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Some Possible Anatomical and Serological Techniques in Diagnosing Stubborn Disease in Citrus

STUBBORN DISEASE is of major importance in Arizona citrus orchards. Carpenter (1) defined 3 categories of stubborn on Marsh grapefruit trees. Type I trees show the most serious symptoms; Type III, the mildest.

The lack of a specific and rapid test for stubborn disease in nursery stock and young trees has been a serious handicap to both commercial growers and research workers. A method of detecting stubborn in leaves (since these are always available) of trees of any age would be of great value. As there is currently no annual host to which this citrus virus can be transferred for rapid determination, a serological method, if one could be developed, would give a rapid means of detecting virus in plant tissues and in identifying different kinds and strains of viruses.

An exploratory project (4) to determine whether or not it would be possible to produce an antiserum to the stubborn disease causal agent was started in December, 1958. A full-scale project was initiated later. Concurrently a search for anatomical differences between stubborn and healthy leaves was undertaken. Some of the information developed in these projects will be reported herein.

Anatomical Study of Citrus Leaves

MATERIALS AND METHODS.—Preliminary experiments on both old and young leaves collected in a random manner, no higher than 6 feet above the ground, indicated that the area of the tree sampled had no effect on experimental results. Leaves were taken from healthy and diseased

Mediterranean Sweet, Koethen Sweet, Valencia and Washington Navel orange trees, and Marsh and Red Blush grapefruit. Samples from diseased trees were taken from branches with and without stubborn fruit. Leaves from seedling trees were also included to serve as a standard. It has recently been brought to the authors' attention that the Frost Nucellar Marsh grapefruit used is now showing stubborn symptoms.

The leaves were cleared and stained, and permanent mounts were prepared as outlined by Foster (2). It was found that the vein endings could best be observed with a trisimplex microprojector rather than a compound microscope, as the projector produces a flatter image.

RESULTS AND CONCLUSIONS.—Veins in infected leaves are somewhat coarser than those in normal ones and their terminals tend to be blunted. These characteristics are especially noticeable in young, first-year leaves. It is not known whether this characteristic is also associated with other citrus diseases.

Healthy citrus leaves were found to contain 3 to 4 times as many calcium oxalate crystals as diseased leaves. The lower crystal content of stubborn-infected leaves supports the finding by McGeorge (3) of lower calcium content of leaves of declining grapefruit. McGeorge was working with "Pink Nose" grapefruit, a condition later associated with stubborn disease. A more extensive program to determine the calcium content of leaves from both healthy and diseased trees is in progress.

The only variety on which Carpenter's (1) three symptom types were studied was Marsh grapefruit. The differences in venation and crystal content held for all Type I and Type II stubborn trees whether leaves were collected from normal-appearing or diseased branches. However, the veinlet endings of leaves of the Type III trees appeared to be the same as those of apparently healthy trees.

Serological Investigations

MATERIALS AND METHODS.—Stubborn diseased and normal fruits were collected from healthy seedling or nucellar lines and diseased Valencia, Koethen Sweet, and Navel orange, and Types I, II, III Marsh, and Redblush grapefruit.

Fruits of each variety and type were peeled, liquified in a blendor, and strained through four layers of cheesecloth. The juice was then centrifuged twice for 15 minutes at 5°C at 10,000 times gravity. The supernatant fluid was centrifuged for 90 minutes at 5°C at 24,500 times

gravity. The resulting pellets were resuspended in 5 ml of sterile distilled water. The suspensions were then mixed with equal amounts of lanolin paste-mineral oil. Three ml of the mixture were injected subcutaneously into the hip of New Zealand rabbits. Injections were made at weekly intervals for 5 weeks. Two weeks after the last injection, the animals were bled and the serum separated and stored in a deep freezer at –16°C until used.

Antiserum, normal serum, and purified juice from healthy and diseased fruits were diluted in a series ranging from 1:4 to 1:128 with distilled water. All possible combinations of these dilutions of juice and sera were tested by Van Slogteren's micro-precipitin test (5).

Results and conclusions.—Results of a typical precipitin test are shown in Figure 1. The results indicate (a) that the same protein is

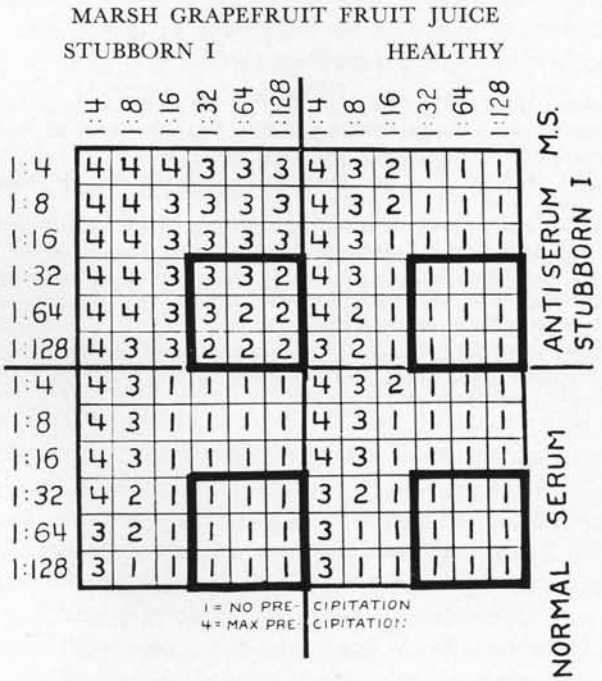

Figure 1. *Typical precipitin test with fruit juice of Marsh grapefruit.*

involved in stubborn disease of Type I and II in Marsh grapefruit, and (b) that a closely related protein is involved in the infections of Red Blush grapefruit. An unrelated protein is involved in the infections in Washington Navel trees. No serologically active proteins different from those in healthy plants were found in stubborn-affected tree of the other citrus varieties tested. It is not known, at this time, whether the proteins reacting in the precipitin test are viruses or metabolic by-products of the diseased tissue.

With the production of a useful antiserum we may be able to examine the proteins related to some stubborn symptoms, and with the electron microscope we may possibly determine their similarity to virus particles.

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