J. Amer. Soc. Hort. Sci. 123(3):357–360. 1998.

5-kD Zinc-binding Protein Accumulation in Macrophylla-decline-affected *Citrus*

K.C. Taylor and H.L. Geitzenauer
University of Arizona, Department of Plant Sciences, Tucson, AZ 85721

Additional index words. *Citrus macrophylla*, sieve tube necrosis

Abstract. Macrophylla-decline (MD)-affected citrus display apparent nutrient deficiencies in a sectorial pattern within the citrus tree canopy. The status of several elements (Ca, Cu, Fe, Mg, Mn, and Zn) was assessed in MD and healthy citrus selected from the same citrus orchards. Leaf and phloem tissues were sampled from mature, reproductive trees. Levels of Ca, Cu, Fe, Mg, and Mn were unaffected by the disorder in leaf or phloem tissues. Zinc was diminished in the leaves of MD citrus, and elevated in the whole phloem tissue (2.57-fold on a dry mass basis). Calcium and Cu were sufficient, while Mg, Fe, and Mn were slightly diminished in the leaf tissue, but phloem levels of these elements were not significantly different from that present in the phloem of healthy trees. Since Zn appeared to be redistributed to the phloem tissue from the leaves, the accumulation of the phloem specific, 5-kD Zn-binding protein (ZBP) was assessed in Macrophylla decline trees relative to healthy trees. The 5-kD ZBP was 4.77-fold greater in the phloem of MD citrus relative to healthy. This appears to account for the 2.4-fold greater level of Zn (on a fresh mass basis) found in the crude phloem extracts of the decline-affected citrus relative to healthy. In the purified ZBP fraction from decline-affected citrus, there was 4.73-fold greater Zn than in the ZBP purified from healthy. However, the ratios of Zn to ZBP were equivalent between MD citrus and healthy citrus, suggesting that phloem Zn accumulation in MD citrus is associated with the 5-kD ZBP.

A disorder in Arizona desert lemons [*Citrus limon* (L.) Burm.] has been described since the 1950s as *Macrophylla* decline (MD) (Schneider, 1956; Schneider, 1960). Trees with MD have phloem abnormalities that occur just below the bud union, i.e., necrosis of the sieve tubes, with callose accumulation in that tissue (Schneider, 1956; Allen et al., 1982). MD is most often observed in 'Allen Eureka' and 'Frost Nucellar Lisbon' lemon scions on *C. macrophylla* Wester. rootstock (Allen et al., 1982; Schneider, 1956). Although some other scion and rootstock combinations have been occasionally reported to have sieve tube necrosis (STN) present in the rootstock (Allen et al., 1977; Allen et al., 1982; California Citrograph, 1977). STN trees appear to decline due to the reduction in photosynthate supply to the roots (Allen et al., 1977), manifested as a loss of fine, new root growth in the upper foot of soil (Allen et al., 1982). The disorder is also characterized by leaf chlorosis patterns typical of micronutrient deficiency (Allen et al., 1982). Previous research on the MD disorder investigated the involvement of mycoplasma-like organisms (MLOs) and viruses as causal agents of the disorder. Neither were demonstrated as causal. The investigators concluded that the cause was incompatibility between rootstock and scion (Allen et al., 1977). We also assessed the possibility that MLOs may cause MD, using MLO-specific PCR primers, and obtained negative results (Taylor and Ellis, 1996). In addition, we explored the possibility that this disorder was caused by the exocortis and cachexia viroids, again obtaining negative data (Taylor et al., 1996b). The incompatibility causes apparent changes in nutrient distribution throughout the tree (Allen et al., 1982). We report here the micronutrient status of MD citrus relative to healthy trees, and demonstrate that the accumulation of a small Zn-binding protein may be responsible for Zn redistribution within the tree.

Materials and Methods

Plant material. Leaf and phloem samples were collected on the Yuma Mesa from fully mature (10 to 25 years old) 'Eureka' or 'Frost nucellar' lemon scions on Macrophylla, a decline-susceptible commercial rootstock in California and Arizona. Healthy and MD candidates were selected. MD candidates had sectored symptom expression of micronutrient deficiency symptoms, twig dieback and leaf loss. Healthy trees lacked these symptoms. Fifteen leaves were sampled from each of ten pairs of MD and healthy trees at one site and from each of eight pairs at another site in Yuma. The fourth leaf back from the tip on current spring shoots were sampled in early June. The trunk phloem tissue was sampled as a 2×5-cm^2 bark patch, taken 20 to 30 cm above the bud union. After micronutrient determinations were assessed, a separate sample of one 5×10-cm^2 patch of phloem was sampled from three trees each of healthy and decline-affected pairs. For phloem sampling, the outer bark was scraped away from the surface with the edge of a stainless steel knife blade, and the remaining tissue was scored down to the xylem-cambial interface. The bark piece was stripped away from the tree and placed into liquid nitrogen. Sampling was accomplished when the bark was slipping (Williams and Albrigo, 1984).

In addition, ten trees each from ≈20-year-old orchards of rough lemon (*Citrus jambhiri* Lush.), and sour orange (*Citrus aurantium* L.) with 'Eureka Lisbon' scions were sampled as described above for leaf and phloem tissue. These were selected as controls for rootstock effect.

Comparisons among rootstocks and between decline and healthy pairs were made by least squares analysis. Statistical differences were only assessed within leaf sample sets and phloem sample sets.

Leaf and phloem tissue digestion. Leaves were washed with 0.1% HCl and soapy water and rinsed just after they were sampled. Phloem samples were cut into 2 to 3-μm slices before drying. They were then dried in a forced air oven at 70 °C for 24 h and ground through a 40-mesh screen. The ground samples were stored dry. A 0.500 ± 0.005 g aliquot of ground tissue was digested in a 550 °C muffle furnace 6 h to overnight. Five milliliters of 25% nitric acid was added to each digest, allowed to sit for 1 h and brought to 10 mL with double-deionized H_2O. The digests were diluted as appropriate to determine Ca, Cu, Fe, Mg, Mn, and Zn. For Ca determination, the digest was diluted with 2.5% lanthanum chloride. The concentrations of these elements were determined by atomic absorption spectrometry.

Phloem protein extraction and chromatographic isolation of

Received for publication 10 June 1997. Accepted for publication 7 Jan. 1998. We thank the Arizona Citrus Research Council for their generous support of this research. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

Table 1. Nutrient data for healthy and decline-affected Eureka lemons on *Macrophylla* rootstock.[z]

Rootstock	Ca	Mg	Cu	Fe	Mn	Zn
	(%)		($\mu g \cdot g^{-1}$)			
Orchard 1						
Leaf						
Healthy	3.51 a	0.29 a	19.6 a	83.5 a	20.7 a	24.6 a
Decline	3.88 a	0.18 b	20.9 a	80.8 a	17.5 a	21.2 a
Phloem						
Healthy	2.94 a	019 a	11.4 a	39.6 a	25.1 a	35.8 b
Decline	3.16 a	0.16 a	9.6 a	46.6 a	17.8 a	71.8 a
Orchard 2						
Leaf						
Healthy	3.59 a	0.38 a	16.8 a	47.5 a	17.9 a	26.5 a
Decline	3.62 a	0.19 b	19.3 a	69.4 a	16.8 a	18.8 b
Phloem						
Healthy	3.46 a	0.18 a	12.9 a	43.4 a	18.3 a	23.6 b
Decline	2.84 a	0.18 a	9.1 a	49.3 a	17.5 a	83.7 a

[z]Comparisons were made among leaf or phloem samples in the healthy and decline condition within each orchard. Similar letters indicate differences in nutrient content at $P = 0.05$, as determined by least squares analysis.

ZN-BINDING PROTEINS. Frozen phloem tissue described above was ground in an electric coffee grinder for 15 to 20 s. Three sets of phloem tissue samples (≈15 g/sample) from each of the healthy and decline-affected pairs were extracted three times each by shaking on ice for 20 min in 40 mL of cold 50 mM Tris-Cl buffer, pH 8.0 with 50 mM β-mercaptoethanol and 5% (w/w) polyvinylpolypyrrolidone. The 150 mL homogenate from each sample was strained through six layers of cheesecloth and centrifuged for 20 min at 36,000 g_{max}. Resulting supernatant was filtered through a 0.45-μm cellulosic membrane and degassed. Phloem extracts were fractionated by ion exchange chromatography (IEC), followed by gel filtration (GF) chromatography as performed previously (Taylor et al., 1996a).

ZINC AND PROTEIN ASSAYS. Total Zn was determined as a measure of $A_{213.9nm}$ in atomic absorption spectrometry. Total protein was determined at A_{595nm} with Quantigold (Diversified Biotech, Newton Centre, Mass.) or with the Bradford assay (Bio-Rad, Hercules, Calif.) according to manufacturers' instructions, depending upon protein concentration, with each stage of purification.

SDS-PAGE. The purification of the 5-kD ZBP was verified by 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified ZBPs (50 μg) were applied to 20% gels in a 25-μL final volume containing 10% sample buffer [50 mM Tris-Cl (pH 6.8) with 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 100 mM dithiothreitol, 0.1% bromophenol blue]. Electrophoresis was carried out for 0.5 h at 80 V, 0.5 h at 150 V, and 1.0 h at 200 V constant voltage, in a pH 8.3 Laemmli buffer system (Laemmli, 1970).

The identity of this 5-kD protein as the 5-kD ZBP isolated previously from blight affected citrus was accomplished by immunoblot analysis using a polyclonal anti-5kD ZBP serum prepared previously (Taylor et al., 1996a). After electrophoresis as above, the gel was then electroblotted for 45 minutes onto 0.45-μm poly(vinylidene diflouride) (PVDF) membrane. The resulting blot was cross-reacted with polyclonal anti-5kD ZBP serum as the primary antibody and alkaline phosphatase (AP) conjugated goat anti-rabbit IgG as the secondary antibody. Color development was accomplished using standard BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro-blue tetrazolium)-AP assay system (King et al., 1985).

PROTEIN AND ZN ACCOUNTING. Throughout purification, fresh mass, volumes, protein concentration, and Zn concentrations were

Table 2. Nutrient data for Eureka lemons on three rootstocks.

Rootstock	Ca	Mg	Cu	Fe	Mn	Zn
	(%)			($\mu g \cdot g^{-1}$)		
Rough lemon						
Leaf	3.43 a	0.24 a	15.8 a	81.9 a	23.3 a	19.3b
Phloem	3.26 a	0.15 b	7.3 a	34.7 b	19.5 a	37.8 b
Macrophylla						
Leaf						
Healthy	3.54 a	0.33 a	18.3 a	67.5 a	19.5 a	25.4 b
Decline	3.75a	0.19b	20.2 a	75.7 a	17.2 a	20.1 b
Phloem						
Healthy	3.21 a	0.19 a	12.1 a	41.3 b	22.1 a	30.4 b
Decline	3.00 a	0.17 a	9.4 a	47.8 ab	17.7 a	77.1 a
Sour Orange						
Leaf	4.25 a	0.28 a	12.1 a	69.6 a	15.9 a	49.3 ab
Phloem	3.47 a	0.18 b	8.5 a	43.8 b	16.5 a	31.7 b

[z]Comparisons were made between leaf or phloem samples among all rootstocks. Similar letters indicate differences in nutrient content at $P = 0.05$, as determined by least squares analysis.

Table 3. Total and concentration of protein and Zn throughout the purification of 5-kD ZBP from *Macrophylla* decline and healthy citrus.

Sample[x]	Total protein (mg)	Protein concn[z] ($mg \cdot g^{-1}$)	Total Zn (μg)	Zn concn[z] ($\mu g \cdot g^{-1}$)	Specific Zn[y] ($\mu g \cdot g^{-1}$)
Healthy					
Crude	30.00	2.00	45.4	3.03	0.002
IEC	1.92	0.15	20.9	1.39	0.011
GF	0.35	0.04	4.1	0.27	0.012
Macrophylla decline					
Crude	30.00	3.27	67.4	7.35	0.002
IEC	5.83	0.39	30.1	3.27	0.005
GF	1.67	0.12	19.4	2.12	0.012

[z]Protein and Zn concentrations were determined on the basis on grams fresh mass.
[y]Specific Zn is expressed as μg Zn μg/protein.
[x]IEC = ion exchange chromatography, GF = gel filtration chromatography.

recorded. These accounts were used to assess our level of ZBP purification and to compare the level of ZBP in MD and healthy citrus material.

Results and Discussion

NUTRIENT STATUS OF MD TREES RELATIVE TO HEALTHY TREES. Ca, Cu, Fe, and Mn were present in leaf tissue at levels within the normal range for those elements (ranging from 2.74% to 4.13% Ca; Cu at 8.4 to 21.7 $\mu g \cdot g^{-1}$; Fe at 44.2 to 85.1 $\mu g \cdot g^{-1}$; and Mn at 14.9 to 22.7 $\mu g \cdot g^{-1}$) (Tables 1 and 2). In contrast, Mg was reduced in leaves of the decline trees sampled (ranging from 0.15% to 0.42% Mg) (Tables 1 and 2) relative to healthy trees. Yet, the concentrations for Mg were never below the critical levels in leaf tissue for this element. Furthermore, the level of Mg was not altered in the phloem tissue.

Zinc was deficient only in the leaves of the trees on the Rough lemon rootstock and in the leaves of the decline-affected trees on Macrophylla rootstocks; while it was sufficient in trees on the sour orange rootstock (Table 1 and 2). Most compelling was the 2.5-fold accumulation of Zn in the phloem tissue taken 20 to 30 cm above the bud union of MD trees. Zinc did not accumulate to this level in the phloem tissue of other rootstocks or in the healthy Macrophylla trees; nor were other elements accumulated in the phloem of decline-affected Macrophylla trees.

The apparent sectorial expression of nutrient deficiency in MD citrus was due to a decrease in the level of Zn in the leaf tissue with a coincident accumulation of the Zn in phloem tissue. The data in Table 1 demonstrate that Zn levels are reduced in the leaves of MD trees relative to healthy, with the significant difference in leaf Zn levels in Orchard 2. Orchard 1 contained trees with absolute levels of Zn that were decreased relative to healthy, but these values were not significantly different from the healthy trees. Because we sampled the tree randomly, without regard to selection of only symptomatic leaves, we likely avoided bias toward only deficient leaves. This may have raised the apparent Zn level for the trees. The trees in Orchard 2 appeared to be more severely affected than trees in Orchard 1, and could account for the more significant difference in Zn of leaves of healthy versus decline trees in Orchard 2. This likelihood is supported by the higher level of Zn in the phloem of MD Orchard 2 trees (Table 1). The phloem of MD citrus in both orchards contained significantly higher levels of Zn relative to healthy trees (Table 1). On average (Table 2) the phloem tissue of decline-affected trees contained 2.54 times more Zn than that of the healthy trees. Generally, 'Eureka' on Macrophylla rootstocks appeared to contain more Zn than 'Eureka' on Rough Lemon and less than 'Eureka' on Sour Orange. But despite the apparent rootstock effect on normal Zn content in phloem tissue, this decline disorder appears to be associated with Zn redistribution to the phloem tissue. We previously characterized a 5-kD protein that is phloem specific and can bind Zn (Taylor et al., 1996a). This protein is present in healthy trees on Rough lemon and Carrizo rootstocks (Taylor et al., 1988). It accumulates in the phloem of blight affected citrus which also displays Zn accumulation in phloem tissue. We assessed whether this protein was present in MD trees at a level higher than that found in the paired healthy trees from the same orchard.

Assessment of ZBP accumulation in Macrophylla decline citrus. Given the 77.1 $\mu g \cdot g^{-1}$ dry mass of Zn in phloem of MD trees relative to 30.4 $\mu g \cdot g^{-1}$ dry mass in phloem of healthy trees (Table 2), we determined the presence of a Zn sequestering component in that tissue. Our accounting results after IEC and GF indicated that 4.73-fold greater Zn (on a fresh mass basis) was present in the extracts of phloem tissue above the bud union of MD trees relative to healthy trees (Table 3). On a total protein basis, there was a 4.77-fold increase in ZBP in the extracts of trunk phloem of trees affected with MD, after the 5-kD ZBP was partially purified by IEC and GF (Table 3). The ZBP fractions from MD citrus contained ≈4.7-fold more Zn than the same fractions from healthy citrus. The specific Zn content of the partially purified Zn-containing fractions after GF were similar whether the phloem tissues were from trees in the healthy condition or affected by MD. Thus, the increase in Zn content in phloem tissues above the bud union appears to be accounted for by the increased accumulation of 5-kD ZBP in that tissue.

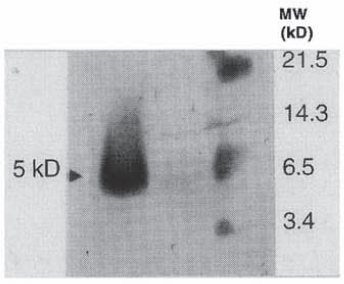

Fig. 1. The 5-kD ZBP isolated from *Macrophylla*-decline-affected trees. Immunoblot demonstrates the positive response of this 5-kD ZBP (>), isolated from healthy and MD citrus, for the anti-5-kD ZBP serum raised to 5-kD ZBP. Lane 1, protein extract from phloem of MD citrus; lane 2, protein extract from phloem of healthy control; lane 3, molecular mass marker; 35-μg protein loaded in each lane.

The 5-kD ZBP isolated from MD citrus was immunologically similar to that purified from blight-affected citrus. In an immunoblot analysis of equally loaded crude phloem protein extracts from MD and healthy trees (Fig. 1), the ZBP from MD trees cross-reacted with the anti-5-kD ZBP serum raised against 5-kD ZBP from blight-affected citrus. The level of ZBP in phloem extracts from healthy trees was below the level of detection in this immunoblot.

A partial sequence of purified 5-kD ZBP was previously obtained (Taylor et al.,

1996a). It shares identity with the chitin binding domain of hevein, wheat germ agglutinin, and several class I chitinases (Taylor et al., 1996a), which is common in many wound induced proteins (Van Parijs et al., 1991). Many chitin-binding proteins are induced by a number of environmental and pathogenic stress conditions, such as drought, heat, heavy metals, viral infection, insect feeding, and fungal infection (Raikhel and Broekaert, 1993). The presence of this type of protein is correlated with plant stress, but does not indicate the source of that stress. Further characterization of the protein and the expression of the gene which encodes it may give us much information about the etiology of the MD disorder. We are currently isolating the gene that encodes this protein. With the gene we will begin functional analyses to ascertain the role of this protein in normal metabolism of citrus.

Conclusions

All rootstocks assessed appeared to have some level of Fe, Mg and Mn deficiency that were not apparently associated with trees affected by decline. Trees affected by MD appear to undergo an abnormal distribution Zn, with an apparent loss of Zn from the leaves and an accumulation in the phloem above the bud union. Leaves had deficient or nearly deficient levels of Zn, yet increased in the phloem tissue above the bud union. The 1:1 ratio of Zn and ZBP in the phloem tissue of MD trees provides evidence that Zn was sequestered in the phloem tissue 20 to 30 cm above the bud union by a 5-kD ZBP. This protein cross-reacts (Fig. 1) with polyclonal anti-serum raised against the 5-kD ZBP isolated from the phloem tissue of blight-affected citrus (Taylor et al., 1996a). The 5-kD ZBP shares identity with wound inducible chitin binding proteins, although the specific role of this protein in citrus or a potential causal agent of Macrophylla decline is unknown. The role of 5-kD ZBP in MD requires further investigation. It is interesting that besides the accumulation of the potentially wound inducible, 5-kD ZBP, callose also accumulates in the phloem tissue above the bud union of MD trees (Allen et al., 1977; Schneider, 1956, 1960). This is the same tissue that sustains necrosis of the sieve elements. By extension, whatever is responsible for sieve tube necrosis, with its callose accumulation (a wound response in plants), may also be responsible for the accumulation for 5-kD ZBP in that tissue and the Zn deficiency that develops in the tree canopy. In contrast, the accumulation of the 5-kD ZBP may be a general response to stress, that might appear broadly in the plant kingdom.

Literature Cited

Allen, R.M., E.L. Nigh, D.R. Rodney, and H. Schneider. 1982. Macrophylla rootstock necrosis affects more trees in Arizona. Calif. Citrograph 67:133–134.

Allen, R.M., D.R. Rodney, and H.H. MacDonald. 1977. Sieve tube necrosis in Arizona. Calif. Citrograph 62:79–80.

California Citrograph. 1977. Ventura lemons: 3,000 acres out, more going. Calif. Citrograph 62:323–325.

King, S.M., T. Otter, and G.B. Witman. 1985. Characterization of monoclonal antibodies against *Chlamydomonas* flagellar dyneins by high resolution protein blotting. Proc Natl. Acad. Sci. USA 82:4717.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Raikhel, N.V. and W.F. Broekaert 1993. Chitin binding proteins, their biology and possible function, p. 407–423. In: D.P.S. Verma (ed.). Control of plant gene expression. CRC Press, Boca Raton, Fla.

Schneider, H. 1956. A new disorder of Eureka lemon trees. Calif. Citrograph 41:387.

Schneider, H. 1960. Bud-Union trouble in *Macrophylla* rootstocks of frost nucellar Eurekas. Calif. Citrograph 46:2, 12.

Taylor, K.C., L.G. Albrigo, and C.D. Chase. 1988. Zinc complexation in the phloem of blight affected citrus. J. Amer. Soc. Hort. Sci. 113:407–411

Taylor, K.C., L.G. Albrigo, and C.D. Chase. 1996a. Purification of two Zn-binding proteins and evidence for structural and compositional change in phloem of blight affected citrus. Plant Physiol. 110:657–664.

Taylor, K.C. and D.R. Ellis. 1996. Mycoplasma-like organisms as the causal agent for Macrophylla decline. Ariz. Citrus Res. Rpt. p. 26–32.

Taylor, K.C., R. Langham, and Z. Xiong. 1996b. Relationship of viroids to Macrophylla decline. Ariz. Citrus Res. Rpt. p. 33–37.

Van Paris, J., W.F. Broekaert, I.J. Goldstein, and W.J. Peumans. 1991. Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta 183:258–264.

Williams, G.L. and L.G. Albrigo. 1984. Some inorganic element changes in trunk phloem of healthy and blight-affected citrus trees. J. Amer. Soc. Hort. Sci. 109:437–440.