Chrysanthemum Foliar Necrosis: Symptoms, Histochemistry, and X-ray Analysis of Leaf Lesions

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ABSTRACT

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Foliar necrosis in the Marble cultivars of *Dendranthema grandiflora* (Tzveler) is characterized by necrotic spotting in the lower leaves with associated chlorosis and premature senescence. The disorder is associated with manganese sensitivity and symptoms are amplified by the addition of supplemental manganese. Symptoms of foliar necrosis were produced in a high percentage of scion grafts of the *D. grandiflora* 'Fanfare' control when rootstocks of the Marble cultivars were treated with supplemental manganese. Marble and control cultivars with manganese-induced necrotic lesions showed degeneration in the palisade and spongy mesophyll cells adjacent to the vascular bundle. Bundle sheath cells were distorted, xylem vessels were occluded, and some sieve elements and companion cells were obliterated. Cell wall appositions were present in bundle sheath and epidermal cells abutting collapsed mesophyll cells. Supplemental manganese enhanced the cytological changes associated with necrosis. Normal, nonnecrotic tissue in the Marble and control cultivars showed obliteration of some sieve elements and companion cells that was enhanced in necrotic Marble and control tissue. X-ray analysis revealed manganese accumulations in necrotic lesions in Marble cultivars with and without supplemental manganese, but not in symptomless tissue. We conclude that foliar necrosis in the Marble cultivars is associated with manganese toxicity.

Additional keywords: Chrysanthemum phloem necrosis, fluorescence microscopy.

Chrysanthemum foliar necrosis (CFN) is a condition associated with the Marble cultivars of *Dendranthema grandiflora* Tzveler, formerly *Chrysanthemum* × *morifolium* Ramat, the florist's chrysanthemum. Foliar symptoms are characterized by necrotic spotting and premature senescence of the lower leaves. The condition has been known for more than 20 yr. Although symptoms vary in severity, many growers have recognized that necrotic spotting of the leaves is common in the Marble cultivars. Floral abnormalities are sometimes also present in which green bracts are intermixed with disk florets. The cause of the symptom(s) is not fully understood.

Foliar symptoms of CFN appear identical to those reported for chrysanthemum phloem necrosis (CPN), a condition in the Marble cultivars reportedly associated with a mycoplasmalike organism (MLO) (11,12). Manganese toxicity also has been known to cause necrotic spotting on lower leaves of many species (4,6), producing symptoms similar to that of CFN. Necrotic spots often are associated with localized accumulations of the element (8).

CPN, which is characterized by necrotic flecking, yellowing, and premature senescence of the lower leaves and by bract formation among disk florets, reportedly can be detected reliably by epifluorescence microscopy (12). Increased fluorescence in phloem using aniline blue, berberine sulfate, ethidium bromide, and acridine orange (12) has been correlated with prominent symptoms of phloem necrosis. Berberine sulfate additionally was used to stain epoxy-embedded sections, allowing for correlation between enhanced berberine sulfate fluorescence and the presence of a MLO detected by transmission electron microscopy (12).

In this study, a comparison was made between histopathogenic effects of manganese-treated and untreated Marble cultivars and manganese-treated and untreated control cultivars. We present experimental evidence that the symptoms of CFN are associated with manganese toxicity. Preliminary reports on these studies have been presented (5,9). In addition, we compare our results with cytopathology reported for CPN (11).

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Source plants and culture. *D. grandiflora* cvs. Florida Marble and Pink Marble with symptoms of foliar necrosis were compared with symptomless cvs. Bonnie Jean, Fanfare, and Vero as controls. Rooted cuttings were grown in a mixture of loam soil, perlite, and peat moss (2:1:1). Plants were fertilized with a proportioner injector every 2 wk with 200 ppm Peters 20-20-20 (N-P-K) starting 2 wk after potting, and they were treated with 100 ml of an aqueous solution of $MnSO_4$ ranging from 0.6 to 4.8 g/L every 2 wk. Foliar symptoms were compared with untreated controls. Each test was repeated at least twice over a period of 2 yr. In most experiments plants were grown in a greenhouse with temperatures fluctuating from 18 to 30 C. During winter, plants were exposed to 14 h of supplemental incandescent light to prevent flowering.

Graft transmission tests. Scions of control cultivars Fanfare and Bonnie Jean were top-grafted to rootstocks of Florida and Pink Marble plants grown in loam soil, perlite, and peat moss (2:1:1). Foliage on rootstocks of Marble cultivars and scion grafts were observed for symptoms over a period of 3 mo. Cuttings from the same Fanfare plants used for scion grafts also were grown on their own roots and compared with symptoms on the scion grafts. All plants were grown in a greenhouse with temperatures fluctuating from 18 to 30 C with an extended 14-h photoperiod in winter to prevent flowering. Experiments were repeated three times over a 2-yr period.

All plants were fertilized with 20-20-20 1 wk after the cuttings were potted. One-half of the Florida and Pink Marble rootstocks and one-half of the Fanfare and Bonnie Jean control plants grown on their own roots were treated with 100 ml of 4.8 g/L $MnSO_4$ at 2 wk and again 4 and 6 wk after potting. Plants were observed for the presence of foliar symptoms.

Effects of $MnSO_4$ and lime on CFN symptoms. Florida Marble cuttings rooted in perlite were transplanted to 4-in. pots in a loam soil, perlite, and peat moss mixture (2:1:1). Plants were fertilized weekly for 3 wk with 200 ppm Peters 20-20-20 (N-P-K). Beginning 3 wk after transplanting, all plants were treated weekly for 2 wk with 30 g of $Ca(NO_3)_2$ + 3 g of KNO_3 per gallon of water (base feed), 100 ml per pot. At the end of the second week of base feeding, one-half of the plants were treated

with 0.8 g of dolomitic lime per pot. Manganese treatments then were initiated with concentrations of 0, 0.3, 0.6, and 1.2 g/L of $MnSO_4$ added to the base feed. Each of 10 pots received six weekly applications of 100 ml of base feed at each specified level of $MnSO_4$ per pot. Five and 6 wk after $MnSO_4$ treatments were initiated, two leaves were sampled from nodes near the base of the plants and rated for the presence of necrotic and chlorotic symptoms.

Tissue sampling for microscopy. Pieces of leaves 2 mm square, including the midvein or secondary veins, were cut from fully expanded leaves of manganese-treated and untreated Marble cultivars and control cultivars. Samples were taken from Marble plants treated with 2.4 g/L of $MnSO_4$ showing necrotic spotting and compared with symptomless tissue from the same plants and from untreated Marble control plants. Similar samples were taken from Vero, Fanfare, and Bonnie Jean plants grown with and without supplemental $MnSO_4$. Tissue was fixed in cold fixative containing 2% glutaraldehyde and 1.5% acrolein in 0.05 M Na_2HPO_4-KH_2PO_4, pH 7.0 (PO_4 buffer). After postfixation in 1% OsO_4, the tissues were dehydrated in a graded series of ethanol and propylene oxide and embedded in LX112. Sections 5 m thick were cut, dried on a glass slide, and stained with methylene blue (0.1 mg/ml in double distilled water) and toluidine blue (0.05% in 0.1% sodium borate) for observation in transmitted light. Fluorochromes included aniline blue (0.1 mg/ml in 0.06 M K_2HPO_4, pH 8.0), acridine orange (10 μg/ml in double distilled water), berberine sulfate (0.25 mM in 2.4 mM $MgCl_2$, pH 5.4), and ethidium bromide (1.0 μg/ml in double distilled water). Methylene blue- and toluidine blue-treated samples were stained for 30 min, rinsed in double distilled water, and mounted in glycerin. Samples treated with aniline blue were mounted in the same stain. Acridine orange-, berberine sulfate-, and ethidium bromide-treated samples were stained for 5–10 min, rinsed, and mounted in double distilled water. Unstained sections were mounted in double distilled water and examined for color by transmitted light and for autofluorescence. Observations were made with a Leitz Ortholux II microscope (Rockleigh, NJ) equipped with a Ploemopak 2.3 fluorescence vertical illuminator. Epifluorescence micrographs were recorded on Kodak Ektachrome 400 film. A mercury vapor lamp (HBO 100) was the light source. Leitz filter block A cube for ultraviolet (excitation filters BP 340-380, dichromatic mirror RKP 400, and supression filter LP 430) and an N2 cube for green (excitation filters BP515-560, dichromatic mirror RKP 580, suppression filter LP 580) wavelengths were used.

Scanning electron microscopy. Pieces of leaves 3 mm square, with and without necrotic lesions, were cut from the same leaves of Florida and Pink Marble with and without supplemental $MnSO_4$ and from Fanfare with and without added manganese. Samples were freeze-dried without fixation by first placing the cut tissue pieces into a freeze-drying bottle and plunging the bottle into a bath containing ethanol and dry ice. The frozen samples were placed under vacuum at about 100 m for 7 h. After sputter coating with gold-palladium, the samples were examined in a JEOL 330A scanning electron microscope (Peabody, MA).

X-ray microanalysis. Freeze-dried leaf pieces of the Marble cultivars and Fanfare with and without necrotic lesions were mounted on carbon stubs and carbon coated. The analysis was made with an energy dispersive analyzer (EDS) Tracor System Series II microanalyzer (Middleton, WI). Samples were analyzed at 0° tilt and 30 kV. Chemical elements concentrated in the area of necrosis were compared with adjacent areas of normal green tissue sampled from the same leaf. In addition, X-ray dot maps were made to assess the distribution of elements in the lesion compared with green tissue adjacent to the border of the lesions.

RESULTS

Source plant symptoms. Lower leaves of Florida and Pink Marble cultivars grown in soil without supplemental manganese showed necrotic spots that expanded as the plants matured (Fig. 1A). As leaves aged, tissue adjacent to the necrotic lesions became chlorotic. No symptoms were present on the lower leaves of Vero, Bonnie Jean, or Fanfare cultivars at the same stage of growth (Fig. 1B). Florida and Pink Marble plants treated with 2.4 g/L of $MnSO_4$ showed an increased number of spots with more extensive necrosis 2–3 wk after the supplemental manganese treatment was initiated. No symptoms were present on the lower leaves of untreated Marble cultivars at this early stage of growth. Small necrotic spots appeared on the lower leaves of untreated Florida and Pink Marble plants 4–6 wk after the spots first appeared on the manganese-treated plants. Leaves later became chlorotic and collapsed. Fanfare and Vero also showed necrotic spotting in the lower leaves, but only when 1.2, 2.4, or 4.8 g/L of $MnSO_4$ was applied. The number and size of the necrotic spots increased with increasing concentrations of $MnSO_4$. Similar spotting was not observed on lower leaves of Fanfare or Bonnie Jean plants not treated with $MnSO_4$. Systemic leaf distortion and chlorosis occurred in Bonnie Jean plants treated with 4.8 g/L of $MnSO_4$.

Graft transmission tests. Some Florida and Pink Marble rootstocks fertilized only with 20-20-20 showed small necrotic spots on lower leaves 2 mo after the cuttings were potted (Table 1). Lower leaves on a few Fanfare scions showed very small necrotic spots, but most leaves were symptomless (Table 1). One month after the initial $MnSO_4$ treatment, a high percentage of Florida

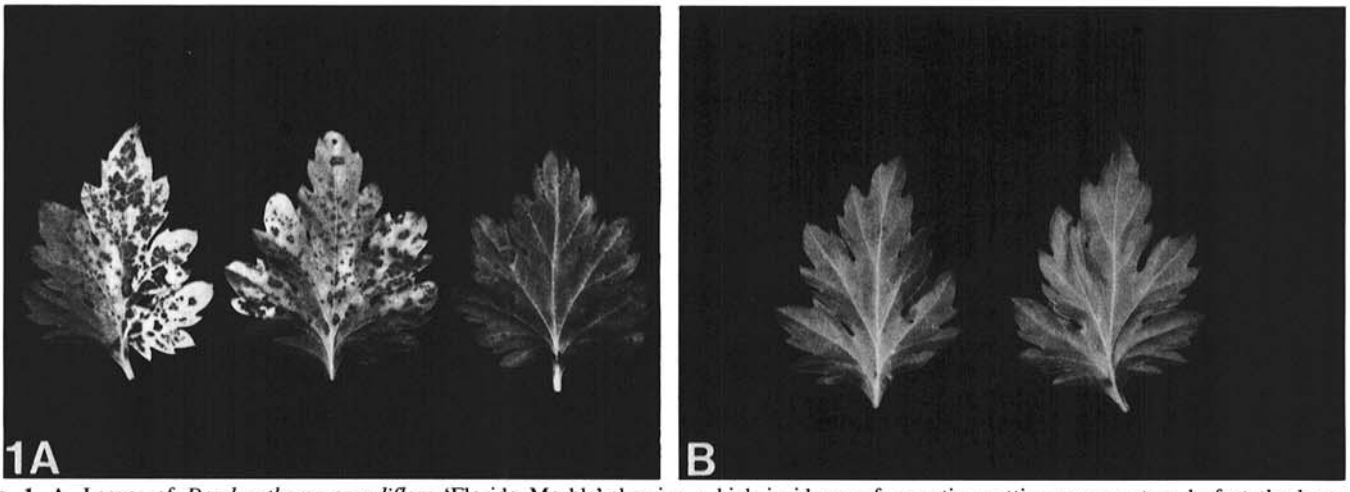

Fig. 1. A, Leaves of *Dendranthema grandiflora* 'Florida Marble' showing a high incidence of necrotic spotting on a mature leaf at the base of the stem (left) with fewer spots on the leaf two nodes higher (middle) with the fewest lesions on the leaf four nodes from the base of the stem (right). Leaves photographed 12 wk after pinching. Plant grown without supplemental manganese. **B,** Symptomless *D. grandiflora* 'Fanfare' leaves from the base of the stem (left) and two nodes higher (right). Leaves photographed 12 wk after pinching. Plant grown without supplemental manganese.

and Pink Marble plants showed numerous necrotic spots on lower leaves. Although only a few lower leaves on Fanfare scion grafts showed necrotic spots 1 mo after the $MnSO_4$ treatment was initiated, most Fanfare scions showed necrotic spots 2 mo after the initial manganese treatment (Table 1). All Fanfare plants grown on their own roots without supplemental manganese were symptomless and 90% of Fanfare plants on their own roots showed many necrotic spots on the lower leaves when treated with 4.8 g/L of $MnSO_4$. Eighty percent of the Fanfare scion grafts on Pink Marble rootstocks and 90% on Florida Marble rootstocks showed necrotic leaf spots with the addition of 4.8 g/L of manganese (Table 1). No necrotic spotting was observed on upper or lower leaves of Bonnie Jean scions grafted onto Florida or Pink Marble grown without supplemental manganese (Table 1). Bonnie Jean scions on Marble rootstocks provided with supplemental manganese failed to show distinct necrotic spotting, although one plant showed a general russetting of lower leaves. Bonnie Jean plants grown on their own roots showed severe systemic leaf distortion and chlorosis with the addition of 4.8 g/L of supplemental $MnSO_4$.

Effects of $MnSO_4$ and lime on CFN. Although soil pH did not differ significantly when a range of manganese concentrations were applied with and without lime, the concentration of manganese in soil treated with 1.2 g/L of $MnSO_4$ and no supplemental lime was more than 150 times higher than in soil with plants treated with the same concentration of $MnSO_4$ that received 0.8 g of supplemental lime per pot (Table 2).

Lower leaves from control plants not receiving supplemental $MnSO_4$ were nearly symptomless with an average of fewer than 10 necrotic spots less than 0.1 mm in diameter (Table 2). An increase in the number and size of necrotic lesions was associated with an increase in the concentration of manganese applied to the soil without any supplemental lime (Table 2). The addition of 0.8 g of lime suppressed development of necrotic lesions in the presence of supplemental $MnSO_4$. The foliar symptom rating was more than 3 times higher in plants receiving 1.2 g/L of $MnSO_4$ without supplemental lime than in those plants receiving lime and 1.2 g/L of $MnSO_4$ (Table 2). In treatments both with and without the addition of 0.8 g of lime, there was a proportional increase in the concentration of manganese in the leaves, regardless of the presence of the added lime. Concentrations of manganese in the foliage of untreated plants and those treated with lime were similar when the plants received the same concentration of $MnSO_4$. The effect of lime on suppression of necrotic symptoms in the foliage was repeated in other experiments. Soil and leaf samples from pots and plants grown with and without supplemental liming and $MnSO_4$ were taken at the end of the experiment when final data on leaf symptoms were recorded. Samples included the soil profiles from four pots with and without $MnSO_4$ and leaves from the lowest two or three nodes that showed necrotic symptoms and leaves from the midportion of the stem. Manganese content was analyzed by the Horticultural Products Division, W. R. Grace & Co.–Conn, Fogelsville, PA.

Brightfield microscopy of symptomless control cultivars and symptomatic Florida Marble. Leaf cross sections of control cultivars showed a well-developed palisade layer, spongy mesophyll, and vascular bundle enclosed with a bundle sheath (Fig. 2A). Larger vascular bundles contained protoxylem with one or two secretory ducts, metaxylem with alternating rows of xylem vessels and parenchyma cells, and protophloem with obliterated sieve elements and companion cells but intact phloem parenchyma cells (Fig. 2B). Xylem parenchyma cells often were occluded with dense-staining material.

Necrotic tissue from an affected leaf of Florida Marble showed collapse and distorted cells in the palisade layer that extended into the spongy mesophyll adjacent to the vascular bundle (Fig. 2C). A pattern of regular organization occurred in the phloem, although many of the sieve elements remained intact (Fig. 2D). Some xylem vessel element lumens were occluded, and an extensive buildup of dense substances was present in the xylem parenchyma (Fig. 2D).

Bundle sheath cells were distorted and many were filled with

TABLE 1. Symptoms on lower leaves of scions of *Dendranthema grandiflora* cvs. Fanfare and Bonnie Jean and rootstocks of cvs. Pink Marble and Florida Marble with and without supplemental manganese sulfate

Treatment[a]	Pink Marble rootstock	Fanfare scion on Pink Marble rootstock	Bonnie Jean scion on Pink Marble rootstock	Florida Marble rootstock	Fanfare scion on Florida Marble rootstock	Bonnie Jean scion on Florida Marble rootstock
No $MnSO_4$	8/10[b]	2/10[c]	0/10[d]	7/10[b]	2/10[c]	0/10[d]
4.8 g/L of $MnSO_4$	10/10	8/10	1/10[e]	10/10	9/10	0/10

[a]Observations were recorded 2 mo after the first $MnSO_4$ treatment.
[b]Number of plants with necrotic spot symptoms/number of plants grown.
[c]Number of Fanfare scions with necrotic spot symptoms/number of Pink Marble or Florida Marble rootstocks.
[d]Number of Bonnie Jean scions with necrotic spot symptoms/number of Pink Marble or Florida Marble rootstocks.
[e]Number of Bonnie Jean scions with russetting on the lower leaves.

TABLE 2. Effect of supplemental manganese and liming on concentrations of Mn in soil and leaves and development of foliar symptoms in *Dendranthema grandiflora* 'Florida Marble'

Supplemental lime (g/4-in. pot)	$MnSO_4$ (g/L)	Soil pH	Soil Mn (ppm)	Leaf Mn (ppm)	Foliar symptoms[a]	
					After 5 wk[b]	After 6 wk[c]
0	0	6.4	0.05	128	1.5	1.8
	0.3	6.7	0.5	310	2.6	3.2
	0.6	6.5	1.3	324	3.5	3.7
	1.2	6.3	8.0	318	6.0	6.0
0.8	0	6.8	0.03	128	1.2	1.6
	0.3	6.7	0.07	247	1.6	1.7
	0.6	6.5	0.12	410	1.7	2.7
	1.2	6.6	0.96	356	1.8	3.5

[a]Symptoms were rated from 1 to 7 based on 1) the absence of symptoms; 2) fewer than 10 localized necrotic spots less than 1 mm; 3) 10–25 or more evenly spread necrotic spots less than 1 mm; 4) fewer than 10 necrotic lesions 3–4 mm in diameter composed of clusters of many small necrotic spots; 5) 10–25 or more necrotic lesions 3–4 mm in diameter composed of clusters of small necrotic lesions; 6) localized chlorotic spots intermixed with no. 5. Ratings were based on the average of two leaves per plant from 10 plants sampled two nodes from the base of the plant. Data are representative of two experiments.
[b]Symptoms after 5 wk of $MnSO_4$ treatments.
[c]Symptoms after 6 wk of $MnSO_4$ treatments.

dense staining substances (Fig. 2D). Increased buildup of osmophilic material also was present in the central vacuoles of vascular parenchyma cells, appearing as extensive granular accumulations (Fig. 2D). Similar substances appeared in all cell types bordering necrosis (Fig. 2D). In addition, a red, nonfluorescent material was present in the walls that may or may not be bordered by fluorescent material. This red material accumulated more frequently in cells of manganese-induced necrotic tissues and was easily distinguished in unstained sections. Its appearance corresponds to the red substances in fresh tissue samples.

Epifluorescence microscopy of symptomless control cvs. Fanfare, Vero, and Bonnie Jean. All fluorochromes except aniline blue produced fluorescence in cell walls. Brightly stained cell walls were outlined against a black background, facilitating observations of gross changes in anatomical structure. Sieve elements in metaphloem had thick nacreous walls and thin outer walls that could be distinguished with acridine orange (Fig. 3A). Bright fluorescence was induced in xylem vessel cell walls and in obliterated protophloem (Fig. 3A) by all fluorochromes (Table 3). Lumens of xylem vessels were clear.

Epifluorescence microscopy of symptomatic cvs. Pink and Florida Marble. Necrosis most frequently occurred in palisade and spongy mesophyll cells located beneath trichomes and stomates and adjacent to vascular bundles. Collapse occurred in tissue between vascular bundles, extending up to, but not including, the bundle sheath cells (Fig. 3B). Fluorescence with aniline blue was observed in metaphloem sieve element cell walls (Fig. 3C). Brightly fluorescing cell wall appositions were present on cell walls adjacent to collapsed tissue. Wall appositions contained fluorescing material as well as a reddish brown substance that did not fluoresce with all fluorochromes. Appositions were never observed in xylem and phloem cells.

Epifluorescence microscopy of symptomatic leaves of control cultivars treated with supplemental manganese. Cytological abnormalities in Vero, Fanfare, and Bonnie Jean were similar to those observed in the Marble cultivars. Necrosis occurred in mesophyll and epidermal cells, usually beneath the trichomes, in guard cells and adjacent to bundles. Aniline blue fluorescence in metaphloem of Fanfare appeared similar to that in the Marble cultivars with some increased fluorescence occurring in the protophloem (Fig. 3D). Obliteration of sieve elements was enhanced in the protophloem as shown in Vero treated with $MnSO_4$ (Fig. 3F), and there was occasional collapse in the metaphloem. Fluorescence from acridine orange (Fig. 3F) and berberine sulfate (Fig. 3G) was enhanced in cell walls of obliterated phloem. Metaphloem collapse was more common in Bonnie Jean than in other cultivars. Xylem vessels contained material that brightly fluoresced with all fluorochromes except aniline blue.

Scanning electron microscopy. Low magnification secondary electron images showed differences in the surface structure of the necrotic lesion area compared with the surrounding leaf tissue. Necrotic tissue in the lesion appeared collapsed in samples without supplemental manganese (Fig. 4A). Two types of trichomes were present on the leaf surface. Uniseriate trichomes with a single-celled foot and three or four stalk cells with a mast cell at the apex formed a T trichome (Fig. 4A). Stalk cells of these trichomes frequently appeared deformed in the lesion area. Biseriate or glandular trichomes also were present (15). These trichomes retained the same surface structure in the necrotic and normal green

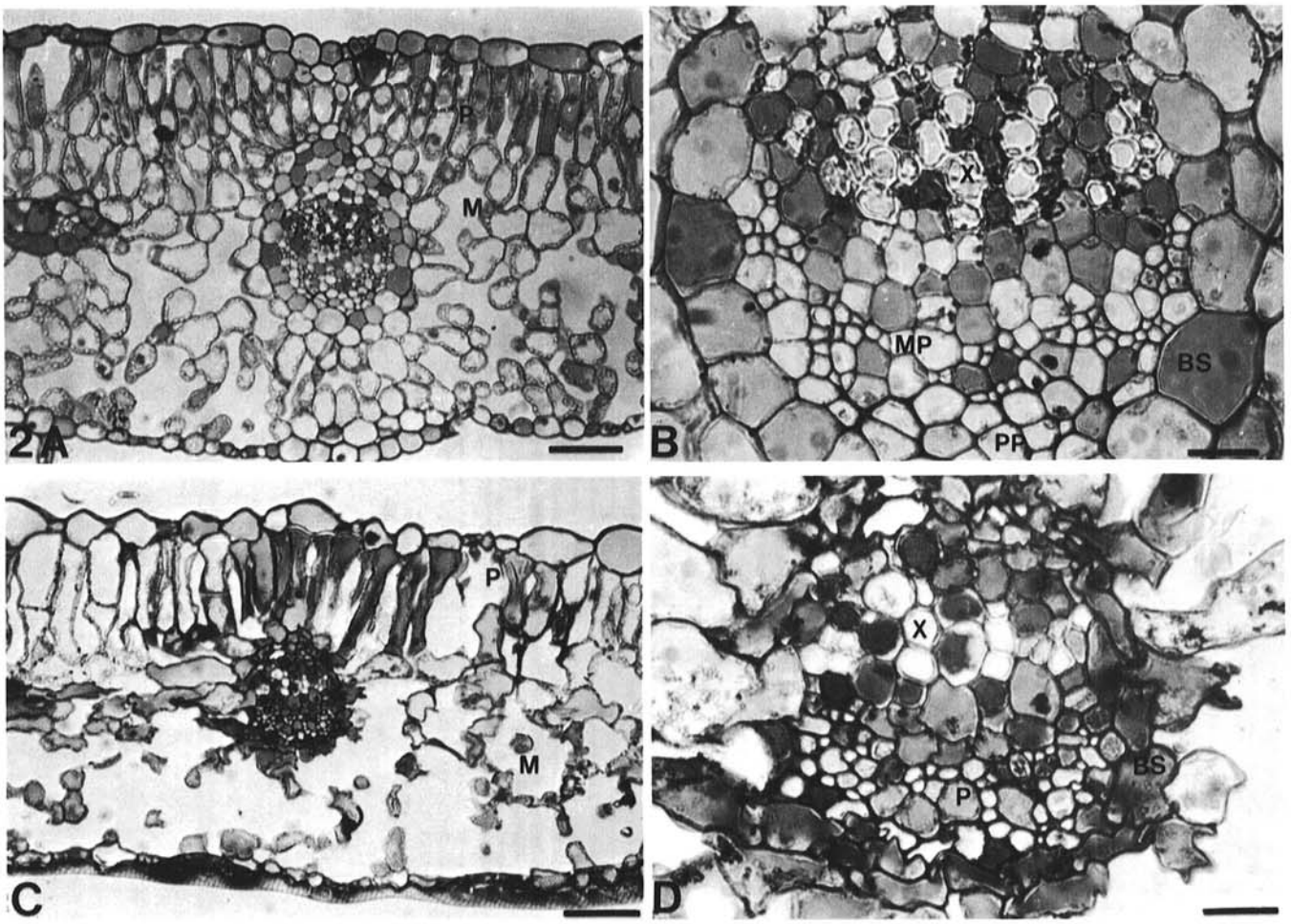

Fig. 2. Cross sections of leaves from symptomless control *Dendranthema grandiflora* 'Vero' (A and B) and symptomatic 'Florida Marble' (C and D) with necrotic lesions stained with methylene blue. **A,** Normal epidermal, palisade, and spongy mesophyll. **B,** Enlarged view of bundle shown in A with normal position of xylem, metaphloem, and bundle sheath cells. Note some obliteration in the protophloem. **C,** Collapsed palisade and spongy mesophyll. **D,** Enlarged view of C showing disorganized xylem, phloem, and distorted bundle sheath cells. Note the dense-staining material in the xylem parenchyma and bundle sheath cells. Many sieve elements remain intact. Bars = 11, 2.8, 11, and 2.8 μm for A, B, C, and D, respectively.

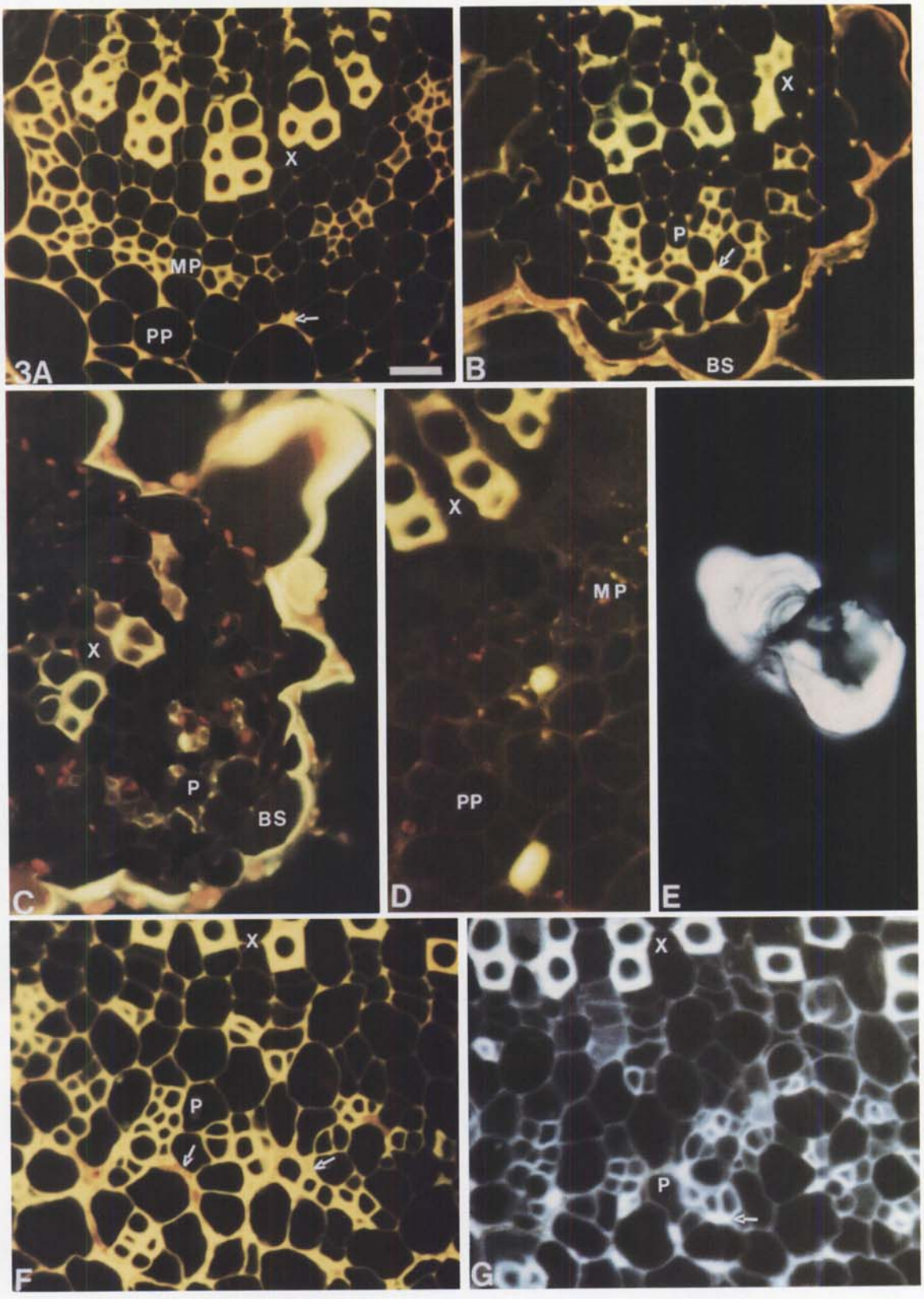

Fig. 3. Fluorescence microscopy of leaf tissue of *Dendranthema grandiflora* (chrysanthemum) cultivars. **A,** Fanfare, manganese untreated. Acridine orange-stained normal vascular bundle from a major vein showing bright fluorescence in a xylem vessel element and metaphloem cell walls and in obliterated sieve elements and companion cells in protophloem. **B,** Florida Marble with necrosis, no manganese treatment. An acridine orange-stained bundle smaller than that in A showing intense fluorescence in obliterated (arrow) phloem and within xylem vessel lumens. Bundle sheath cells distorted with thickened walls abutting mesophyll. **C,** Florida Marble with necrosis, no manganese treatment. Aniline blue-stained vascular bundle showing fluorescence in xylem vessel and sieve element cell walls in the phloem. Bundle sheath cells contain fluorescent appositions on walls adjacent to mesophyll. **D,** Fanfare, manganese treated. Aniline blue-stained vascular bundle with fluorescence in metaphloem similar to that in Florida Marble. **E,** Bonnie Jean, manganese treated. Aniline blue-stained mesophyll cell showing a wall apposition with striations of fluorescing and nonfluorescing material. **F,** Vero, manganese treated. Acridine orange staining bright fluorescence in areas of increased phloem obliteration (arrows). **G,** Vero, manganese treated. Berberine sulfate-stained section showing the same bundle as F with an overlapping field of view. Intense fluorescence in sieve element and vessel cell walls and in collapsed sieve elements in phloem (arrow). X, xylem vessel; MP, metaphloem; PP, protophloem; P, phloem; BS, bundle sheath. Bar = 17 μm A–G.

tissue.

In addition to the collapsed appearance in the necrotic tissue, the secondary electron image of the lesion surface produced more signal and the separation between cells became less distinct (Fig. 4B). Imaging with a backscattered detector showed a difference in contrast between cells within the lesion compared with adjacent normal tissue (Fig. 4C). This difference was particularly evident at the border of the lesion where accumulations or deposits of material were observed (Fig. 4C).

X-ray microanalysis. In manganese-treated and untreated Pink Marble and in manganese-treated Fanfare, X-ray dot mapping revealed a higher concentration of manganese in necrotic lesions than in surrounding green tissue. The greatest accumulation of manganese was at the border of the lesion (Fig. 4D). Localization of manganese at the margin of the lesion corresponded to the material revealed in the backscattered image. In addition, a dot map analysis of calcium showed increase in calcium concentration in the lesion compared with the adjacent normal green tissue.

X-ray spot analysis of the margin of a necrotic lesion from a leaf of Florida Marble grown with supplemental manganese showed a spectral peak for the presence of manganese (Fig. 5A). A similar peak was not produced from other areas analyzed within the lesion. This was apparently due to the lower concentration of manganese in the center of the lesion. Similarly, spot analysis at the lesion margin showed an increased concentration of calcium compared with the necrotic areas in the center of the lesion. The concentration of manganese in the green tissue adjacent to the necrotic lesion was low and could not be detected by spot analysis (Fig. 5B).

DISCUSSION

Etiology of foliar necrosis. Necrotic spotting and flecking on the lower leaves of the Marble cultivars have been recognized by growers for many years. Foliar necrosis and premature deterioration of the lower leaves have been associated with a disease described as chrysanthemum phloem necrosis (CPN) (11). The cause of the disease has been attributed to a mycoplasmalike organism (11,12).

Symptoms of prominent veinal discoloration and necrosis of the lower foliage associated with CPN were graft transmissible (10). Based on CPN reports it is not possible to assess the efficiency of graft transmission or the relationship between symptoms on the rootstock and scion grafts. Symptoms reportedly occurred 3–4 wk after grafting, but specific data were not presented. In this and the following report, we describe foliar necrosis on Fanfare scion grafts on Marble rootstocks, but failed to associate foliar necrosis with a mycoplasmalike agent or other biotic organism. We conclude that the low incidence of symptoms on Fanfare scions grafted to Marble rootstocks of plants grown in soil without supplemental $MnSO_4$ results from more efficient transport of manganese from the Marble understock to the Fanfare scion than occurs by direct uptake of manganese through roots of Fanfare control plants.

The addition of supplemental $MnSO_4$ to Marble rootstocks results in transport of manganese into Fanfare scions with the development of necrosis. We conclude that Fanfare is not as sensitive to manganese as the Marble cultivars but Fanfare scions do show symptoms of manganese toxicity when supplemental manganese is provided in the Marble rootstocks.

Bonnie Jean scions grafted to Pink Marble and Florida Marble rootstocks failed to show necrotic symptoms without supplemental manganese, and only a slight russetting was observed when manganese was added. We conclude that the cultivar Bonnie Jean is less susceptible to manganese than the Marble cultivars or Fanfare. In addition, the presence of necrotic spotting on the leaves of rootstocks of Marble cultivars and the absence of these spotting symptoms on Bonnie Jean scions is evidence that no

TABLE 3. Reactions of symptomless and necrotic leaf tissue of *Dendranthema grandiflora* (chrysanthemum) cv. Florida Marble and control cvs. Vero, Fanfare, and Bonnie Jean to various histochemical stains

Cytological feature	Stain used to produce the histochemical reaction shown below[a]							
	Toluidine blue	Methylene blue	Osmium	Acridine orange	Berberine sulfate	Ethidium bromide	Aniline blue-N cube	Aniline blue-A cube
Symptomless tissue[b]								
Mesophyll-cell walls	BL,V	BL	NR	GR,O	BL	OY	V,NR	YG,NR
central vacuole	BL,V,ND	BL,ND	BR,ND	NR,ND	NR,ND	NR,ND	NR,ND	NR,ND
Bundle sheath-cell walls	BL	BL	NR	GR	BL	OY	V,NR	YG,NR
central vacuole	V,ND	BL,ND	BR,BK,ND	NR,ND	NR,ND	NR,ND	NR,ND	NR,ND
Xylem								
Parenchyma-cell walls	BL	BL	NR	GR	BL	OY	V	YG,NR
Central vacuole	BL,V	NR	BR	NR	NR	NR	NR	NR
Vessel-cell walls[c]	BL,GR	BL,G	NR	GR	GR	OY	BL,V	GR,YG
Lumens	ND	ND	ND	ND	ND	ND	ND	ND
Protophloem								
Parenchyma-cell walls	BL	BL	NR	GR,O	BL	OY	BL	YG,NR
Central vacuole	NR,ND	NR,ND	BR,ND	NR,ND	NR,ND	NR,ND	NR,ND	NR,ND
Oblite sieve elements and companion cells	BL	BL	BR	O	BL	O	BL	YG
Metaphloem								
Parenchyma/companion cells-cell walls	BL	BL	NR	GR,O	BL	O	BL	YG
Central vacuole	BL,V,ND	NR,ND	BR,ND	NR,ND	NR,ND	NR,ND	NR,ND	NR,ND
Sieve elements-cell walls	BL,V[d]	BL	NR	O,GR[d]	BL	O	BL	YG
Lumen content	BL,ND	BL,ND	NR,ND	NR,ND	NR,ND	NR,ND	NR,ND	NR,ND
Necrotic tissue[e]								
Cell wall appositions	RU,BL,V	BL,RU	RU,R,BR	Y,O,GR,RU	GR,RU,BL	Y,O	BL	YG,GR
Cell walls	RU,BL,G	G,BL	RU,R	Y,O,GR,RU	GR	Y,O	BL,NR	GR
Xylem vessel content	V	BL	BR	Y	BL,GR	O,G	NR	NR
Cytoplasm	BL	G,B	BR,RU	Y,GR,RU,O	BL	O	NR,BL	GR
Phloem	BL,RU	BL,RU	BR,RU	Y,RU	BL	O	BL	Y

[a] Histochemical reactions: BL = blue; RU = rust; BR = brown; O = orange; V = violet; Y = yellow; BL = black; YG = yellow green; GR = green; OY = orange yellow; R = red; NR = no reaction; ND = no content detected.
[b] Includes symptomless Marbles.
[c] Faint autofluorescence present.
[d] Second color = color of nacreous cell wall.
[e] Necrotic leaf tissue of Marble cultivars without supplemental Mn.

graft-transmissible agent is present in Marble cultivars that can be transmitted to Bonnie Jean, irrespective of the presence of manganese.

We have correlated symptoms of necrotic spotting on Marble cultivars with increasing concentrations of supplemental $MnSO_4$. Without the addition of manganese, only a few pinpoint necrotic spots were observed. There was an increased number and size of spots formed as the concentration of manganese increased.

The addition of lime to Florida Marble plants decreased the amount of manganese measured in the soil. However, leaves from plants treated with a high concentration of supplemental manganese and lime also contained a high level of manganese. Manganese-tolerant alfalfa clones contained lower concentrations of manganese in the tops and higher concentrations of calcium and manganese in the roots (14). These investigators concluded that calcium uptake regulated manganese toxicity by reducing the transport of manganese to plant tops. The addition of silicon to the nutrient solution prevented development of necrotic spots in barley by redistributing manganese in the plant rather than reducing uptake (16). Partial suppression of necrotic symptoms in Florida Marble plants receiving both lime and a high concentration of manganese may result from an effect of calcium

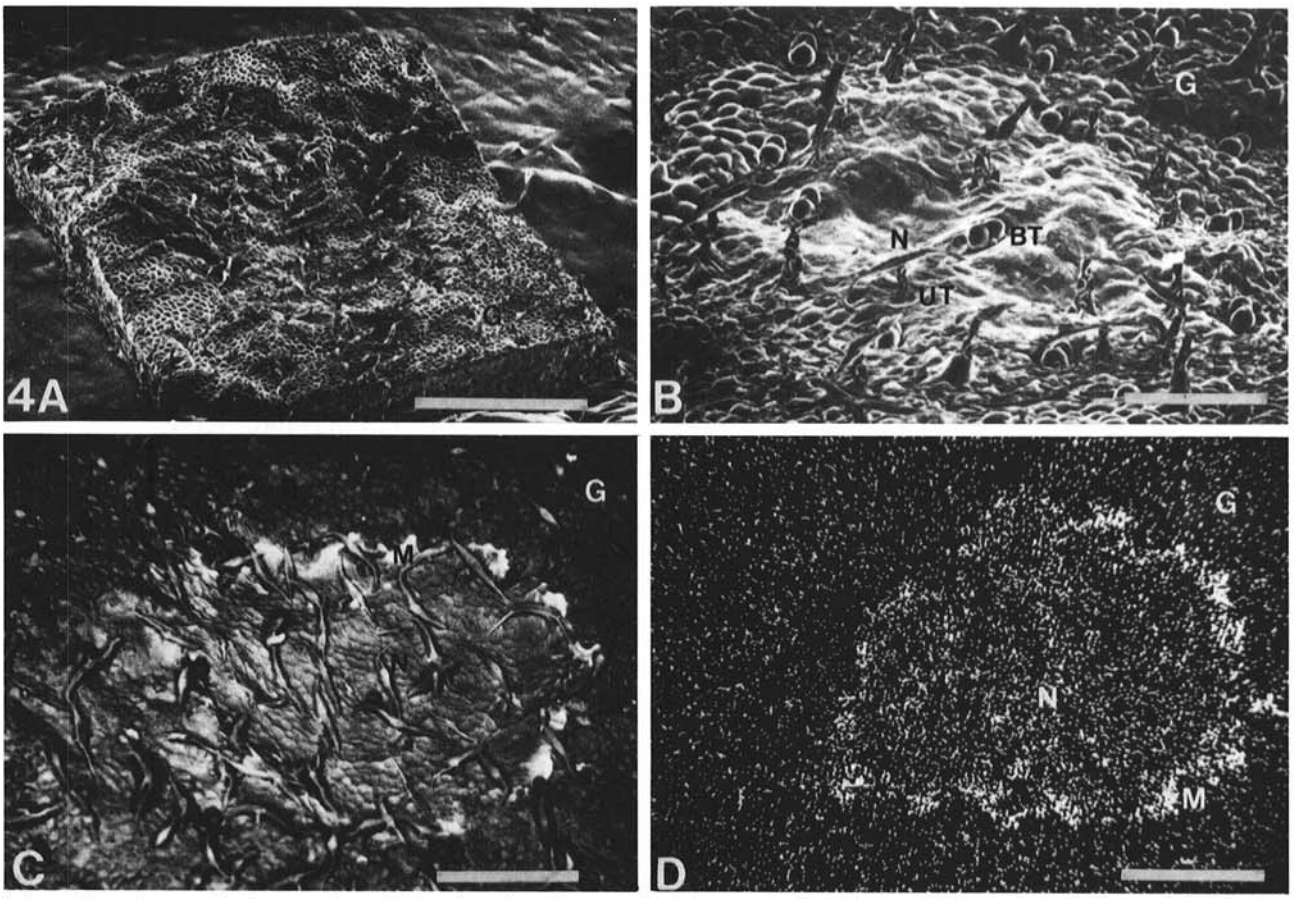

Fig. 4. Secondary electron, backscattered, and X-ray imaging of a necrotic lesion on the leaf of *Dendranthema grandiflora* 'Florida Marble' without supplemental manganese. **A,** Secondary electron image showing area of necrosis in the center of the sample. The necrotic area is surrounded by normal green tissue. **B,** Necrotic lesion with uniseriate trichomes and biseriate trichomes. The cells in the necrotic lesion appear collapsed and epidermal cells not as prominent as in adjacent green tissue. **C,** Backscattered image of a necrotic lesion showing an increase in the signal from the necrotic portion of the sample and the margin of the lesion indicating the accumulation of material compared with the adjacent green tissue. **D,** X-ray analysis for manganese in a dot map showing an increased number of counts in the necrotic tissue and at the margin of the lesion compared with the normal green tissue. N, necrosis; G, green tissue; UT, uniseriate trichomes; BT, biseriate trichomes; M, margin. Bars = 1, 0.5, 0.5, and 0.5 μm for A, B, C, and D, respectively.

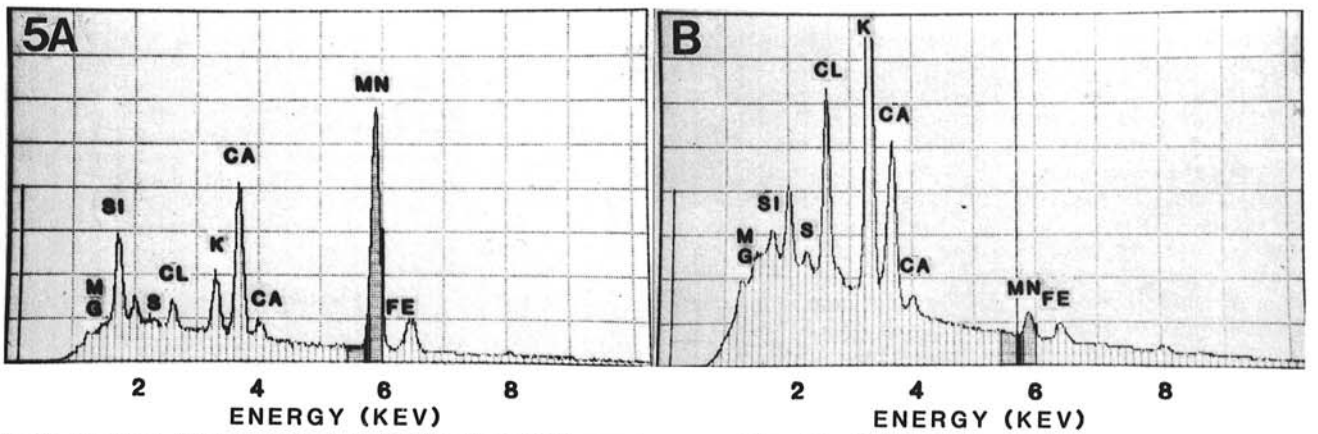

Fig. 5. Relative elemental composition in leaf surface tissue of *Dendranthema grandiflora* 'Florida Marble' grown without supplemental manganese. **A,** Spot analysis at the margin of the lesion with a manganese Kα energy peak at approximately 5.9 kev. **B,** No manganese energy peak was observed in adjacent green tissue.

on redistribution on manganese that prevents the formation of toxic tissue reactions.

Cytopathology in the Marble cultivars. Cytological changes associated with chrysanthemum foliar necrosis are similar to many of the observations associated with CPN (11,12). Our interpretation of these observations do, however, differ significantly. We have duplicated many of the cytological changes that are reportedly specific to CPN by treating cultivars Vero, Fanfare, and Bonnie Jean with supplemental manganese. We observed enhanced obliteration of protophloem with collapse of sieve elements in necrotic Marble tissue as well as in the control cultivars with necrotic symptoms.

Necrosis sometimes occurs in association with the vascular bundle, but not consistently or specifically in the sieve elements of necrotic tissue from Marble cultivars with symptoms of foliar necrosis. Although some disruption occurs in phloem, most mature sieve elements remain unaffected. We conclude that necrosis in the sieve tubes does not commonly occur. When it does occur, it is found in necrotic tissue of both the Marble and control cultivars.

Epifluorescence diagnosis of CPN was conducted primarily on fixed and unfixed freehand petiole tissue sections (12) while our observations were from fixed and embedded tissues. McGovern et al (12) did stain osmicated, epoxy-embedded leaf tissue with berberine sulfate and reported that collapsed sieve elements with enhanced staining occurred in Pink Marble petioles, but not in Bonnie Jean (12). We observed collapsed phloem and enhanced berberine sulfate staining both in Marble cultivars and in Vero plants receiving supplemental manganese. McGovern et al interpreted increased berberine sulfate staining in collapsed sieve elements as indicative of the presence of DNA and RNA in MLOs (12). An alternative interpretation is that increased staining results from an increased accumulation of phenolic materials. Berberine sulfate, ethidium bromide, and acridine orange all have an affinity for lignin in addition to RNA and DNA. Acridine orange and ethidium bromide also may react with phenolic substances.

We observed a general increase in fluorescence of cell walls in necrotic tissue stained with acridine orange and berberine sulfate that was frequently accompanied by some thickening. Although none of the fluorochromes are specific for lignin, increased staining of the walls may reflect the accumulation of polyphenolic substances.

Elevated levels of phenolic materials were found in vascular and nonvascular tissue, and lignin formation and callose accumulation was observed in the phloem of CPN-affected but not in unaffected chrysanthemum cultivars (11). Synthesis of callose and lignin and the formation of phenolic compounds may be a reaction to infectious organisms or to chemical or mechanical injury (1,2). Large appositions, apparently composed of callose, were reported in CPN-affected tissue (12). We observed similar structures stained with aniline blue in cells bordering necrotic tissue. The appositions sometimes showed the layered structure associated with CPN. In addition, increased fluorescence occurred in phloem of necrotic tissue stained with aniline blue. Similar reactions were present in the necrotic tissues of manganese-treated control cultivars in addition to the Marbles. Increased fluorescent staining in the phloem and staining of well appositions with aniline blue may represent the accumulation of callose. Accumulation of callose is apparently the result of manganese-induced necrosis and results from physiological stress and cell death rather than the presence of a mycoplasmalike organism. Callose deposition has been associated with manganese toxicity in the leaves of cowpea showing necrotic lesions (17).

Manganese toxicity. Necrotic lesion formation, first on the lower leaves, such as observed in the Marble cultivars, is a typical symptom of manganese toxicity (4,6,7) on a wide range of different species. Chlorosis of young expanding leaves, irregularly shaped brown spots near the midrib and main veins of leaves, and browning of the veins also have been reported (7). These symptoms, as well as those reported for CPN and foliar necrosis syndromes described in this report, resemble classic symptoms of manganese-induced toxicity.

Manganese oxides accumulate as dark granules in epidermal cells of *Phaseolus vulgaris* in small black leaf spots induced by manganese toxicity (3). Precipitated manganese compounds were localized primarily in the cell wall in the vicinity of xylem vessels (8). The accumulation of this material may correspond to the red material we have observed in the walls of tissues exhibiting manganese-induced necrosis. X-ray dot mapping revealed the deposition of manganese in epidermal cells, but not in palisade, spongy parenchyma, or vascular bundle cells (13). We have shown that manganese is present in necrotic lesions and on the margin of lesions in greater concentration than in adjacent green tissue. The development of some necrotic lesions and their expansion on older leaves may be associated with increased sensitivity of the Marble genotypes compared with control cultivars, or it may be associated with the interaction of other elements that influence the translocation and form of manganese in the leaves. The sensitivity of control cultivars described in this report shows, however, that other chrysanthemum cultivars not typically expressing foliar necrosis may be induced to produce a toxic reaction when elevated concentrations of manganese are provided.

Based on observations of the plants we examined, we conclude that foliar necrosis in the Marble cultivars is a nutritional disorder and is not caused by a biotic agent. Although we attempted to obtain cuttings from the same Marble and control cultivars reportedly affected by the CPN agent, they were not made available for comparative evaluation. We must, therefore, conclude that foliar necrosis and CPN may be different until the material is provided for a side-by-side comparison.

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