Positive Diagnostic Tests for Declinio on Plants Root-Graft Inoculated in Brazil

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ABSTRACT. In 1984, a transmission trial was begun in an orchard affected by declinio. Two of four healthy nursery plants (receptors), were approach-root-grafted to each of 10 adult donor trees. The other two plants of each group were kept as controls to monitor soil effects and natural spread of declinio. Periodically, declinio diagnostic tests were applied to the receptors and controls. The tests used were: water uptake by syringe injection, accumulation of zinc in the trunk wood, percentage of amorphous plugs and observation of visual symptoms. The first evaluation was made in February 1986 when the plants were at the original site. At this time all trees tested negative for declinio. In 1986 all the plants were moved to the Bebedouro Experiment Station. In May 1987 at Bebedouro, the diagnostic tests were still negative, but by December 1987, two of the inoculated plants showed visual symptoms of declinio. In March 1989, visual symptoms were present and all of the declinio diagnostic tests were positive for the root-grafted receptor plants. Tests on the control trees were normal. These results showed that declinio can be transmitted by approach-root-grafting in Brazil.

Declinio is a disease of unknown etiology and one of the most serious problems in the Brasilian citrus industry. The losses due to this disease were estimated to be about 10 million trees in 1988 (A. A. Amaro, personal communication). Declinio shows the same characteristics of blight in Florida, i.e., reduced water conductivity of the trunk and root xylem, high zinc levels in the trunk wood, and the presence of occlusions in the xylem vessels (3). Declinio research studies are slow because the trees do not manifest symptoms and characteristics until they are generally at least 5 yr old. Diseases with similar symptomatology have been found in South Africa, Cuba, Venezuela, Argentina, Uruguay and Surinam (1, 2, 3, 7). Blight was first reported in Florida by Swingle and Webber in 1896 (12). The symptoms observed were wilting, sparse foliage, foliar zinc deficiency symptoms, small fruit, and reduced yield. Declinio was first observed in the State of São Paulo, Brazil in 1968, when a disease affecting adult trees in the region of Cajobi was described as "definhamento" (9). The affected trees were removed and the cause of the problem was not determined (8). In 1970, a similar disease was seen for the first time in the State of Bahia and again in São Paulo, and was named declinio (8, 10).

The first successful transmission of citrus blight was reported in Florida in 1984. Symptoms appeared three years after a series of apparently healthy mature trees were approach-root-grafted to mature blighted trees (13). Similar results were obtained in a second such experiment, however, in this case, symptoms developed on the receptor trees within two years after the root-graft-inoculation (6). Transmission of blight has also been achieved in an experiment in which healthy mature trees on Carrizo citrange were root-graft inoculated using only pieces of roots collected from blight affected trees (6).

Several experiments involving root-grafting were set up to demonstrate experimental transmission of declinio (11). In this report we describe one of these experiments which provide the first evidence of declinio transmission in Brazil.

MATERIAL AND METHODS

Fourteen-yr-old trees with moderate declinio symptoms (leaves small and often flaccid with symptoms of zinc deficiency, canopy sparse with some twig dieback) and healthy Valencia orange on Rangpur rootstock, were chosen as donors in a commercial orchard in the Barretos region, northern São Paulo State. The follow-

ing diagnostic tests were used to identify the healthy and the diseased donor trees: visual symptoms, water uptake into the trunks, accumulation of zinc in the wood and percentage of amorphous plugs in the xylem vessels.

Approach root grafts. In May 1984, four 1-yr-old nursery plants of the same scion-rootstock combination as the donor trees were planted around each of 10 donor trees which were located randomly in the commercial block. Three to five secondary roots about 0.5 cm in diameter of two of the nursery plants were grafted to the roots of the diseased donors and designated RG. The other two plants were ungrafted controls, and were designated C_2. Four nursery trees were similarly planted around each of five healthy orchard trees. The root-grafted ones were designated C_1 and the ungrafted ones C_3. In February 1986 the root-grafted and ungrafted nursery trees were replanted to the Bebedouro Experiment Station. Not all the trees survived transplanting. The remaining 12 RG, 2 C_1, 8 C_2 and 2 C_3 nursery trees were planted directly, at Bebedouro Experiment Station and periodically observed for declinio symptoms. Nine additional healthy trees of the same rootstock-scion combination (designated C_4) were planted as additional control trees.

Diagnostic tests. The nursery plants (receptors) were evaluated for declinio using standard blight diagnostic tests (4). The following tests were applied: a) canopy symptoms were rated on a scale of 0 = healthy, 1 = mild (small leaves with symptoms of zinc deficiency, short internodes, slight wilt but little or no thinning of foliage), 2 = moderate (small leaves, often flacid, with symptoms of zinc deficiency, sparse canopy with some twig dieback), and 3 = severe (thin canopy, substantial twig dieback, trunk sprouts common); b) zinc concentration in the trunk wood (14); c) water uptake by syringe injection (5); and d) occurrence of amorphous plugs in the xylem vessels (2).

Sample processing. Core samples 5 mm in diameter by 6 cm long were removed from the trunk about 20 cm above the bud union with a Haglof increment borer and fixed in 3% glutaraldehyde. Sections 20 μm thick were transversely sectioned from the portion of the core between 2 and 3 cm from the cambium using a Leitz Cryostat. Sections were observed microscopically and amorphous plugs typical of those found in blighted and declinio-affected trees (1, 2) were counted in a sample of 200 vessels for each tree.

The core samples from the trunk were prepared for scanning electron microscopy (SEM) and viewed to confirm the results obtained using light microscopy. The methodology described by Merida *et al.* (7) was used, in which the samples were fixed in 2% formaldehyde for 12 hr. Then the samples were dried under vacuum with 380 mm Hg pressure for 48 hr in a glass cube containing silica gel. The specimens were mounted on SEM stubs, sputter coated with 100 Å gold-palladium and viewed with a Hitachi S-500 microscope at 20KV.

RESULTS AND DISCUSSION

Root-grafting. Twelve months after grafting, most unions between the receptor and the donor plants were examined and about 90% of the roots had taken with no secondary infection.

Evaluation of the plants. When the donor adult trees were chosen, they were first evaluated by the standard blight diagnostic tests. The trees selected as declinio donor trees showed all the characteristics of the disease, whereas tests for the healthy donor trees were all negative.

In February 1986, 20 months after the root-grafting, the receptor plants were evaluated visually prior to transplanting to the Bebedouro Experiment Station. At the same time,

the young trees (RG, C_1, C_2, C_3 and C_4) were checked for water-uptake by syringe injection tests in the trunk and for the occurrence of amorphous plugs in the xylem vessels. Water uptake was normal, and no amorphous plugs in the xylem vessels were seen (4). Another evaluation was conducted in May 1987, 35 months after root-grafting and 14 months after transplanting to the Bebedouro Experiment Station. These results were also negative.

In December 1987, 42 months after grafting, some of the receptor plants that were root-grafted to diseased donor trees began to show visual symptoms of declinio. The diagnosis tests were then applied to all the young plants of the experiment (Table 1). All the RG plants were showing leaf zinc deficiency symptoms with some of them very intense. Zinc deficiency was also observed in two of the C_4 and three of the C_2 plants. The visual symptoms of the C_2 and C_4 plants disappeared a few months later, whereas, the symptoms remained in the plants of the RG group. Water uptake in almost all the RG plants was minimal, whereas the controls, C_1, C_2, C_3 and C_4, had normal water-uptake (Table 1). Zinc accumulated in the trunk wood of the RG plants, whereas the controls, C_1, C_2, C_3 and C_4, had normal zinc concentrations in the trunk (Table 1). Amorphous plugs were found in the RG plants but were not detected in the controls (Table 1).

Manifestation of visual symptoms. Visual symptoms of declinio increased from grade 1 to 3 in RG plants, and by March 1989, 10 of the 12 plants that were root-grafted to diseased donor trees had small leaves with symptoms of zinc deficiency, a thin canopy, twig dieback, and small-sized fruit. These symptoms were absent in the controls. The RG plants also showed reduced growth when compared with the other treatments. The diagnostic tests were positive, leading to the conclusion that declinio was transmitted for the first time in Brazil by approach-root-grafting. Nothing abnor-

TABLE 1
RESULTS OF DECLINIO DIAGNOSTIC TESTS ON VALENCIA ORANGE TREES ON RANGPUR ROOTSTOCK INVOLVED IN A ROOT-GRAFTING EXPERIMENT[z]

Treatments	No. of trees tested[y]	No. of trees with Zn deficiency	Water uptake (ml/sec)[x]		Zn concn (μg/g)[w]	Amorphous Plugs (%)
(RG) Root-grafted to declinio donor	12	12	Range	0-0.13	3.00-9.13	3.92-50.05
			Mean	0.03a[u]	5.54a	29.80a
(C1)root-grafted to healthy donor	2	0	Range	0-0.30	2.00-3.00	0
			Mean	0.15	2.50	0
(C2) Non-root grafted around declinio donor	8	3	Range	0.03-0.20	1.25-2.50	0
			Mean	0.12b	1.67b	0b
(C3) Non-root grafted around healthy donor	2	0	Range	0.20-0.55	0.75-1.75	0
			Mean	0.37	1.25	0
(C4) Healthy nursery plants	9	2	Range	0.03-0.28	1.00-2.00	0
			Mean	0.13b	1.69b	0b

[z]This study was initiated with nursery trees and the data obtained 42 months later. All receptor trees were transplanted from the field to a new location after 20 months.
[y]The number tested differs from the original number because of tree loss during transplanting.
[x]By syringe injection test.
[w]Determined in trunk-wood segments above bud union.
[u]Mean separation by Student's "t" test, P≤0,05; data of treatments C1 and C3 were not statistically analyzed.

mal was observed in the control plants, even in those which had been planted around the declinio donor trees to monitor soil and enviromental factors. Therefore, it is probable that the transmission occurred by the root-graft union, illustrating the likely presence of a systemic pathogen. While pathogen might be found in any part of the trees, however, root-grafting has been the only way demonstrated so far to transmit declinio. Other methods of transmission are now being tested.

The transmission by approach-root-grafting described here was somewhat different than the procedures done in Florida, where the mature trees that were used as receptors remained root-grafted to the donor trees during the entire experiment. With the declinio transmission experiment described here, the nursery plants (receptors) had an acquisition period long enough to acquire the disease, and were then removed from the donor source.

Blight and declinio have the same diagnostic characteristics, and have now both been transmitted similarly by approach-root-grafting, further indicating that the cause of the two diseases is the same. The results also suggest that the hypothesis concerning physiological, nutritional or soil condition problems may not be tenable as a direct cause of declinio, but might be important factors that accelerate the development of the disease by stressing the trees and enhancing their susceptibility to the pathogen.

The ability to experimentally induce declinio will help in the search for a causal agent and permit a better evaluation of rootstocks in relation to the disease. Any method which also reduce the period of time to less than 7 yr for the trees to show symptoms will make a meaningful contribution to studying the disease and developing control strategies.

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