Proteins Associated with Transmission of Citrus Declinio in Bahia, Brazil

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ABSTRACT. Citrus declinio was experimentally transmitted from declinio-affected trees to healthy sweet orange trees by root-graft inoculations. Inoculated trees developed conspicuous visual symptoms after 1 yr and reacted with significantly reduced water uptake after 18 mo. Vacuum extracts from roots of trees naturally-infected by citrus declinio, experimentally inoculated, and healthy sweet orange trees were analyzed by SDS-polyacrylamide gel electrophoresis. Extracts from rootlets of infected plants contained several proteins that were diagnostic for the disorder, since they were not observed in healthy controls. Other proteins appeared to have their synthesis increased by declinio. Some inoculated, symptomless trees contained some of the novel proteins, indicating that they could be used for early diagnosis. Inoculated trees reacted positively in dot immuno-binding assays with antiserum against a 12 kDa protein derived from blighted trees in Florida, while non-inoculated control trees were negative. This is additional evidence that citrus declinio and blight are etiologically related disorders.

Declinio of citrus is a serious disorder which was first observed in the state of Bahia, Brazil in 1970 (18). Affected trees display zinc deficiency foliar symptoms due to zinc accumulation in the outer wood, and reduced water uptake caused by amorphous xylem plugging (16, 17). It is now also present in São Paulo, Rio de Janeiro and Minas Gerais, and has been responsible for the loss of millions of trees. Transmission attempts were unsuccessful for many years, and the causal agent remains unknown. Bacteria have been suggested by some as being the cause of blight (9, 11), a disease very similar or identical to declinio (10). However, it has been difficult to establish a causal relationship between these bacteria and blight or declinio. Furthermore, bacteria observed in affected trees are also present in healthy trees (9). Isometric virus particles have also been isolated from the roots of blight-affected trees, but there is, as yet, no evidence that they are involved in the etiology (6). Both citrus blight (20, 21) and declinio (19) have now been transmitted by approach root grafting.

In addition to characterization of the causal agent, research is presently aimed at disease management including selection of tolerant rootstocks and early diagnosis. The present study was conducted to assess the presence of declinio-specific proteins (8) in inoculated trees for their possible use in early diagnosis.

MATERIALS AND METHODS

Baianinha sweet orange was budded onto the following rootstocks: Rangpur lime; Valencia and Caipira sweet orange; Volkamer lemon; Florida rough lemon; local selection of rough lemon; Orlando tangelo; Cleopatra mandarin; trifoliate orange; and Swingle citrumelo with 10 plants per selection, as part of a larger experiment on rootstock tolerance to declinio. When trees were 3-yr-old, five were graft-inoculated with three to four root pieces from diseased trees. These donor trees had a water uptake rate of 0.025 ml/10 sec and an average 13 ppm zinc level in the outer trunk wood, compared to 2.8 ml/10 sec and 11 ppm zinc for healthy trees; these determinations were conducted as previously described (14, 16). Five trees of each rootstock type were left uninoculated as controls. After 1 yr (April

1995), the grafts were inspected, dead ones replaced and the total number root grafts increased to 6-8 per tree. A second check and reinoculation was carried out in August 1995. Inoculated roots were wrapped with grafting tape, covered with soil and marked to allow for easy access. Water uptake determinations were conducted on these trees in October 1994 and again in October 1995.

Sampling of rootlets for physiological affects of declinio was conducted from healthy and inoculated trees 1 yr after the initial inoculation. Protein extraction was done according to Derrick et al. (7), except that PBS (0.8% NaCl, 0.02% KH_2PO_4 0.02% KCl, 0.02% NaN_2, 0.29% $NaH_2PO_4 \cdot 12H_2O$, pH 7.4, containing 1% 2-mercaptoethanol) was used as extraction medium. Using a vacuum pump at 5-10 in. Hg/5 min, 500 µl of PBS were pulled through root pieces and collected in chilled Eppendorf tubes. Samples were diluted 1:1 with SDS-buffer (20% glycerol, 0.01% bromophenol blue, 10% stacking gel buffer, 2% 2-mercaptoethanol, 2% SDS) (1), denatured for 5 min at 100°C and stored at -20°C until use. Discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (13) with few modifications. For total protein analyses, pieces of roots, bark and leaves were powdered in liquid nitrogen, extracted with PBS, subjected to low speed centrifugation (20 min, $10{,}000 \times g$ and $20{,}000 \times g$), denatured in SDS-buffer and separated by SDS-PAGE. Gels were silver stained according to Blum and Gross (4).

For dot immuno-binding assays (DIBA), 10 leaves were harvested per tree, and a composite sample was analyzed using antiserum against the 12 kDa protein found in blighted trees in Florida and following the method of Derrick et al. (8).

RESULTS AND DISCUSSION

All inoculated trees developed typical declinio symptoms 12-14 mo. after inoculation; whereas all of the control trees remained healthy. This is a shorter reaction time than has been reported previously for declinio (19) and for blight (15, 20), and may have been due to the increased number of root grafts per tree. There was no decrease in water uptake 1 yr after inoculation, but, after 18 mo., all the inoculated trees showed reduced uptake (Table 1).

When Baianinha sweet orange plants were analyzed by DIBA for the 12 kDa blight-associated protein, 19 of the 50 trees were positive, with clear rootstock differences: Rangpur lime, Florida rough lemon and Orlando tangelo (80%); local rough lemon (60%); Valencia sweet orange

TABLE 1
WATER UPTAKE BY DECLINIO-INOCULATED AND HEALTHY BAIANINHA SWEET ORANGE TREES ON DIFFERENT ROOTSTOCKS

Rootstock	Declinio (D) or Healthy (H) trees	Water uptake (ml/10 sec)	
		Oct. 1994	Oct. 1995
Rangpur lime	D	1.1	0.4
	H	1.8	1.0
Florida rough lemon	D	1.8	0.3
	H	1.5	1.4
Valencia sweet orange	D	1.8	0.4
	H	2.6	1.5
Trifoliate orange	D	1.9	0.3
	H	1.8	1.0

(40%); and Caipira sweet orange and Volkamer lemon (20%). Inoculated trees on Cleopatra mandarin, Swingle citrumelo, and trifoliate orange gave negative serological results, although several had visual symptoms.

Total protein analysis of extracts from naturally diseased and experimentally inoculated trees showed no differences from healthy trees, in contrast to studies on blight in Florida (2,3). However, vacuum extracts from xylem showed striking differences between inoculated and non-inoculated trees. In 10% gels (Fig. 1), a protein that migrates very closely to 14 kDa was observed in both naturally diseased and experimentally inoculated plants, but it was absent in healthy trees. Weak bands of approximately 15.5 to 16.5, 22, 23, 27, 38 and 42 kDa were also detected in diseased samples only (Fig. 1). In 18% gels, the apparently same 14 kDa protein was determined as a 12.7 to 13 kDa protein (Fig. 2). This protein may be the same as the 13 kDa protein reported by Bausher and Sweeney (3) and the 12 kDa protein detected by Derrick et al. (7), but positive identification will have to await immunoblot assays. Other proteins can also be seen in 18% gels; a 34 kDa and a 17 kDa protein were also present in diseased and experimentally inoculated trees. The latter was observed faintly from one apparently healthy tree, so it may be an early response to the declinio disorder.

It has been argued that proteins in these size ranges may be produced in response to diverse stress factors, such as the 13 to 15.8 kDa protein in tobacco mosaic virus-infected tobacco (5) and the 15 to 18 kDa heat shock protein in soybean (12). However, these arguments are not consistent with the fact that the 12.7 to 13 kDa protein was not detected in declinio-free trees exposed endemically to citrus tristeza virus and to the same abiotic stress factors. It is, therefore, concluded that the 12.7 to 13 kDa protein is related to the inoculum

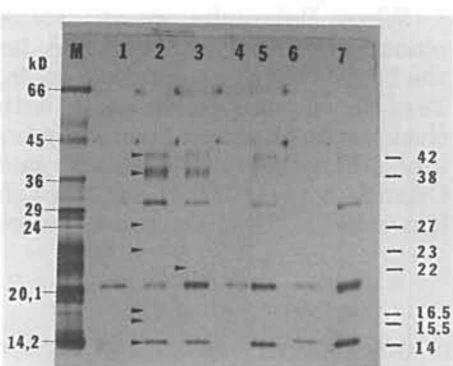

Fig. 1 SDS-PAGE (4% stacking, 10% separating) of proteins from declinio-affected and healthy orchard citrus trees. M = molecular weight markers; lanes 1 and 4, healthy controls (non-inoculated Pera sweet orange on Rangpur lime); lanes 2 and 3, declinio-affected Pera sweet orange on Rangpur lime; lanes 5,6 and 7, experimentally inoculated Baianinha sweet orange on Valencia sweet orange, trifoliate orange and Rangpur lime respectively. Arrows indicate declinio-specific bands.

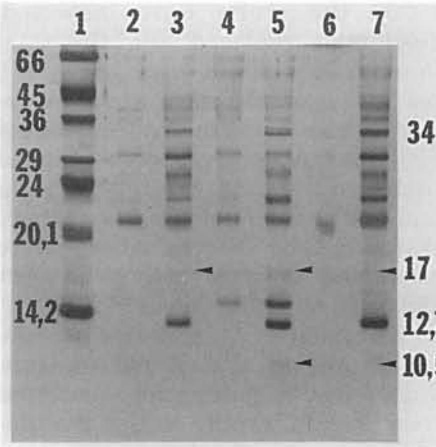

Fig. 2. SDS-PAGE (4% stacking, 18% separating) of proteins from declinio-affected citrus trees. Lane 1, molecular weight markers; lanes 2 to 7, Baianinha sweet orange, respectively non-inoculated control and symptomatic inoculated plants on Florida rough lemon (2 and 3), trifoliate orange (4 and 5) and Rangpur lime (6 and 7).

contained in root pieces and does not appear to be induced by abiotic stress factors.

Research is continuing to characterize these declinio-associated proteins and to investigate their possible use in early detection of the disease.

ACKNOWLEDGMENTS

We thank Dr. Julia Beretta (Instituto Biológico, São Paulo) for conducting the DIBA, and Mr. Antonio Teixeiro Filho for his expert assistance in the field.

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