On The Etiology of the Citrus Sudden Decline in Venezuela

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ABSTRACT. Since 1985, a decline has been observed in sweet orange trees budded on Volkamer lemon. Diagnostic techniques have suggested similarities with the blight-declinio disease group. Isolates of *Fusarium* spp. from roots and shoots of plants were grown in potato-dextrose broth. Fungal structures were separated from the medium by centrifugation at low temperature. The supernatant was passed through a 0.2 μm filter and infiltrated for 3 days into 1-yr-old sweet orange plants budded on Volkamer lemon. Toxin treated plants had a greater number of amorphous plugs and a higher zinc content than control plants. All plants showed some twig dieback, but some of the infiltrated plants showed leaf flaccidity and zinc deficiency. In other experiments, soil was collected from the field near roots from healthy and affected trees and sweet orange on Volkamer lemon were potted with steam-sterilized and nonsterile soil from both sources. After one yr, all plants were evaluated for visible symptoms, the presence of xylem plugs and the zinc content in the wood. It is possible that the Fusarium toxins play a role in the disease, but there was no transmission through soil in the pot test.
Index words. Citrus blight, declinio, toxins, *Fusarium* spp.

The sudden decline or "decaimiento repentino" represents a serious threat to Venezuelan citriculture. A previous diagnosis has demonstrated the similarity between the "decaimiento repentino" and the blight-declinio disease group, according to visible canopy symptoms, lack of water absorption by the plants, presence of amorphous plugs in the xylem and high zinc content in the trunk (10, 11, 12, 15). Up to 1980, more than 1,500 trees had died in the commercial grove where the disease was found initially, and about 5,000 plants are estimated to affected in the Carabobo and Yaracuy States. In 1987, Venezuela had 56,174 ha of citrus about 11,459,496 trees, of which 6,875,698 are budded on Volkamer lemon (3, 7). Estimating a 3% annual incidence, 206,270 trees will affected in the near future, represening a considerable economic loss. The etiology of this group of diseases is still unknown, although recent root-grafting experiments indicate that an infectious agent is involved (2, 6, 13). Some authors have suggested the possibility that *Fusarium* toxins could be the cause of blight. Nemec, *et al.* and Bender, *et al.* (1, 8, 9) argue that the obstructions are formed as a response of the plant to the presence of *Fusarium solani* and its toxins, the former author concluded that *F. solani* is a primary pathogen in citrus roots and the primary cause of plugging in blighted trees. On the other hand, Graham, *et al.* (4), using scanning electron microsoopy (SEM) concluded that *F. solani* is not the primary cause of blight. Citrus roots infected by the fungus did not show the typical amorphous plugs present in trees suffering from blight (4). *Fusarium solani* only colonized citrus roots after starch reserves had been depleted (4). In Cuba and Venezuela, diseased citrus trees had fewer fibrous roots and at times wood necrosis of the main roots, from which *Fusarium* spp. was isolated consistently (14, 15).

The objectives of this study were to evaluate the effect of *Fusarium* toxins on trees on Volkamer lemon rootstock; and to attempt soil transmission of the disease.

MATERIALS AND METHODS

Isolation of the fungus and toxin production. Root and twig samples were collected from 6-yr-old Valencia sweet orange plants budded on Volkamer lemon rootstock in three localities and taken to the laboratory. *Fusarium* sp. isolated consistently from the samples in potato dextrose agar medium were grown in a potato-

dextrose broth for 16 days. The fungal structures were separated from the liquid phase in three steps. First, the cultivated fungus was filtered through sterile cheesecloth. The mycelium obtained was used in pathogenicity tests and the liquid fraction was centrifuged at 1800 x *g* for 15 min in a CRV-5000 Damon/TEC Division Model Centrifuge. The supernatant was filtered using a 0.2 μm. filter. The absorbancy of each filtrate was measured using a Leitz, Inc., Model M colorimeter (filter N° 45).

Types of inoculations. Pieces of sterile cotton impregnated with mycelium of *Fusarium* were placed between the cortex and the wood of 1-yr-old sweet orange plants budded on Volkamer lemon. Control plants were inoculated only with sterile distilled water. All treated plants were placed in a humid chamber for 15 days and were evaluated montly.

Ten-day-old Volkamer lemon seedlings were placed in small glass bottles containing the toxins and were incubated in a growth chamber at 20 C under fluorescent light for 15 days. After that time, the dry weight of roots and stems (without cotyledons) was measured.

Volkamer lemon cuttings 30 cm tall and 0.5 cm in diameter were obtained by excising the roots and disinfesting the cut ends with a 3% sodium hypochlorite solution for 1 min and washing immediately 3 times with sterile distilled water. The cuttings were placed in test tubes containing the toxins. The mouth of the tubes was closed with cotton to avoid excess evaporation. The treated cuttings were placed at first in a shade house, at 32-34 C for 24 hr to accelerate the absorption of the toxins. After this time, they were moved to laboratory conditions at 25-27 C. Eight days later, the number of plugs were counted, under the light microscope, in stem slices (20 μm thick), cut with a Leitz freezing microtome and stained with fast green and safranin.

One-yr-old Valencia sweet orange plants budded on Volkamer lemon were infiltrated with *Fusarium* toxins as follows. A hole was made, using a drill in such a way as to hold a pasteur pipette, filled with the toxin and recharging it for 3 days. The wounds were then sealed with wood tar. One year later, the plants were evaluated, according by visible symptoms, presence of amorphous plugs in the xylem using SEM and wood zinc content (5, 16) (Fig. 1C).

All previous experiments used a completely randomized design, with six treatments (isolates) and three replicates.

Transmission through soil. One-yr-old Valencia sweet orange plants budded on Volkamer lemon were grown in pots, containing sterilized and non sterilized soil, the latter collected near the roots of affected and healthy trees in the field. The soil was sterilized by hot vapour for 5 hr. One year later, the plants were evaluated, according to visible symptoms, presence of amorphous plugs in the xylem using SEM and dry weight of plants. A completely randomized design with four treatments and four replicates was used.

RESULTS

After 3 months, the plants inoculated with the mycelium of *Fusarium* showed necrosis in the stems and the fungus was isolated from these lesions. The control plants showed no symptoms.

There were no differences in the external appearance, dry weight of roots and stems, among the Volkamer lemon seedlings placed in the *Fusarium* filtrates broth or water. None of the filtrates induced phytotoxicity, probably because the seedlings were still utilizing the cotyledonary reserves. Nevertheless, the growth of contaminants only in the broth suggested the presence of toxic substances in the other treatments that inhibited saprophytic microbial growth (Table 1). On the other hand, Volkamer lemon cuttings inoculated with *Fusarium* toxins began to show

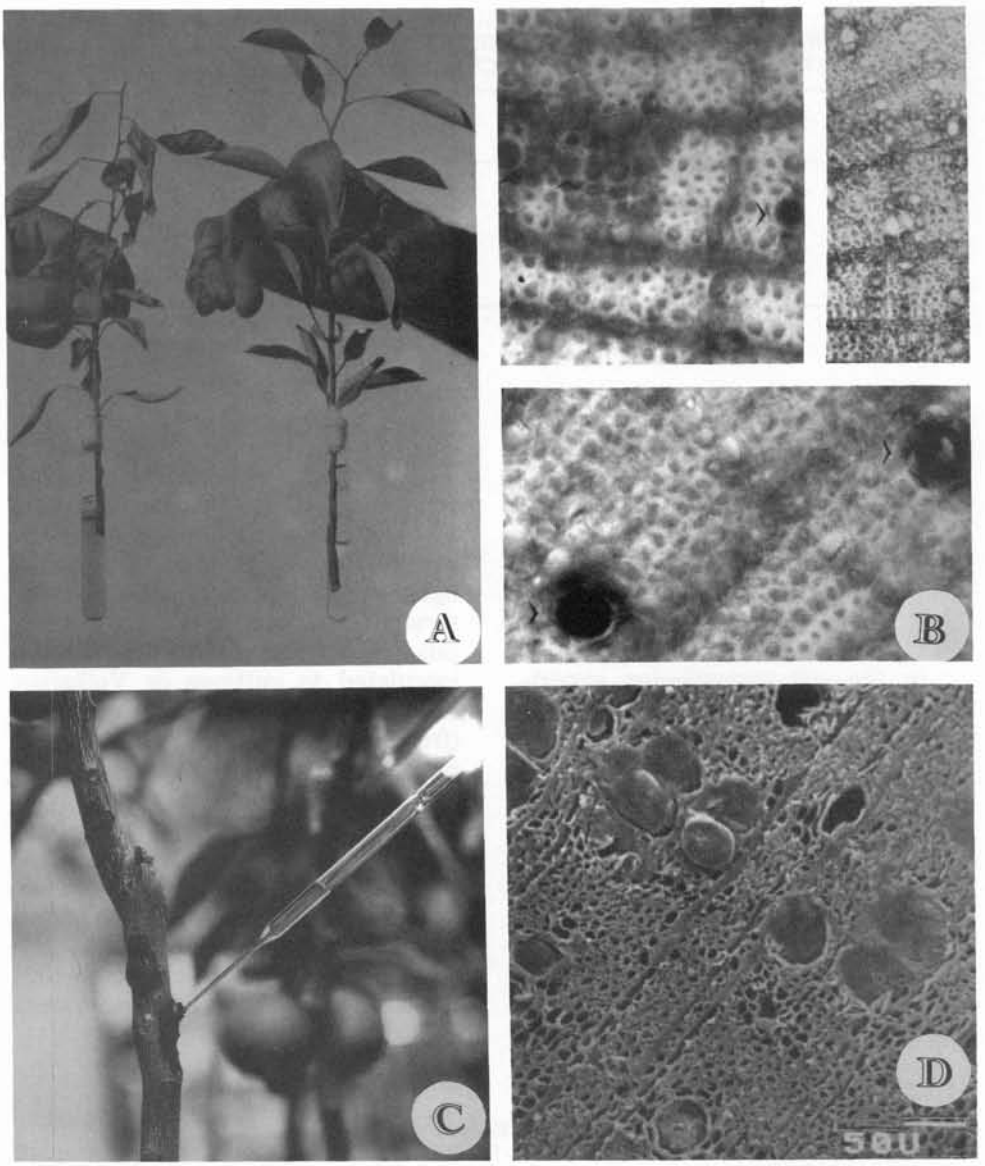

Fig. 1. A. Volkamer lemon cuttings inoculated with *Fusarium* toxins (left) and with sterile water (right). B. Stem slices (20 mμ thick) from Volkamer lemon cuttings showing xylem plugs (arrows) and control (right corner). C. *Fusarium* toxin infiltration, in 1-year old citrus plants. D. Xylem amorphous plugs in toxin infiltrated plants.

leaf flaccidity symptoms in 24 hr and completely wilted after 8 days (Fig. 1A). The toxins induced between 0.43 to 24.6% of plugs in the xylem (counting 200 vessels/treatment (Fig. 1B). In the filtered broth, sterile broth and sterile water treatments no symptoms or plugs were induced.

Sweet orange plants budded on Volkamer lemon infiltrated with the toxins showed dry ends of twigs and some leaf flaccidity and zinc deficiency symptoms. Control plants had only dried twig ends. There were significant differences between the control and treated plants, the latter had higher number of amorphous plugs in the xylem (Fig. 1D) and higher wood zinc content (Table 2). Some of the samples had filamentous and wound-gum plugs.

TABLE 1
AVERAGE DRY WEIGHT OF ROOT AND SHOOT OF VOLKAMER LEMON SEEDLINGS WITHOUT COTYLEDONS TREATED WITH CULTURAL FILTRATES OF *FUSARIUM* SPP.

Cultural filtrates	Root wt (mg)	Shoot wt (mg)	Presence of contaminants
GMM_4FA	15.0	38.6	no
MM_8	12.0	30.7	no
86044	13.3	35.3	no
Filtered broth	13.4	32.8	yes
Distilled water	11.8	32.9	no

The controls showed few amorphous and filamentous plugs.

There were differences in absorbancies among cultural filtrates obtained from *Fusarium* isolates. These had higher percentages of absorbancy than water (0%) or the broth (20%). The MM8 isolate had the highest value (55%) (Table 2).

In the soil transmission experiment, there was only a significant difference between dry weights of plants grown in sterile and nonsterile soil. Probably the sterilization procedure changed the chemical composition and microflora of the soil, as lower zinc contents were obtained in plants grown in sterilized soil (Table 3). Plants showed dry ends in all treatments, without zinc deficiency symptoms or flaccidity of the leaves.

DISCUSSION

This study corroborates previous reports about the pathogenicity of *Fusarium* as a primary agent in citrus plants. Histological studies using the light microscope and SEM demonstrated the formation of numerous plugs when *Fusarium* filtrates were inoculated to cuttings or Volkamer lemon plants, in a short period of time (from days to a year). The amorphous plugs induced in sweet orange on Volkamer lemon rootstock were morphologically identical to those observed in field affected plants. Toxin treated plants also showed higher levels of zinc in the trunk and some had zinc deficiency symptoms and flaccidity of leaves. All these characteristics are similar to those found in plants

TABLE 2
EFFECTS OF *FUSARIUM SPP.* CULTURAL FILTRATES ON VALENCIA SWEET ORANGE ON VOLKAMER LEMON PLANTS AFTER ONE YEAR

Isolates	Amorphous plug index[z]	Zn content (μg/g)	Absorbance of cultural filtrates
MM_1FC_1	0.73a[y]	49.8	0.30
86044	0.69a	31.2	0.33
GM_1FA	0.67a	47.1	0.29
MM_8	0.67a	35.0	0.55
GM_4FB	0.58ab	45.0	0.28
MM_1FC_2	0.45b	45.8	0.24
Control (Without injury)	0.16c	22.0	—
Control (Water injected)	0.12c	24.0	0

[z]Number of amorphous plugs / total number of plugs.
[y]Mean separation in columns by Duncan's multiple range test, $P \leq 0.05$.

TABLE 3
ATTEMPS TO TRANSMIT SUDDEN DECLINE THROUGH SOIL TO VALENCIA ORANGE ON VOLKAMER LEMON PLANTS POTTED IN THE INDICATED TREATMENT AND EVALUATED ONE YEAR LATER

Treatment	Dry wt (g)	Zn content (μg/g)	Amorphous plugs
Soil from beneath healthy plants			
Sterilized	118bc[z]	22.37	no
Non sterilized	369a	39.49	no
Soil from beneath diseased plants			
Sterilized	262ab	19.31	no
Non sterilized	330a	34.93	no

[z]Mean separation in columns by Duncan's multiple range test, $P \leq 0.05$.

affected with "decaimiento repentino" in the field.

Soil transmission tests considering long term evaluations need to be conducted to discard or associate soil factors with the "decaimiento repentino".

A survey about the distribution and incidence of the disorder is being conducted. This will suggest the possible mechanisms involved in the disease.

Experiments at field level and biochemical studies of the interaction between the host and the toxin are necessary to draw a conclusion about the role of *Fusarium* toxins in the etiology of citrus sudden decline in Venezuela.

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