Toxicity of Fungicidal Copper in Soil to Citrus Seedlings and Vesicular-Arbuscular Mycorrhizal Fungi

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ABSTRACT

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Carrizo citrange (*Poncirus trifoliata* × *Citrus sinensis*) seedlings were potted in a sand soil (pH 6.8) amended with eight rates of copper (0–300 $\mu g \cdot g^{-1}$ of soil) as basic copper sulfate ($CuSO_4 \cdot 3 Cu(OH)_2 \cdot H_2O$). Double-acid-extractable Cu concentrations in soil ranged from 3 to 248 $\mu g \cdot g^{-1}$. Growth of seedlings and colonization by the mycorrhizal fungus *Glomus intraradices* were reduced logarithmically with Cu concentration. Minimum toxic amounts of Cu ranged from 19 to 34 $\mu g \cdot g^{-1}$ of soil. Leaf P content decreased linearly with Cu for mycorrhizal seedlings without added P but not for nonmycorrhizal plants with supplemental P. The Cu-induced reduction in P uptake of mycorrhizal plants was more closely related to the inhibition of hyphal development outside of the root than to development of vesicles and arbuscules in the root. Thus, Cu-induced P deficiency was attributed to inhibition of P uptake by mycorrhizal hyphae in soil. In a citrus orchard soil with double-acid-extractable $Cu > 80 \mu g \cdot g^{-1}$ and $pH < 5$, replanted trees were stunted and had less mycorrhizal colonization than unaffected trees.

Additional key words: Cu toxicity, foliar disease control, iron chlorosis.

Presently in Florida, copper-containing fungicides are the most efficacious and economical fungicides for controlling certain fruit and foliar diseases of citrus (27,28). Copper sprays are also the only effective means for controlling citrus bacterial canker (23). If canker becomes established in Florida citrus groves, additional sprays might be needed to control the disease. This would accelerate the accumulation of Cu in orchards, and especially in those with long histories of fungicide application.

Studies in the early to mid-1950s (15) indicated a relationship between the age of an orchard, high concentrations of Cu in soil due to yearly fertilizer/fungicide application, and poor growth of citrus plants in pot and field tests. The toxic effects of Cu on citrus trees are greatest in acid soils and can be reduced by applying lime to raise the pH (14,16). At pHs between 6 and 7, Cu accumulates in the surface horizon of the soil (15) and may form complexes with organic matter (15,24). Although the toxic effects of Cu in soils with low pH are well documented (15), there is little information on the impact of Cu in soil when the pH is relatively high. Trees growing in limed soils with a high level of total Cu appear to be as healthy and productive as trees growing in soils with much lower Cu content (1).

Observations of root stunting and Fe-deficiency symptoms in leaves of trees affected by Cu toxicity suggest that Cu is having an impact on root function and mineral nutrition (18–22). Since vesicular-arbuscular mycorrhizal (VAM) fungi are instrumental in root health and nutrient uptake by citrus (9,10), the fungicidal effects of Cu on VAM fungi need to be identified and distinguished from any direct effects on the host plant.

In a pot study, we investigated the impact of a Cu fungicide, applied to soil, on growth of citrus seedlings and on root colonization and hyphal development by the mycorrhizal fungus, *Glomus intraradices* Schenck & Smith. Observation of fungitoxic effects in the greenhouse led us to examine the mycorrhizal status of roots of trees exhibiting symptoms of Cu toxicity in a citrus orchard in Florida.

MATERIALS AND METHODS

Soil and treatments. The soil used in these experiments was Candler fine sand (Typic quartzipsamments; 96.5% sand, 2% silt, 1.5% clay) that was unfertilized and therefore low in Cu and P. It had a pH of 6.8, organic matter content of 1.0%, and double-acid (0.05 M HCl in 0.05 M H_2SO_4)-extractable (8) concentrations of Cu 2.7, P 3.5, Ca 156, Mg 8, K 4, Fe 3.6, Mn 0.92, and Zn 0.84 $\mu g \cdot g^{-1}$ (University of Florida Soil Testing Laboratory). The soil was autoclaved (121 C, 1.1 $kg \cdot cm^{-2}$) for 6 hr to eliminate indigenous VAM fungi. Basic copper sulfate [$CuSO_4 \cdot 3 Cu(OH)_2 \cdot H_2O \cdot$ (53% Cu) was mixed with air-dry soil in a cement mixer at eight rates: 0, 25, 50, 75, 100, 150, 200, and 300 μg total Cu per gram of soil. Cu-amended soils contained in 2-L plastic pots were watered to field capacity (17% moisture), and the pots were wrapped in a plastic bag for 1 mo prior to further treatment to allow the Cu to equilbrate in moist soil. Subsequently, double-acid-extractable Cu in the soil was determined monthly during the course of the experiment. Extractable Cu at each rate did not decrease appreciably over the 4-mo growing period; however, an effort was made not to leach the soil when watering the pots.

Plants and plant treatments. Seeds of Carrizo citrange [*Poncirus trifoliata* (L.) Raf. × *Citrus sinensis* (L.) Osbeck] were germinated in autoclaved Candler soil and the seedlings were watered weekly with Hoagland's solution minus P. After 2 mo, 20 seedlings of uniform size were transplanted into each of the Cu-amended soils. For VAM inoculation, 10 seedlings per treatment received 0.1 g of Sudan grass (*Sorghum bicolor* L. Moench. var. *sudanense*) roots containing approximately 700 chlamydospores of *G. intraradices*. Inoculum was placed below the roots at the time of transplanting. Ten uninoculated seedlings received an inoculum water extract to establish the microflora associated with the inoculum in nonmycorrhizal treatments. Seedlings were grown for 4 mo in the greenhouse under a maximum photon flux density of 800 $\mu Ein \cdot m^{-2} \cdot sec^{-1}$ and average day/night temperatures of 33/25 C. Inoculated plants received a weekly application of Hoagland's solution (0.1 μg of Cu per milliliter) minus P, and uninoculated plants received Hoagland's plus P (as KH_2PO_4) equal to 250 mg total P per pot (1,500 cm^3 of soil per pot). This amount of P was sufficient to produce nonmycorrhizal plants of similar size and P-status as VAM-inoculated plants grown in untreated soil without added P or Cu.

At harvest, the root systems of ten 6-mo-old seedlings per treatment were washed free of soil and the total feeder root length was estimated by a line-intersect technique (25). After the roots were dried at 60 C for 48 hr and weighed, the entire root system for each of five seedlings per treatment was cut into 2-cm lengths. Root segments were cleared in hot KOH, stained with trypan-blue in lactophenol (12), and randomly distributed under a grid of 1 mm squares. At least 100 squares were examined for the presence of vesicles and arbuscules in roots (percentage root colonization) and the occurrence of large-diameter hyphae (>5 μm) outside of the roots (percentage hyphae).

For each treatment, the mineral content of plant tissue was determined for five leaf samples (10 plants) and two root samples (four plants), each sample a composite of two plants. Tissue was digested in perchloric-nitric acid. P was determined by the vanadate-molybdate method (26) and other minerals by atomic absorption.

This experiment was conducted twice with similar results. The data from the second experiment are presented.

Field observations. Three-year-old replants of grapefruit (*C. paradisi* Macf. 'Marsh') on sour orange (*C. aurantium* L.) rootstock were studied in an established orchard in Indian River County near Vero Beach, FL, with a history of Cu fungicide application. Ten trees were selected in an area where trees were growing poorly and had iron chlorosis leaf symptoms. Five trees were located in an adjacent area where trees lacked these symptoms. Trees were visually rated as follows: 0 = healthy, 1 = mild iron chlorosis on leaves, 2 = moderate iron chlorosis and stunting of the canopy, and 3 = severe iron chlorosis and dieback of the canopy. Spring flush leaves were collected in December for leaf analysis. Soil cores to a depth of 20 cm were taken at four points around each tree at the dripline and were combined into one composite sample for soil analysis. Fibrous roots were collected in the surface 20-cm of soil under the tree canopy and stained for evaluation of VAM colonization as previously described.

RESULTS

Greenhouse study. Double-acid-extractable Cu was linearly correlated with the rate of added basic copper sulfate (Fig. 1). This method extracted from 75 to 80% of the applied Cu. Since double-acid extraction is the method currently used to predict Cu toxicity by the University of Florida Soil Testing Lab, further comparisons are based on the double-acid-extractable Cu at each rate averaged over the course of the experiment.

Growth of Carrizo citrange seedlings both uninoculated and inoculated with *G. intraradices* was reduced logarithmically with
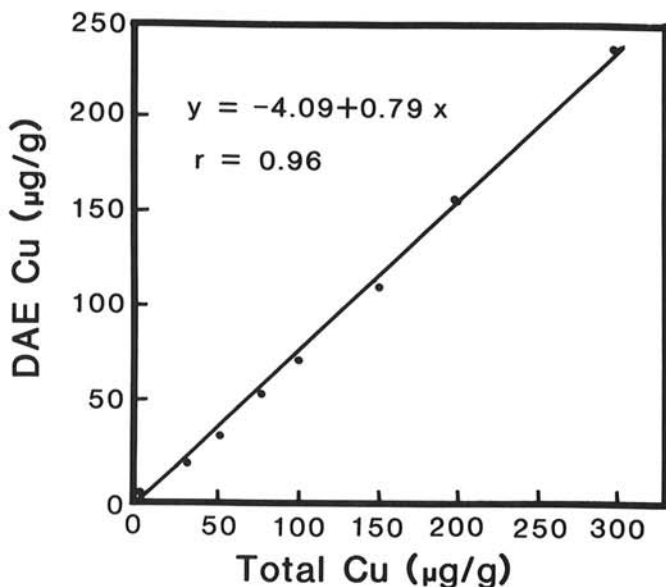
Cu concentration (Fig. 2A and B). Dry weight of inoculated plants was more greatly affected by Cu than that of nonmycorrhizal seedlings, as indicated by the significantly steeper slope of the VAM curve. The minimum toxic amount of Cu was 19 μg·g^{-1} for inoculated plants and 37 μg·g^{-1} for nonmycorrhizal plants. Shoot dry weight was affected similarly; i.e., inoculated plants were more greatly inhibited by Cu than were uninoculated plants (Table 1). The reduction in root dry weight and root length was also logarithmically related to Cu concentration, but the slope of the curves did not differ significantly between VAM and nonmycorrhizal treatments. At Cu ≥ 37 μg·g^{-1} root length was less, and more, short, unbranched roots were evident. No swelling and stunting of root apices was observed.
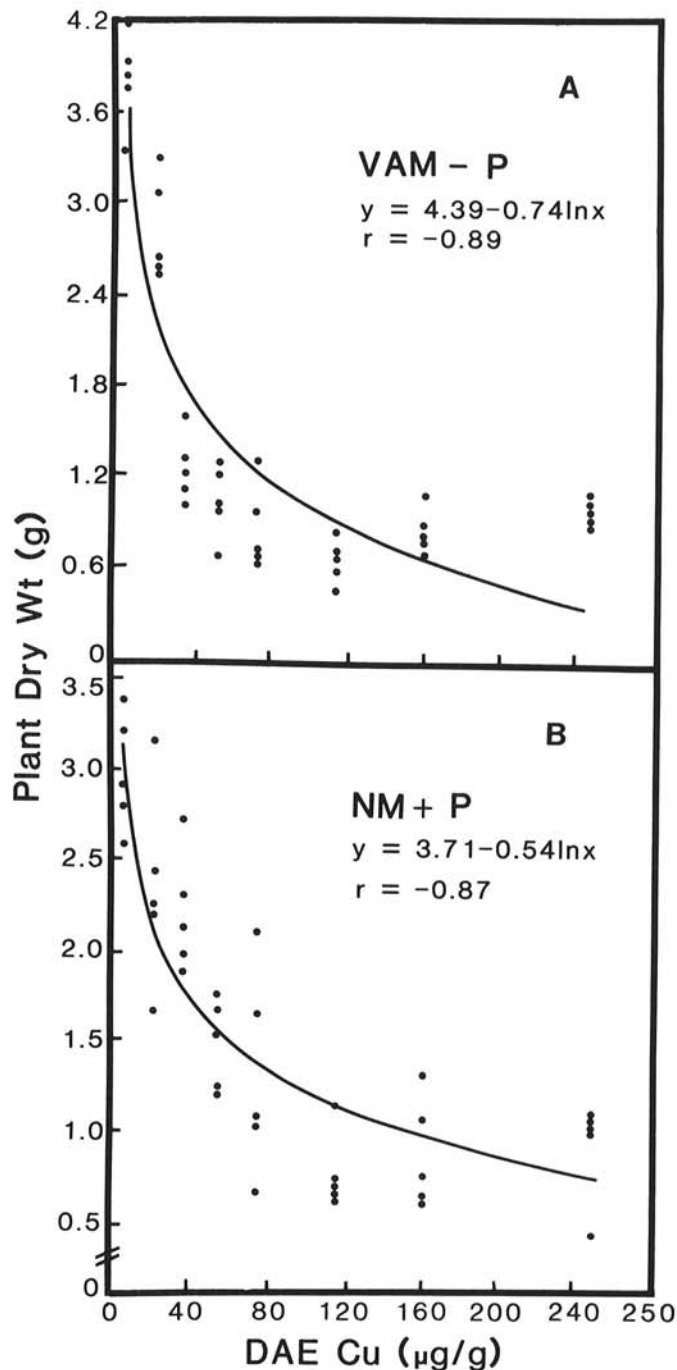
Fig. 2. Regression analysis of double-acid-extractable (DAE) copper concentrations in a low-P soil versus growth of Carrizo citrange seedlings either **A**, inoculated with *Glomus intraradices* (VAM-P) or **B**, fertilized with P (NM + P). Correlations are significant at $P < 0.01$. Regression equations have significantly different ($P < 0.05$) slopes according to Student's *t*-test.

Fig. 1. Relationship between rate of copper amendment as basic copper sulfate and double-acid-extractable (DAE) levels in a low-P sand soil.

In the low-P soil, there was a decrease in leaf P which was strongly correlated with the log Cu for inoculated seedlings but not for uninoculated plants (Table 1). Regression analysis revealed a negative logarithmic correlation between VAM colonization in the root, or hyphal development outside of the root and Cu (Fig. 3A,B). There was a significant linear relationship between VAM colonization or hyphal development, and the decrease in either leaf P content or plant growth (Table 2). The correlations were stronger for hyphal development than for VAM colonization. Leaf P was deficient ($<0.1\%$ P) in VAM seedlings at $Cu \geqslant 37~\mu g \cdot g^{-1}$, whereas plants in all nonmycorrhizal treatments were P-sufficient.

A relationship between Cu rate, mycorrhizae, and uptake of micronutrients was also apparent (Table 3). The content of Cu in roots of VAM and nonmycorrhizal plants was proportional to the amount added to soil. In contrast, the Cu concentration in leaves increased gradually up to soil Cu at $114~\mu g \cdot g^{-1}$ and then rose sharply at the two highest Cu rates. At the two lowest levels of Cu amendment, mycorrhizae significantly increased Cu content of roots but not of leaves. Uptake of other micronutrients supplied at nutritional levels in Hoagland's solution were only significantly affected by mycorrhizae at the lower Cu rates. There was a notable increase in root content of Zn in VAM and nonmycorrhizal plants at all rates of added Cu compared to plants with no added Cu. Iron, supplied in chelated form, was not deficient in leaves and its concentration increased in leaves but not in roots at the higher Cu rates.

Field observations. Soil analysis indicated that there were high levels of double-acid-extractable Cu in both the area with symptomatic trees and apparently healthy trees but pH was one unit lower and concentrations of P, Ca, Mg, Fe, and Zn were significantly lower in soil from the affected area (Table 4). Soil pH, Ca, Mg, and P were higher in the unaffected area probably because the soil contained deposits of calcareous marine materials. Fibrous roots of the affected trees were dark brown and moribund compared to those of the nonsymptomatic trees. VAM colonization of symptomatic roots was significantly less than for roots of healthy trees (Table 5). In spring flush leaves, Cu was in excess and Fe was deficient for the affected trees, and calcium was deficient for trees from both locations (7).

DISCUSSION

The inhibitory effect of Cu on growth of citrus seedlings is well established (13,16,19-21,22). In our study, Cu logarithmically
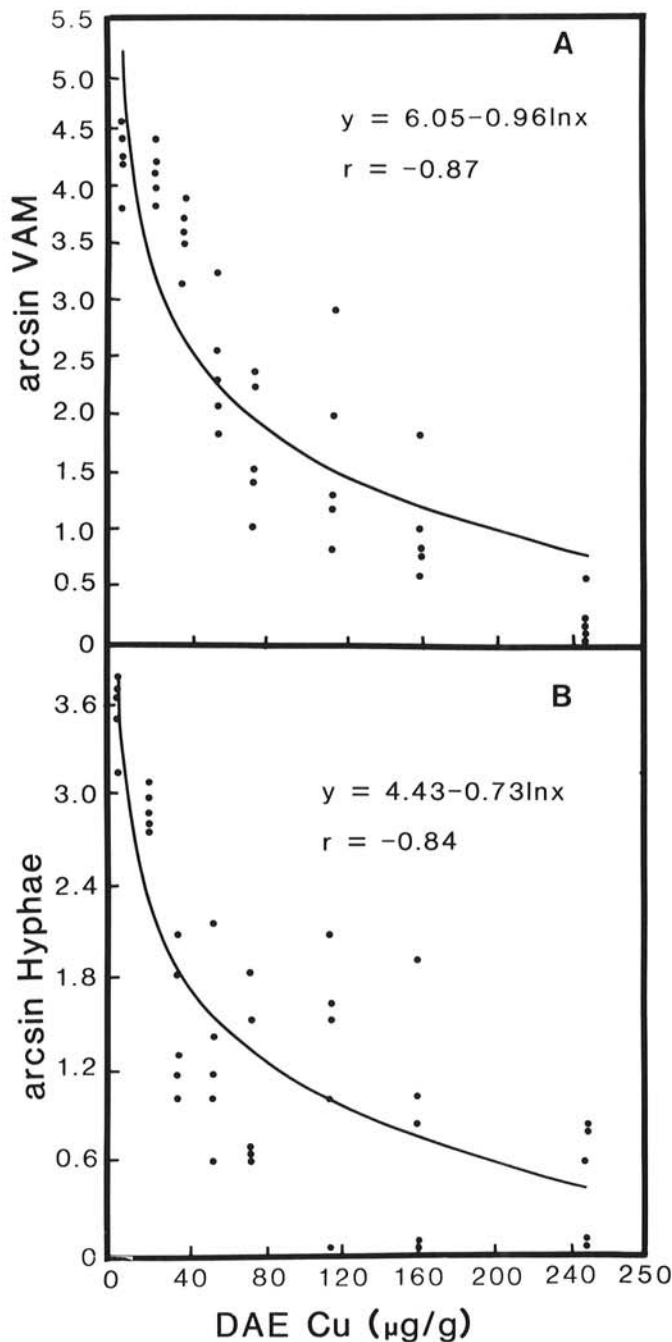

Fig. 3. Regression analysis of double-acid extractable (DAE) copper concentrations in a low-P soil versus **A,** percentage root colonization (VAM) and **B,** percentage hyphal development outside of roots (hyphae) of Carrizo citrange seedlings by *Glomus intraradices*. Percent root colonization and hyphal development data were arcsin transformed to stabilize the variance. Correlations are significant at $P < 0.01$.

TABLE 1. Regression analysis of double-acid-extractable Cu in a low-P soil amended with eight rates of basic copper sulfate versus root and shoot growth and P content of Carrizo citrange seedlings either inoculated with *Glomus intraradices* and not fertilized with P (VAM − P) or uninoculated and fertilized with P (NM + P)

Factor	Treatment	Regression equation	Correlation coefficient (r)[a]
Dry weight (g)			
Shoot	NM + P	$y = 2.31 - 0.36 \ln x$[b]	−0.89
	VAM − P	$y = 2.76 - 0.50 \ln x*$	−0.89
Root	NM + P	$y = 1.40 - 0.17 \ln x$	−0.88
	VAM − P	$y = 1.63 - 0.23 \ln x$	−0.81
Root length (m)	NM + P	$y = 10.3 - 1.45 \ln x$	−0.82
	VAM − P	$y = 8.82 - 1.33 \ln x$	−0.72
Leaf P (%)	NM + P	$y = 0.25 - 0.014 \ln x$	−0.50
	VAM − P	$y = 0.23 - 0.040 \ln x*$	−0.81

[a] All correlations are significant at the $P < 0.01$ level.
[b] VAM−P regression equations followed by (*) have significantly different ($P < 0.05$) slopes from NM + P according to Student's t-test.

TABLE 2. Regression analysis of percentage root colonization (% VAM) or hyphal development of the root (% hyphae) by *Glomus intraradices* versus leaf P content (% leaf P) and growth (plant dry wt) of Carrizo citrange seedlings in a low-P soil amended with eight rates of basic copper sulfate

Factors[a]	Regression equation	Correlation coefficient (r)[b]
% VAM versus % leaf P	$y = 0.02 + 0.03~x$	0.73
% hyphae versus % leaf P	$y = 0.02 + 0.04~x$	0.80
% VAM versus plant dry wt	$y = 0.28 + 0.54~x$	0.73
% hyphae versus plant dry wt	$y = 0.24 + 0.82~x$	0.84

[a] Root colonization and hyphal development data were arcsin transformed with the arcsin transformation to stabilize variance.
[b] All correlations are significant at the $P < 0.01$ level.

reduced shoot and root growth, and root length. We observed an inhibition of root extension and branching at double-acid-extractable Cu concentrations exceeding 37 $\mu g \cdot g^{-1}$. Similar effects were reported by Smith and Specht (18–20) in vermiculite or liquid cultures when Cu ranged from 0.1 to 0.25 $\mu g \cdot ml^{-1}$ in the nutrient solution. In our greenhouse test, iron chlorosis of leaves was not apparent and the foliar Fe content was not reduced by added Cu where Fe chelate was applied. This is consistent with the results of previous studies (19,21) in which uptake of chelated forms of Fe was unaffected by high levels of Cu. In nonchelated form, Fe is taken into the root but not translocated from high-Cu roots (21). Our observations confirmed that orchard trees with symptoms of Cu toxicity are deficient in foliar Fe.

Cu accumulated in roots in proportion to the amount added to soil. Smith (18) reported that Cu ranged from 30 to 50 $\mu g \cdot g^{-1}$ in healthy roots, up to 600 $\mu g \cdot g^{-1}$ in roots of stunted plants, and 600 to 800 $\mu g \cdot g^{-1}$ in dead roots. We found up to 2,400 $\mu g \cdot g^{-1}$ in roots that appeared to be alive but not actively growing. As previously reported (19–22), even though Cu accumulated to high levels in roots, foliar levels were not in excess (7) until double-acid-extractable Cu exceeded 160 $\mu g \cdot g^{-1}$. Leaf analysis standards did indicate that symptomatic trees in the orchard had excess Cu compared to high levels in apparently healthy trees.

The toxicity of Cu to plants inoculated with a mycorrhizal fungus in P-deficient soil differed from the effects on nonmycorrhizal seedlings fertilized with P. Colonization and hyphal development by G. intraradices were severely reduced by as little as 50 $\mu g \cdot g^{-1}$ of double-acid-extractable Cu. The poor growth of the VAM plants at the higher rates of Cu was primarily due to P deficiency induced by the inhibition of mycorrhizae by Cu.

Our results are supported by the observations of Spencer (22) on the effect of Cu on P uptake by Cleopatra mandarin seedlings in an unfertilized, nonsterile Florida soil similar to the one we used. He found that Cu greatly reduced P uptake in the absence of P fertilizer but not when P was added to the soil. He concluded that Cu interfered with P uptake from the soil. In the present study, we noted no effect of Cu on P uptake by nonmycorrhizal plants fertilized with P. We conclude that seedlings in Spencer's study were P-sufficient in the low-P soil because they were colonized by

indigenous VAM fungi, and that P uptake and growth were reduced because colonization was inhibited by Cu.

The reduction in extramatrical hyphal development outside of the root was more closely correlated with decreased P uptake and plant growth than with intramatrical vesicle and arbuscule development in the root. The fungicides benomyl (3,6) and parachloronitrobenzene (5) also reduce P uptake by mycorrhizal roots. The inhibitory effect of fungitoxicants on P uptake by roots with intramatrical, but not extramatrical, development emphasizes the primary role that hyphae play in uptake and translocation of P to the root. Nemec (11) reported that Cu added at 224 kg·ha^{-1} (112 $\mu g \cdot g^{-1}$) to a soil with properties similar to the one we studied, reduced growth of sour orange but not VAM colonization by G. etunicatum Becker & Gerd. The reduction in growth in low-P soil is again indicative of Cu inhibition of P uptake by mycorrhizal hyphae, but this level of total Cu also severely inhibited root colonization by G. intraradices in our study.

Although it is relatively insoluble compared to other Cu fungicides (4), the immediate toxic effect of a single application of basic copper sulfate to soil was anticipated. Seedlings planted immediately after soil amendment with Cu were killed by total concentrations of 250 $\mu g \cdot g^{-1}$ (J. H. Graham and L. W. Timmer, unpublished). Apparently, Cu became less bioavailable after 1 mo of equilibration, although the amount extracted did not decrease even after several months. The double-acid method extracted a high proportion of the total added Cu and therefore may not be an appropriate measure of Cu bioavailability from basic copper sulfate.

In contrast to direct application to soil in the greenhouse study, in the orchard Cu fungicide is periodically applied to leaves which eventually fall to the soil and decompose. Cu associated with organic matter is thought to form complexes (15,24). When pH is maintained near neutrality, Cu bioavailability is apparently low because orchard trees are productive (1) and have normal populations of VAM fungi in roots (J. H. Graham, unpublished) where total Cu is as high as 500 kg·ha^{-1}. At pHs below 5.0, however, we observed symptoms of Cu toxicity and reduced VAM colonization in roots compared to those of nonsymptomatic trees growing at pHs above 5.0. Because Cu appears to be fungitoxic to VAM fungi in orchard soils, mycorrhizal deficiency should be

TABLE 3. Effect of basic copper sulfate in a low-P soil on leaf and root micronutrient composition of Carrizo citrange seedlings either inoculated with *Glomus intraradices* and not fertilized with P (VAM − P) or uninoculated and fertilized with P (NM + P)

| DAE Cu[a] | Leaves ($\mu g \cdot g^{-1}$) | | | | | | | | Roots ($\mu g \cdot g^{-1}$) | | | | | | | |
| | Cu | | Fe | | Zn | | Mn | | Cu | | Fe | | Zn | | Mn | |
($\mu g \cdot g^{-1}$)	NM+P	VAM−P[b]	NM+P	VAM−P[b]	NM+P	VAM−P[b]	NM+P	VAM−P[b]	NM+P	VAM−P[b]	NM+P	VAM−P[b]	NM+P	VAM−P[b]	NM+P	VAM−P[b]
2.7	8	10	186	198	38	42	67	92*	57	84**	1,650	2,350*	160	155	1,850	1,900
18.8	10	11	180	150*	34	52**	85	146**	275	410**	1,600	1,650	585	700**	2,350	3,250**
34.0	11	13	188	160*	36	41	76	95	495	555*	1,650	1,500	465	495	2,500	3,550*
52.0	13	13	176	182	34	43	83	102	660	580	1,250	1,300	315	375	2,500	2,350
72.0	12	13	180	222	34	50	72	140	690	720	1,300	1,300	425	560	2,000	2,850
114.0	14	14	184	220	44	51	89	134	910	850	1,400	1,500	350	415	2,200	2,750
160.0	22	16	246	236	51	53	164	156	1,000	1,200	1,250	1,250	505	350	2,400	2,600
248.0	45	22	304	278	60	56	142	148	2,400	1,900	1,300	1,200	355	400	1,900	1,950

[a] Double-acid extractable Cu.
[b] VAM−P followed by (*) or (**) significantly different from NM+P at the $P < 0.05$ and $P < 0.01$, respectively, levels according to Student's t-test.

TABLE 4. Soil characteristics in two areas of an orchard with replants of grapefruit on sour orange rootstock showing symptoms of leaf iron chlorosis, stunting, and twig dieback

| Tree Area | Tree rating[a] | pH | Double-acid-extractable ($\mu g \cdot g^{-1}$) | | | | | | |
			Cu	Fe	Zn	Mn	P	Ca	Mg
1	0.0	5.8	98	46	56	19	184	1,296	211.
2[b]	2.5	4.8**	84	23**	26**	18	51**	500**	87**

[a] Visual rating: 0 = healthy, and 1–3 mildly to severely affected (see text).
[b] Means for area 2 differ significantly from means for area 1 at the $P < 0.01$ level (**) according to Student's t-test.

TABLE 5. Percentage of root length colonized by mycorrhizal fungi (VAM) and leaf mineral composition of grapefruit on sour orange rootstock showing symptoms of leaf iron chlorosis, stunting, and twig dieback in two areas of the same orchard

| Area | Tree rating[a] | VAM (%) | Leaf content ($\mu g \cdot g^{-1}$) | | | | Leaf content (%) | | |
			Cu	Fe	Mn	Zn	Ca	Mg	P
1	0.0	47	18	80	25	18	1.5	0.46	0.19
2[b]	2.5	15**	28*	58**	33	28	1.6	0.42	0.16

[a] Visual rating: 0 = healthy, 1–3 mildly to severely affected (see text).
[b] Means for area 2 differ significantly from means for area 1 at the $P < 0.01$ level (**) or the $P < 0.05$ level (*) according to Student's t-test.

considered a potential problem, particularly when nonmycorrhizal trees are outplanted into low-pH soils high in Cu. In the greenhouse study, Cu inhibition of VAM colonization was clearly associated with P deficiency in low-P soil; in the orchard soil P was more than adequate for growth of citrus which masked nutritional effects of mycorrhizal deficiency.

Although the double-acid-extractable Cu from both areas of the orchard site corresponded to levels in the greenhouse study that were clearly toxic to the plant and the fungus, trees appeared to be healthy in the area where soil pH was higher. Double-acid-extractable concentrations of Fe, Zn, Ca, Mg, and P also were significantly greater in this soil. High levels of soil P have been shown to reduce Cu toxicity (17,22). Thus, it is difficult to clearly attribute the lack of symptom development to pH alone. Furthermore, the proportion of Cu and other elements extracted by the double-acid-extractable method may vary with pH (2). The failure of this method to correlate Cu availability with biological effects in the greenhouse and field indicates a need to develop a soil extraction method which more accurately predicts Cu toxicity.

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