Study of Three Enzymes from Stubborn-affected Citrus Foliage

C. W. Meister, R. M. Allen, and R. L. Caldwell

Citrus-indexing programs in areas where stubborn disease occurs need quick, inexpensive, and reliable methods for testing sources of budwood for presence of the causal entity. Many of the symptoms associated with stubborn disease also result from other causes. Furthermore, stubborn-affected citrus trees may be symptomless, or nearly so, and propagations from such plants often result in high percentages of infected nursery stock. In Arizona, infected trees often produce malformed or mottled leaves during hot weather, but during cooler seasons, foliage appears normal and symptomless. Acorn-shaped or otherwise malformed fruit, those with stylar-end greening, and those containing aborted seeds (*2*) are specific for stubborn disease. These may vary from season to season, with seed abortion probably being the most reliable symptom.

This paper reports results of a search for a quick and reliable test for stubborn disease. Selected enzymes extracted from infected leaves, with and without symptoms, were compared by electrophoresis with those extracted from apparently healthy leaves.

MATERIALS AND METHODS

Hinckley, Madam Vinous, and Pineapple sweet orange seedlings were inoculated by budding or side-grafting with tissue taken from a virus-free, stubborn-affected navel tree located at Yuma, Arizona. Inoculated plants were grown in 2-gallon pots in a greenhouse at Tucson; these plants showed leaf malformation and mottling, vein yellowing, leaf-margin chlorosis, and stunting. Control plants of the same cultivars that had received tissues from a healthy navel tree remained symptomless for more than three years. Young and mature leaves harvested from stubborn-affected and healthy trees of each cultivar were extracted and processed at the same time under identical conditions.

Leaves (1.0–2.5 gm) were ground in 8 ml of buffer (0.1 M Tris, pH 7.8, containing 0.001 M 2-mercaptoethanol and 0.5 M sucrose), with an Omnimixer (Sorvall). Resulting suspensions were squeezed through cheesecloth, ground further in a glass homogenizer, and clarified by centrifugation at 28,700 *g* for 45 minutes. The supernatant was stored at about 4° C. Protein, in an aliquot of each extract, was precipitated with 12 per cent trichloracetic acid, and measured spectrophotometrically as described by Lowry *et al.* (*3*). Extracts were then diluted with Tris buffer so that each contained equal amounts of protein per ml.

Aliquots (0.2 ml) of each extract containing 500 μg of protein were layered onto separate 8 per cent polyacrylamide gel columns and subjected to electrophoresis with Tris-glycine buffer, pH 8.2 (*1*), for 2.5 hours. Individual gels were then removed from their glass tubes and placed in the reaction mixtures described below. Gels containing extracts derived from leaves of stubborn-affected and healthy plants were placed in the same reaction mixture so that direct comparisons could be made.

Catalase activity was measured by submerging individual gels in 0.03 per cent hydrogen peroxide in a fermentation tube. Oxygen evolving from each gel during the resulting reaction was trapped inside the tube and measured visually.

Isoenzymes of starch phosphorylase

were detected by placing gels in 20 ml of 1.0 M sodium acetate buffer, pH 5.7, containing 80 mg glucose-1-phosphate, 12 mg adenosine triphosphate, 80 mg sodium fluoride, and 2 ml of 95 per cent ethanol, and incubating them for 5 hours at 25°C. A solution of 4 per cent potassium iodide and 2 per cent iodine was then used to stain the bands of starch produced by the enzyme reaction.

General acid phosphatases in gels were located by incubating them in 20 ml of 0.4 M sodium acetate buffer, pH 5.0, containing 8 mg α-naphthyl acid phosphate and 9 mg fast-red TR salt (4-chloro-o-toluidine diazotate).

RESULTS

Young and mature, symptomless leaves and those leaves exhibiting symptoms of stubborn disease were harvested periodically from stubborn-affected sweet orange trees over a two-year period. Comparable leaves were taken each time from a healthy tree of the same cultivar. Catalase activity in extracts from leaves showing disease symptoms was only one half to two thirds that of extracts from healthy leaves.

One, and sometimes two, starch phosphorylase isoenzymes from leaves showing symptoms were usually more active than those obtained from corresponding healthy leaves. Two of three samples of leaves collected from each stubborn-affected Hinckley, Madam Vinous, or Pineapple sweet orange tree yielded greater starch phosphorylase activity than did those from healthy trees; the other three samples showed no differences.

Leaves exhibiting symptoms of stubborn disease yielded electrophoretic acid phosphatase patterns that were usually different from those of leaves from healthy trees. The isoenzyme reaction product designated band number 1 in figure 1 stained intensely in gels containing extract from stubborn-affected leaves. The same band stained less intensely in gels containing extract from healthy leaves. Bands 2 and 3 were more intense in healthy than in stubborn-affected extracts. These apparent isoenzyme differences were observed in gel-column patterns from four samples from paired healthy and diseased Hinckley trees, three of five samples from paired Madam Vinous trees, and from one of two samples from paired Pineapple trees.

Most (80 per cent) of the extracts taken from symptomless leaves harvested from these same stubborn-affected trees yielded isoenzyme patterns identical with those extracted from healthy leaves.

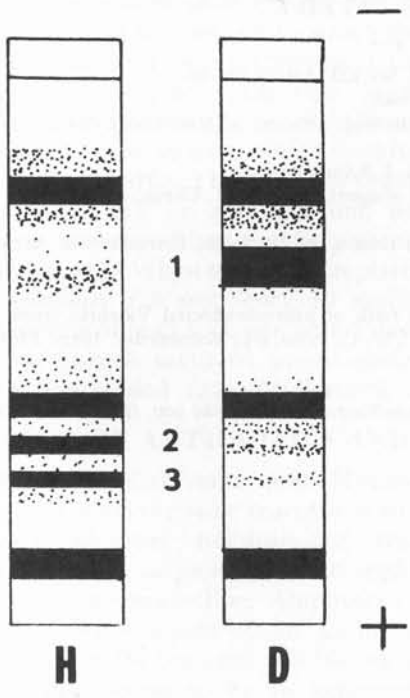

Fig. 1. Electrophoretic patterns of general acid phosphatase extracted from leaves collected from healthy (H) and stubborn-affected (D), greenhouse-grown Madam Vinous sweet orange trees. The enzyme was electrophoresed at pH 8.3.

DISCUSSION AND CONCLUSIONS

Catalase, starch phosphorylase, and general acid phosphatase extracted from most leaves showing symptoms of stubborn differed quantitatively from the enzymes obtained from leaves from healthy trees. However, decreases in activity of catalase have also been noted for extracts from leaves of psorosis-affected (*5*) and tristeza-affected (*6*) citrus. Thus, reduced activity of catalase appears to be a general phenomenon of disease, and will therefore be of limited value for the diagnosis of stubborn disease. Differences in the activities of isoenzymes of starch phosphorylase and general acid phosphatase appear to be induced only in leaves showing visible symptoms of stubborn disease. Infected but symptomless leaves, healthy leaves, and leaves of citrus plants showing symptoms of drought-induced wilt, psorosis A, and crinkly leaf yielded identical starch phosphorylase and general acid phosphatase isoenzyme patterns (*4*). The observed quantitative differences apparently reflect changes in metabolism of citrus leaves caused by the stubborn disease pathogen and appear to be associated directly with symptoms.

Since differences in enzyme activities were found only for leaves already showing stubborn symptoms, this method of differentiating stubborn-diseased and healthy trees appears of limited use.

(Arizona Agricultural Experiment Station Journal Paper No. 1974.)

LITERATURE CITED

1. Davis, Baruch H.
   1964. Disc electrophoresis. II. Ann. New York Acad. Sci. **121**, Art. 2: 404–36.
2. Carpenter, J. B., E C. Calavan, and D. W. Christiansen
   1965. Occurrence of excessive seed abortion in citrus-fruit affected with stubborn disease. Plant Dis. Reptr. **49**: 668–72.
3. Lowry, O. H., N. J. Rosenbrough, A. L. Farr, and R. J. Randall
   1951. Protein measurement with the folin-phenol reagent. Jour. Biol. Chem. **193**: 265–75.
4. Meister, C. W.
   1972. Enzyme composition of virus infected citrus tissues. Ph.D. thesis, University of Arizona, Tucson.
5. Monselise, S. P., and R. Goren
   1965. Some physiological properties of leaves and bark of psorosis-affected Valencia trees. *In:* Proc. 3rd Conf. Intern. Organ. Citrus Virol. (W. C. Price, ed.) Gainesville: Univ. Florida Press, pp. 295–98.
6. Trippi, V S., and J. P. Mesias
   1957. Alguna deferencias entre plantas sanas y possiblemente enfermas con tristeza en citrus. Rev. Indust. Agr. (Tucuman) **41**: 29–32.