Virus-*Glomus etunicatum* Interactions in Citrus Rootstocks

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ABSTRACT

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Sour orange, *Citrus macrophylla*, and Duncan grapefruit seedlings were blind-bud-inoculated with tristeza virus isolates T-3 and T-1 and citrus leaf rugose virus (CLRV-2), respectively, after growing in a greenhouse for 3 mo in low-phosphorus (P) soil (9–12 μg P/g) amended with *Glomus etunicatum* or in soil amended with 210 μg P/g only. Controls were virus-free plants grown in these soils. Growth of *C. macrophylla* and Duncan grapefruit was not significantly reduced by virus infection in the *G. etunicatum*- or phosphorus-amended soil 98 days after inoculation, but growth of sour orange infected with tristeza virus isolate T-3 was significantly reduced in both soils compared with that in the nonvirus treatments. In growth chamber studies with Duncan grapefruit inoculated with CLRV-2, growth was significantly reduced in both soils compared with the controls because temperatures (27 C day/21 C night and 32 C day/21 C night) were cool enough to favor increased expression of virus symptoms. More root degeneration occurred in virus-inoculated plants in both soils than in controls. In greenhouse studies, fungus chlamydospore numbers and percentage of infection generally were higher in the nonvirus treatments than in virus treatments. Mycorrhizal infection of sour orange did not minimize the pathogenic effects caused by tristeza.

Few studies of virus-infected mycorrhizal plants have been reported, but a review of this subject (2) has indicated that plant resistance to viruses was reduced by the limited number of mycorrhizas studied. The number of local lesions was greater in tomato and tobacco infected with *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe and tobacco mosaic virus than in controls (8,9). In another study (1), virus titer was higher in leaves and roots of mycorrhizal plants than in those of control plants. In the latter study, tomato infected with tomato aucuba mosaic virus and potato virus X and *Petunia violacea* Lindl. 'Rose of Heaven' or *Fragaria chiloensis* Duch. var. *ananassa* Bail 'Talisman' infected with arabis mosaic virus all contained higher virus titer when also infected with *G. macrocarpum* var. *geosporum* (Nicol. & Gerd.) Gerd & Trappe. Schöenbeck and Schinzer (9) and Daft and Okusanya (1) suggested that increased viral activity in mycorrhizal plants was due to the enhanced nutrition associated with mycorrhizal plants.

In this study, we evaluated the potential of mycorrhizal citrus rootstock seedlings to protect against growth suppression by viruses. *G. etunicatum* Becker & Gerd., chosen as the test symbiont, is common in Florida soils and is one of the most efficient in promoting citrus growth (5). Virus isolates included citrus leaf rugose virus, a mild tristeza isolate (T-1), and a severe tristeza isolate (T-3).

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MATERIALS AND METHODS

Seeds of *Citrus macrophylla* Wester, *C. paradisi* Macf. 'Duncan' (grapefruit), and *C. aurantium* L. (sour orange) were sown in flats of a low-phosphorus (P) subsoil phase of Astatula fine sand (hyperthermic, uncoated typic quartzipsamments) (9–12 μg P/g) amended with *G. etunicatum* and in Astatula fine sand amended with triple superphosphate to give 210 μg P/g. Inoculum of the fungus added to flats was increased on sudangrass (*Sorghum sudanese* (Piper) Stapf) and consisted of spores, infected root fragments, and extramatrical mycelium. This inoculum was mixed with steam-pasteurized sand and added to the flats before seeds were planted. The amount of inoculum added to flats was equivalent to about one chlamydospore per gram of inoculated

Table 1. Growth, root rot, and development of *Glomus etunicatum* in soil and roots of *Citrus macrophylla* inoculated with citrus tristeza virus T-1, Duncan grapefruit inoculated with citrus leaf rugose virus (CLRV-2), and sour orange inoculated with tristeza isolate T-3 and grown in low-phosphorus (P) soil inoculated with *G. etunicatum* or in P-amended soil

Treatment	Plant growth: Stem diam. (mm)	Plant growth: Fresh wt (g) Top	Plant growth: Fresh wt (g) Root	Root rot[a]	Fungal development: Chlamydospores per 25 g soil	Fungal development: Root infection[b] (%)
Citrus macrophylla						
G. etunicatum	4.7	17.8	16.7	0.4**	159	81***
G. etunicatum + tristeza	5.1	19.6	14.4	1.4	177	55
P-amended control[c]	6.1	27.1	24.6*	0.3*	...	...
P-amended + tristeza[c]	5.9	24.1	18.5	1.0	...	...
Duncan grapefruit						
G. etunicatum	6.7	30.1	30.6	0.3*	498	64
G. etunicatum + CLRV-2	6.5	29.6	19.9	1.3	153	64
P-amended control[c]	7.6	33.9	30.8	0.4*	...	...
P-amended + CLRV-2	7.7	35.4	27.0	1.3	...	...
Sour orange						
G. etunicatum	5.9	33.8***	38.2***	0.0***	616***	50***
G. etunicatum + tristeza	6.4	22.8	22.8	1.3	112	22
P-amended control[c]	6.4	31.7***	35.1***	0.3***	...	...
P-amended + tristeza	6.4	19.3	20.8	1.9	...	...

[a] Root rot rating on a scale of 0–5, where 0 = no root rot and 5 = extensive epidermal and cortical sloughing.

[b] Infection evaluated in 30 1-cm-long root pieces per plant. Dunnett's test used to compare virus treatments with nonvirus treatments (* = $P = 0.05$, ** = $P = 0.01$, and *** = $P = 0.001$).

[c] Soil amended with 210 μg P/g.

Table 2. Growth, root rot, and development of *Glomus etunicatum* in roots of Duncan grapefruit inoculated with citrus leaf rugose virus (CLRV-2) and grown in low-phosphorus (P) soil containing *G. etunicatum* or in P-amended soil at two temperature regimes

Treatment	Plant growth				Root rot[a]	Root infection (%)
	Stem diam. (nm)	Plant ht (cm)	Fresh wt (g)			
			Top	Root		
27 C Day (12 hr)/21 C night (12 hr)						
G. etunicatum	3.4	46.4**	18.0*	21.8	0.8**	7.8
G. etunicatum + CLRV-2	2.5	33.8	12.4	17.4	4.0	16.3
P-amended control[b]	3.5***	54.2*	26.2***	28.6**	0.8**	0.0
P-amended + CLRV-2	1.7	26.8	11.4	11.3	4.0	0.0
32 C Day (12 hr)/21 C night (12 hr)						
G. etunicatum	6.1**	62.9	44.6**	39.9	0.8*	26.0
G. etunicatum + CLRV-2	2.9	42.5	20.1	28.4	3.3	26.6
P-amended control[b]	6.0**	53.8	30.4**	33.1*	0.0**	0.0
P-amended + CLRV-2	3.1	48.5	15.2	16.9	2.0	0.0

[a] Root rot rating on a scale of 0–5, where 0 = no root rot and 5 = extensive epidermal and cortical sloughing.
[b] Soil amended with 210 μg P/g. Dunnett's test used to compare virus treatments with nonvirus treatments (* = $P = 0.05$, ** = $P = 0.01$, and *** = $P = 0.001$).

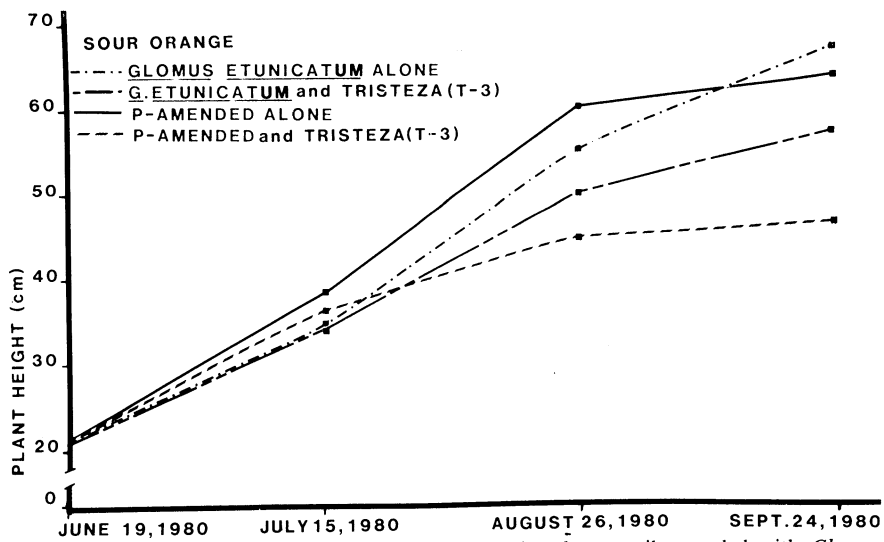

Fig. 1. Height of sour orange plants grown in low-phosphorus soil amended with *Glomus etunicatum* alone, in soil amended with phosphorus (210 μg/g) alone, and in both soils in which sour orange was inoculated with tristeza virus isolate T-3.

soil, an amount in excess of that normally used to ensure early optimum infection. When the resulting seedlings were 5 mo old and about 15 cm tall, plants grown in mycorrhiza-infested soil were transplanted to steamed low-P sand and uninoculated seedlings were transplanted to steamed sand amended with 210 μg P/g in clay pots. Experiments were set up in a greenhouse and in growth chambers.

In the greenhouse studies, plants were grown in 15-cm-diameter clay pots on benches for 3 mo and fertilized monthly with a 12-0-6 (NPK) liquid fertilizer. Three months were sufficient for the mycorrhizal plants to become established in pots and develop a stem of suitable caliper to bud. After 3 mo, seven-pot replicates each of sour orange, *C. macrophylla*, and Duncan grapefruit grown in mycorrhiza-infested soil and P-amended soil were blind-bud-inoculated with tristeza virus isolates T-3 and T-1 and citrus leaf rugose virus (CLRV-2), respectively. Mycorrhizal plants were not disturbed to assay for root infection when they were virus-inoculated. Virus cultures were maintained in systemically infected citrus seedlings in a greenhouse before use. Controls were virus-free plants grown in these soils. All plants were grown and fertilized monthly with 12-0-6 fertilizer. After an additional 98 days, the plants were evaluated for root rot, fungus sporulation and infection, and mineral content.

The viruses chosen for this study produce distinct symptoms in their hosts. Tristeza isolate T-1 is mild in all hosts but can produce some stem pitting in *C. macrophylla*, tristeza isolate T-3 causes severe yellows and stunting in sour orange seedlings, and CLRV-2 causes severe shock reactions of stunting and chlorosis in Duncan grapefruit.

In the growth chamber studies, nonmycorrhizal Duncan grapefruit seedlings were transplanted to low-P sand amended with *G. etunicatum* or to sand amended with superphosphate to give 210 μg P/g. They were grown in 20.5-cm clay pots in a greenhouse for 100 days to allow the fungus to become established in the roots. Then half of the plants in each treatment were double-bud-inoculated with CLRV-2. Seventeen days later, five-pot replicates of each treatment inoculated with CLRV-2 and the appropriate nonvirus *G. etunicatum*- and P-amended controls were moved to two growth chambers. One growth chamber was set at 27 C day (12 hr) and 21 C night; and the other was set at 32 C day (12 hr) and 21 C night. Lighting was the same in both chambers (250 μE m^{-2} sec^{-1}).

The chamber with the coolest day setting was designed to approximate temperatures ideal for foliar symptom development. When plants were placed in the growth chambers, all were topped to within two buds of the inoculation point and one bud was forced to develop a single terminal shoot. All plants were fertilized with the same 12-0-6 fertilizer used in the greenhouse studies. After 77 days, plants were evaluated for stem diameter, height, top and root fresh weight, root rot, fungus development in roots, and mineral content of leaves.

Root rot was rated on a scale of 0–5, where 0 = no root rot and 5 = extensive epidermal and cortical sloughing. In the greenhouse study, fungus chlamydospores were wet-sieved from 25-g soil samples and counted on the stage of a dissecting microscope. Chlamydospore counts were not made in the growth chamber study. In the greenhouse and growth chamber studies, root infection by *G. etunicatum* was determined as follows. Roots of each plant were chopped into pieces 1 cm long, stained in hot lactophenol solution containing acid fuchsin or aniline blue,

and destained in lactophenol (4). Percentage of infection was based on the presence or absence of hyphae or vesicles in 30 root pieces per plant.

Mineral content of Duncan grapefruit and sour orange leaves was determined by extraction with hydrochloric acid and atomic absorption analysis (3).

RESULTS

At the end of the greenhouse tests, only mild chlorosis had developed in leaves of virus-infected Duncan grapefruit compared with the nonvirus controls; no leaf symptoms developed in virus-infected *C. macrophylla*. Growth of *C. macrophylla* and Duncan grapefruit was unaffected by virus inoculation except for slight suppression of root mass in *C. macrophylla* (Table 1), even though virus-induced root degeneration occurred on both mycorrhizal and nonmycorrhizal plants. This root degeneration did not significantly affect fungus sporulation, but it was related to a significant reduction in fungus infection in *C. macrophylla*. Tristeza inoculation of sour orange resulted in a significant reduction ($P = 0.01$) in height of both mycorrhizal and nonmycorrhizal plants compared with their respective controls (Fig. 1). Virus inoculation of mycorrhizal sour orange caused a decrease in infection and chlamydospores produced.

Plant growth in both nonvirus treatments of each day/night regime was similar in the growth chamber study (Table 2). Inoculation with CLRV-2 in *G. etunicatum*- and P-amended treatments caused a marked expression of leaf shock symptoms, a significant reduction in growth, and an increase in root rot but no apparent change in the status of fungal development compared with the respective controls (Table 2). Virus symptom development was expressed more severely in the chamber programmed for the coolest day, but a better comparison of symptom expression with the control occurred at the warmest day temperature because of the more nearly normal growth of the control at that temperature.

Mineral content of leaves was significantly less in Duncan grapefruit infected with the virus plus fungus than in the mycorrhizal control in the greenhouse study (Table 3). Similar data were obtained in the growth chamber study (Table 4). This change in mineral content did not occur in leaves of plants infected with CLRV-2 in P-amended soil (Table 3) and was not clearly evident in the same treatment in the growth chamber study (Table 4). In general, leaves of sour orange infected with the virus plus fungus had a lower mineral content than the fungus control (Table 3). The virus plus fungus treatment and fungus treatment alone resulted in improved Cu content in leaves compared with the P-amended controls in both studies (Tables 3 and 4). Mineral content of leaves, except for manganese in the greenhouse study, was in a satisfactory range for citrus. Manganese was lower than the 20–50 μg/g considered satisfactory (6).

DISCUSSION

In all reported studies, resistance of plants to viruses appeared to be reduced by the mycorrhizas (2). The number of local lesions was increased in mycorrhizal plants and so was the virus titer in leaves and roots (7). Immunofluorescent techniques indicated that the tobacco mosaic virus in mycorrhizal host roots was mostly bound to cells containing the arbuscular stage of the endophyte (10). In this study, the most pronounced virus effects on the host were caused by tristeza on sour orange and by CLRV-2 on grapefruit in the growth chamber. Effects were similar with both fungus infection and optimum soil P, which indicates that mycorrhizal roots did not protect the hosts against virus infection and disease development.

The suppression of growth of sour orange by tristeza virus in the greenhouse might have been even more striking if the plants had been topped to force new shoots where symptoms are usually pronounced and if the greenhouse temperatures had more closely approximated temperatures suitable for symptom expression. Tristeza virus, as well as CLRV-2, causes symptoms at temperatures cooler than those used in the greenhouse during these tests. These greenhouse experiments were run through early and late summer and were timed to afford an environment optimal for mycorrhizal fungus activity. Nevertheless, *G. etunicatum* did not protect against the effects of the virus.

Root rot on tristeza-inoculated *C. macrophylla* and sour orange was a manisfestation of virus-induced phloem dysfunction. The same mechanism may account for the high level of root rot in the CLRV-2-inoculated grapefruit grown in growth chambers. Roots starved in this way may support only a low level of fungus infection and sporulation. In the case of grapefruit, root degeneration and reduction of mycorrhizal activity probably disrupted mineral uptake, which could account for the lower mineral content in

Table 3. Mineral content in leaves of Duncan grapefruit and sour orange inoculated with citrus leaf rugose virus (CLRV-2) and tristeza virus T-3, respectively, and grown in low-phosphorus (P) soil containing *Glomus etunicatum*, or in P-amended soil

Treatment	Leaf mineral content (ppm)				
	Fe	Mn	Cu	Zn	P
Duncan grapefruit					
G. etunicatum	68***[a]	6.4**	6.6***	59***	1754*
G. etunicatum + CLRV-2	52	4.9	4.6	24	1540
P-amended control[b]	69	11.0	3.2	30	1920
P-amended + CLRV-2	65	9.8	3.5	28	1843
Sour orange					
G. etunicatum	51	6.0**	6.0	27	1481*
G. etunicatum + tristeza	44	4.5	5.2	27	1923
P-amended control[b]	61**	12.0	2.7	26***	2079
P-amended + CLRV-2	48	11.0	2.7	30	1745

[a] Dunnett's test used to compare virus treatments with nonvirus treatments (* = $P = 0.05$, ** = $P = 0.01$, and *** = $P = 0.001$).
[b] Soil amended with 210 μg P/g.

Table 4. Mineral content in leaves of Duncan grapefruit inoculated with citrus leaf rugose virus (CLRV-2) and grown in low-phosphorus (P) soil containing *Glomus etunicatum* or in P-amended soil in a growth chamber set at 32 C day (12 hr) and 21 C night (12 hr) for 77 days

Treatment	Leaf mineral content (ppm)							
	Ca	Mg	K	P	Cu	Fe	Mn	Zn
G. etunicatum	20,250***[a]	2175	11,225	1850	13.3	172	27.3	41.3***
G. etunicatum + CLRV-2	15,780	1960	13,360	1760	12.8	125	24.2	24.0
P-amended control[b]	23,800	1725	11,875*	1850	6.7	128	30.0	37.0**
P-amended + CLRV-2[b]	19,800	1260	10,000	2160	7.8	179	29.0	24.0

[a] Dunnett's test used to compare virus treatments with nonvirus treatments. (* = $P = 0.05$, ** = $P = 0.01$, and *** = $P = 0.001$).
[b] Soil amended with 210 μg P/g.

the leaves. The fact that leaf mineral content was not significantly affected by CLRV-2 in P-amended soils signifies the important role mycorrhizal fungi have in mineral transport.

Citrus inoculated with mycorrhizal fungi in low-P sands grew well in this and many other studies. Addition of P to uninoculated sand at the rate of 210 μg/g resulted in growth similar to that of inoculated plants in low-P sand. However, these experiments were not concerned with mycorrhizal replacement by P, and statistical comparison between these treatments was considered invalid. Low-P nonmycorrhizal fungus treatments were not included in these studies because nonmycorrhizal citrus grows very little in P-deficient soils (5).

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