 0.14, 0.2, and 0.18, respectively, compared to a value of 1.8 for *P. tracheiphila* under the same conditions.

The nature of antigens in the acetone precipitate was investigated by fractionation on a Sepharose 6B column (Fig. 1). The elution pattern of the fractions which reacted positively in the ELISA test and Lowry reagent respectively, suggests that the antigen preparation contained heterogenous protein molecules with an estimated molecular weight of $0.5\text{--}1.5 \times 10^6$.

The appearance and accumulation of antigens during growth of *P. tracheiphila* is illustrated in Fig. 2. A low concentration of antigens was detected in culture filtrates during the initial phase of growth, a sharp increase was detected after 10 days, and a maxi-

mum was reached in the 17th day.

Detection of *P. tracheiphila* antigens in infected tissue. Extracts of mal secco-infected and healthy lemon stems were tested by ELISA for the presence of antigens (Fig. 3). A positive ELISA value of 0.75 was obtained with infected tissue at a buffer/tissue ratio of 1:1,250, but ELISA values of 0.25 were obtained with healthy tissue at a buffer/tissue ratio of 1:50. ELISA values for homogenates from infected viable green wood and infested dead wood did not differ significantly (Fig. 3). Agar double diffusion tests of extracts from heavily infected stems detected the antigens only at a dilution of 1:10 (w/v), and those from infected leaves were not detected at all.

Stem and leaf samples from trees of various lemon cultivars, some showing chlorosis or necrosis, were collected in several orchards. The samples were examined by ELISA, microscopy, and cultural techniques to determine the presence of mal secco disease. All samples in which *Phoma tracheiphila* was identified either by

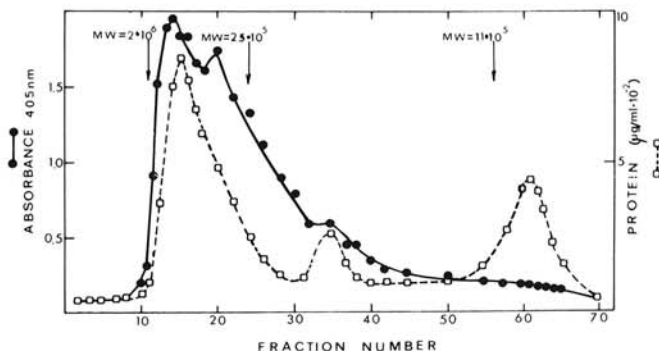


Fig. 1. Distribution of antigens during chromatography of the antigen preparation on a Sepharose-6B column. Acetone precipitate from 400-ml of filtrate was applied to the column as previously described (7). For determining enzyme-linked immunosorbent assay (ELISA) values, the fractions were diluted $\times 10$ in PBS solution. Protein was determined according to Lowry et al (6). Molecular weights of the eluted fractions were estimated as previously described (7).

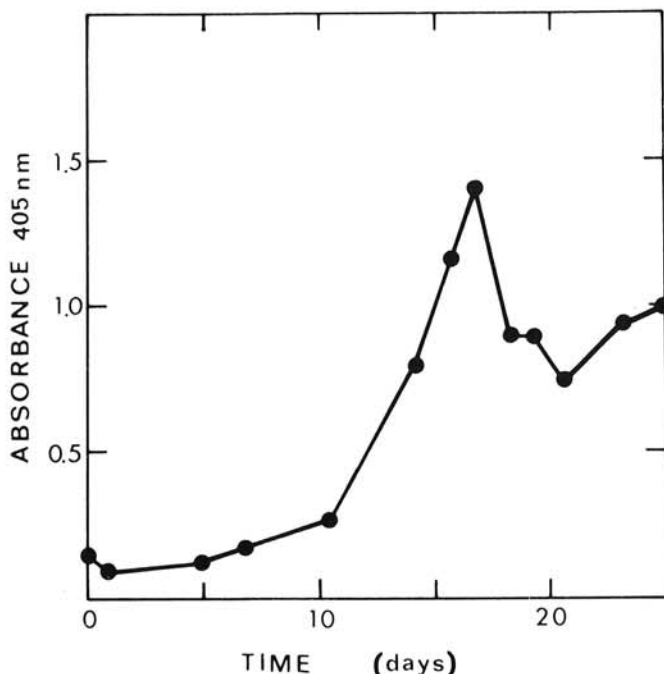


Fig. 2. Secretion of antigens into the culture medium during growth of *Phoma tracheiphila*. Samples (2 ml) were taken at intervals and the mycelium was removed by filtration. The supernatant was diluted $\times 100$ and used for enzyme-linked immunosorbent assay.

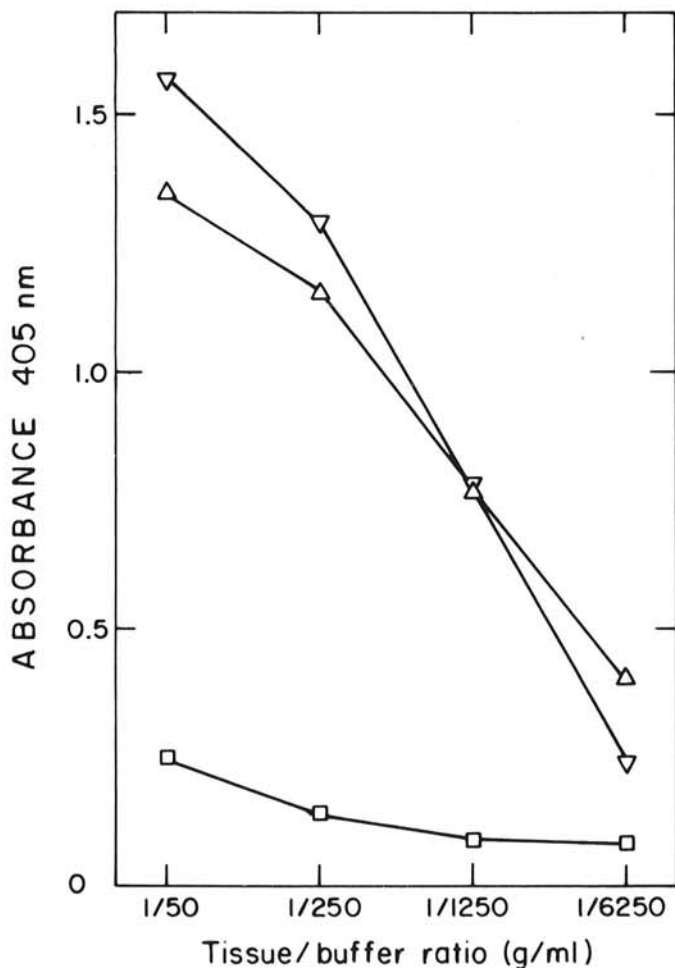


Fig. 3. Enzyme-linked immunosorbent assay (ELISA) value ($A_{405 \text{ nm}}$) of *Phoma tracheiphila* antigens in extracts of lemon tissues prepared with different ratios of extracting buffer to tissue. The necrotic part of a mal secco affected lemon stem (▽). The viable green part of an infected lemon stem (△). Healthy lemon stems (□).

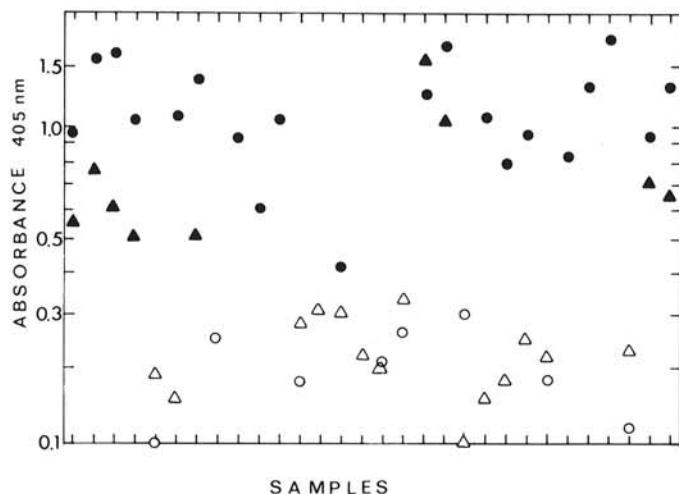


Fig. 4. Enzyme-linked immunosorbent assay values of randomly sampled mal secco affected and healthy lemon leaves and branches. Samples were measured at a dilution of tissue/buffer ratio of 1:10. Symbols: △ healthy leaves; ○ healthy twigs; ● infected twigs; ▲ infected leaves.

microscopy or culturing gave ELISA values above 0.42 (Fig. 4). The mean ELISA values for infected stems and leaves was 1.15 ± 0.34 and 0.68 ± 0.27 , respectively; those for leaves free of *P. tracheiphila*, chlorotic leaves, wounded leaves, and partly necrotic leaves colonized by saprophytic fungi were significantly lower, 0.25 ± 0.10 , 0.21 ± 0.07 , and 0.21 ± 0.09 , respectively. Generally, the differences in color intensity of the ELISA reaction between the infected and healthy samples were sufficient to permit visual scoring.

DISCUSSION

Results presented here indicate that extracellular antigens of *P. tracheiphila* can be detected readily and specifically by enzyme-linked immunoassay. The sensitivity of the technique was at least 10^3 – 10^5 times greater than that of the double diffusion test. Similar results also were reported for other nonfungal antigens (2,5). The extreme sensitivity of the ELISA test enabled the detection of *P. tracheiphila* antigens in crude plant extracts. ELISA values for noninfected tissue were low; those for infected tissues were usually at least 0.2 units higher and also could be determined visually. The latter suggests that a simplified version of ELISA might be used to conduct a rapid field test for mal secco.

Samples of infected branches often yielded higher ELISA values than those of leaves (Fig. 4) apparently due to shedding of heavily infected leaves. Tissue samples as small as 0.5–1.0 g were adequate for ELISA testing. It is noteworthy that the ELISA test detects filtrate antigens secreted by the pathogen. Since these antigens might be secreted in advance of the growing hyphae, an early detection of mal secco can be made in uninvaded tissue at sites distant from the pathogen. The relative simplicity and high sensitivity of the ELISA procedure, and its suitability for use with crude plant extracts suggest that it might be of great benefit for rapid diagnosis of mal secco in epidemiological studies as well as for quarantine purposes. Quarantine is of special significance since mal secco disease is restricted to the Mediterranean and Black Sea areas (9).

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