Induced Tolerance to Mal Secco Disease in Etrog Citron and Rangpur Lime by Infection with the Citrus Exocortis Viroid

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ABSTRACT

Solel, Z., Mogilner, N., Gafny, R., and Bar-Joseph, M. 1995. Induced tolerance to mal secco disease in Etrog citron and Rangpur lime by infection with the citrus exocortis viroid. Plant Dis. 79:60-62.

Etrog citron and Rangpur lime plants were each inoculated with one of four isolates of the citrus exocortis viroid (CEVd) and incubated for 1 yr, during which time the viroid spread systemically. CEVd caused severe leaf curling and short internodes in Etrog citron but not in Rangpur lime. After leaf inoculation with *Phoma tracheiphila,* typical mal secco disease symptoms developed on leaves of both cultivars, regardless of prior infection by CEVd, but growth of the mycelium from the infected leaves into the branches was greatly affected by CEVd infection. In Etrog citron, *P. tracheiphila* was isolated from only 9.1% of the branches sampled from CEVd-infected plants, compared with 100% from CEVd-free plants. In Rangpur lime, the incidence of branches containing *P. tracheiphila* varied in CEVd-infected plants from 16.7 to 50% among CEVd isolates, compared with 70% in the viroid-free controls. This is believed to be the first report of systemic resistance induced in a woody plant by prior inoculation with a viroid.

Additional keywords: biological disease control, induced systemic resistance

Mal secco, caused by the fungus *Phoma tracheiphila* (Petri) Kantsch. & Gik., is a severe vascular disease of various citrus cultivars in the Mediterranean region (22). After penetration via wounds, the pathogen colonizes the xylem elements of the leaf, causing veinal chlorosis, wilt, and shedding of leaves. The fungus progresses systemically in the xylem elements into the branch wood (24), causing withering and dieback of branches and eventually debilitating the tree. No satisfactory genetic or chemical control measures are available for this damaging disease (22). Tolerant lemon cultivars have been selected in Italy, but the quality of their fruit is not acceptable for the export market, and their productivity is low (22). Preventive sprays and sanitation may limit the severity of the disease to some extent (22).

Field observations in Sicily and Israel have indicated that nucellar lines of lemon, free of viroids, are more susceptible to mal secco than "old" lines of the same cultivars (14,23). Combinations of old-clone citrus cultivars grafted onto Rangpur lime (*Citrus limonia* Osbeck), Palestine sweet lime (*C. limettoides* Tan.), and sweet orange (*C. sinensis* (L.) Osbeck) rootstocks were found to be tolerant to foot rot caused by *Phytophthora citrophthora,* whereas similar combinations grafted with viroid-free nucellar scions succumbed to the disease soon after planting (1,19). Furthermore, combinations of these rootstocks grafted with viroid-free budwood, obtained by the shoot tip grafting method, were also susceptible to Phytophthora root rot in conditions under which plants infected by the citrus exocortis viroid (CEVd) were tolerant (M. Bar-Joseph, *unpublished*).

Old-clone lemons and other cultivars were found to be infected by a range of citrus viroids (CVds) (4). Molecular and biological properties of the known CVds suggested their separation into five groups (5). The type members of four groups have been characterized and found to consist of CEVd (21), hop stunt viroid (15,20), and two natural chimeric viroids (2,16).

The objective of this study was to examine our hypothesis that CEVd infection of citrus cultivars susceptible to mal secco augments their tolerance to this fungal disease.

MATERIALS AND METHODS

CEVd isolates. Two isolates, CEVd-Y and CEVd-225-sm, were separated from the graft-transmissible dwarfing complex (GTDC)-225, which is composed of five different CVds (9), by mechanical inoculation of tomato (*Lycopersicon esculentum)* and *Gynura aurantiaca* plants, respectively. Two severe CEVd isolates, CEVd-76 and CEVd-P3, were separated from GTDC #Sho, originating from a Eureka lemon tree at Shokeda in the northern Negev, by passage through *G. aurantiaca.* RNA extracts from the herbaceous host plants were slash inoculated onto Etrog citron seedlings, and the presence of the CEVd RNA band of 370 nt was assayed by sequential polyacrylamide gel electrophoresis (s-PAGE) (9,18).

Plants and CEVd inoculation. Etrog citron (*C. medica* L.), grafted onto Volkamer lemon rootstock (*C. volkameriana* Pasquale), and Rangpur lime seedlings were grown in 10-L plastic bags in a greenhouse. At the age of about 1 yr, 16 plants of each cultivar were inoculated with the four CEVd isolates (Y, 225-sm, 76, and P3), and four uninoculated plants served as controls. Four plants were graft inoculated with each isolate by budding two chips from infected plants. The seedlings were pruned and grown in a screenhouse for about 1 yr until the following summer. Then the plants were pruned to induce growth of new shoots for assessment of tolerance to mal secco.

Testing the presence of CEVd in plants. Leaves were collected from CEVd-infected seedlings, and the presence of viroids in RNA extracts was determined by s-PAGE (9,18).

Inoculation with *P. tracheiphila.* A young, fully expanded leaf at the shoot apex of each plant was inoculated at two points by injecting an aqueous conidial suspension (10^6/ml) of *P. tracheiphila* through the lower epidermis by using a hypodermic syringe with a fine needle. This process created minute wounds, often over small veins, and the spore suspension was infiltrated into an area surrounded by bigger veins. Most conidia were retained close to the injection point. The inoculated plants were maintained in a temperature-controlled greenhouse at approximately 23 C. Disease symptoms, expressed as parenchymal chlorosis over the veins, developed within 2–4 wk after inoculation and were recorded.

Systemic development of *P. tracheiphila.* Three months after the inoculation, randomly selected shoots each with an infected leaf were removed and assayed for colonization by the pathogen. Surface-sterilized sections (2–3 mm), cut from various locations along the shoot, were plated on potato-dextrose agar and examined for development of *P. tracheiphila.*

RESULTS AND DISCUSSION

Leaf and shoot samples from all the plants inoculated by the four CEVd iso-

Contribution 1306-E, 1994 series, from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel.

Accepted for publication 18 July 1994.

Table 1. Rate of leaf infection and isolation of *Phoma tracheiphila* from the wood of shoots each with a symptomatic leaf after inoculation with one of four citrus exocortis viroid (CEVd) isolates[a]

	Etrog citron				Rangpur lime			
	Leaves (no.)		Branches (no.)		Leaves (no.)		Branches (no.)	
CEVd isolate	Inoculated	Infected	Examined[b]	Infected	Inoculated	Infected	Examined	Infected
P3	18	17	8	0	14	12	6	2
Y	21	21	3	0	15	12	6	3
76	21	20	7	2	14	14	5	1
225-sm	13	9	4	0	22	21	6	1
Viroid-free control	48	48	9	9	25	24	10	7

[a]Each isolate was inoculated onto four Etrog citron plants and four Rangpur lime plants.
[b]Cultures were made from five to 15 2-mm segments sampled along the branch.

lates were found to carry the viroid. The infected shoots of Etrog citron showed severe curling of the leaves and short internodes. Rangpur lime, although infected by CEVds, did not exhibit distinct morphological changes at the time of the assay.

Leaves of Etrog citron or Rangpur lime inoculated with *P. tracheiphila* became infected by the fungus regardless of whether the plants were infected with CEVd (Table 1). Three months after the inoculation, it became evident that only the CEVd-free plants revealed severe defoliation and drying of shoots (Fig. 1A and C), whereas none of the CEVd-infected seedlings showed any systemic mal secco symptoms (Fig. 1B and D). Mal secco symptoms occur when the pathogen colonizes the xylem vessels of the branches and leaves.

The systemic advance of the mycelium from the infected leaves into the branches was strongly affected by CEVd infection in both citrus cultivars (Table 1). *P. tracheiphila* was isolated from all (Etrog) or almost all (Rangpur) of the examined branches of the uninoculated control plants. In contrast, the fungus could not be isolated from the branches of the Etrog citron infected by CEVds, except for two branches in a plant infected by CEVd-76 (Table 1). Likewise, in Rangpur lime seedlings, an inhibition of the systemic development of the pathogen was observed in CEVd-infected plants (Table 1). The incidence of branches containing *P. tracheiphila* varied in CEVd-infected plants from 16.7 to 50% among CEVd isolates, compared with 70% in the viroid-free controls (Table 1). Lack of systemic growth of *P. tracheiphila* indicates that the fungus is contained in the original infected leaves, apparently because of inhibitory factor(s) induced by CEVd infection in Etrog and Rangpur lime plants. The fungus was viable in the inoculated leaves, regardless of whether they exhibited mal secco symptoms.

The mechanism by which infection by CEVds induces resistance of citrus plants to *P. tracheiphila* is not yet known. Induced systemic resistance of plants to fungal pathogens conferred by infection with viruses (10,11,13), by a virus attenuated by satellite RNA (17), or by other pathogens (11) has been described in herbaceous plants. Our study appears to be the first report of systemic resistance induced in a woody plant by prior inoculation with a viroid. This study confirms field observations of the relative disease tolerance of old clones, apparently the result of a viruslike entity, compared with that of nucellar ones (14,23).

Recently, the de novo synthesis of

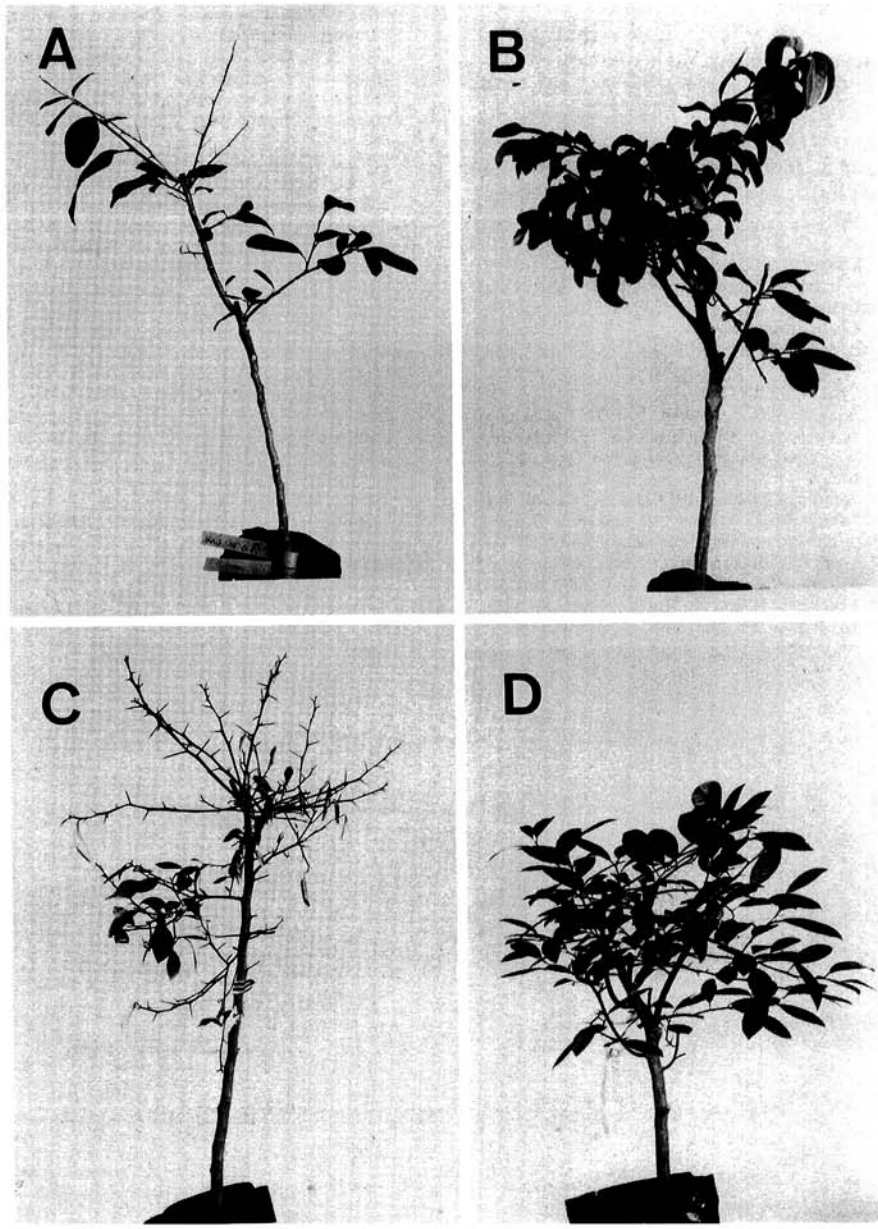

Fig. 1. Severity of mal secco symptoms 3 mo after leaf inoculation with *Phoma tracheiphila:* Etrog citron plants **(A)** not infected and **(B)** infected and Rangpur lime plants **(C)** not infected and **(D)** infected with citrus exocortis viroid.

pathogenesis-related (PR) proteins in infected plants and their role in resistance to diseases has been widely reported and reviewed (6,12). In one report, tomato plants inoculated with CEVd developed systemic infection, which induced synthesis and accumulation of a set of 10 major different cationic PR proteins (8). One of these proteins, P23, was characterized and shown to have an antifungal activity. Chitinase, a widely investigated PR protein, was shown to promote resistance to *Rhizoctonia solani* (3). Recently, the involvement of chitinase in resistance to mal secco has been conjectured (7).

This study has shown that CEVd infection of citrus plants enhances their tolerance to mal secco disease. The practical use of this effect for protecting citrus crops susceptible to mal secco, such as lemons and tangeloes, and the mechanism(s) involved need to be explored.

ACKNOWLEDGMENTS

We thank Miriam Kimchi and J. Ben Shalom for technical assistance. This study was supported by the Citrus Marketing Board, The Chief Scientist's Fund, and GIARA.

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