

Ultrastructure of Chrysanthemum Stunt Virus-Infected and Stunt-Free Mistletoe Chrysanthemum

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ABSTRACT

The American source of *Chrysanthemum morifolium* 'Mistletoe' infected with chrysanthemum stunt virus (CSV) shows chlorotic spots on leaves of plants grown in the greenhouse in both summer and winter. Chlorotic spots are also present on fully expanded leaves of CSV-free American Mistletoe in the greenhouse in summer, but the spots disappear from expanding leaves in winter. Chlorotic spots on leaves of CSV-infected and CSV-free American Mistletoe were not modified with the addition of supplemental iron chelate. A source of CSV-free Mistletoe from Great Britain shows a few chlorotic leaf spots

on unexpanded and fully expanded leaves in summer, but not in winter. No viruslike particles were observed in chlorotic spotted or green leaf tissue of CSV-infected Mistletoe. Chloroplasts in chlorotic leaf tissue from CSV-infected and CSV-free plants contain electron-dense inclusions resembling phytoferritin. Inclusions were also observed in chloroplasts of palisade and mesophyll cells from green tissue of CSV-infected plants, but were less numerous than in chlorotic tissue. No inclusions were observed in these cells in green tissue from healthy plants. Phytopathology 61:653-656.

The Mistletoe cultivar of *Chrysanthemum morifolium* (Ramat.) Hemsl. is used as an index host for Chrysanthemum stunt virus (CSV) in the United States and Europe. Mistletoe is one of the few chrysanthemum cultivars that shows chlorotic spots on the leaves of plants infected with CSV. Spotting also occurs on the leaves of CSV-free American Mistletoe grown in high light and high temperature. This nonspecific spotting limits the usefulness of the American source of this variety in a program of stunt indexing.

In England, the senior author observed CSV-free Mistletoe which shows no chlorotic leaf spots during the summer. Leaves from American and English Mistletoe were of similar shape, but flowering tests showed that these two sources of Mistletoe were different. The American Mistletoe flower is yellow, and the English Mistletoe flower is nearly white. CSV-infected American Mistletoe, however, produces white flowers.

The purpose of this investigation was to localize chrysanthemum stunt virus in thin sections, and to compare the ultrastructure of chlorotic and green leaf tissue of diseased and healthy chrysanthemum plants. In addition, we wanted to determine the influence of light, temperature, and iron nutrition on chlorotic leaf spotting of CSV-infected and CSV-free American and English Mistletoe selections, and to compare chlorotic spotting on leaves of healthy plants of the American and English types. We also wanted to determine if the two selections are genetically different. Some of our findings have been previously reported (4).

MATERIALS, METHODS, AND RESULTS.—*Effect of light and temperature on development of chlorotic spots.*—We compared chlorotic spotting on plants of CSV-free American and English Mistletoe grown under 1,200 ft-c fluorescent illumination and 18-23 C in fall and winter, and in light ranging from 400-2,500 ft-c and 23-32 C in summer. We also compared the spotting reaction on

plants at 21 and 27 C constant temperatures grown in rooms with controlled fluorescent illumination of 500 ft-c on an 18-hr day. Spots on CSV-infected American and English Mistletoe were compared in the greenhouse in summer under natural conditions and in the winter without supplemental illumination.

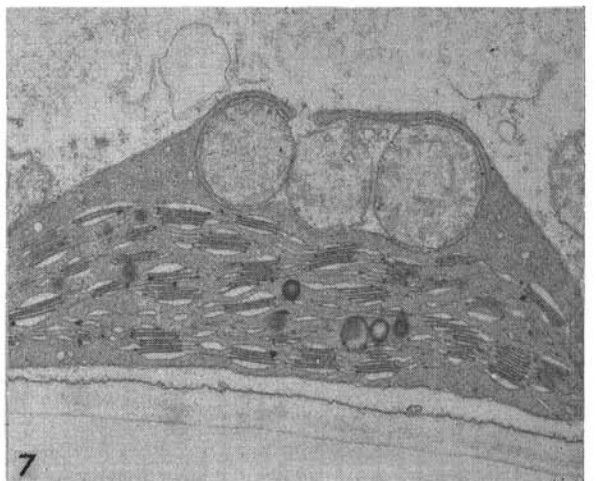
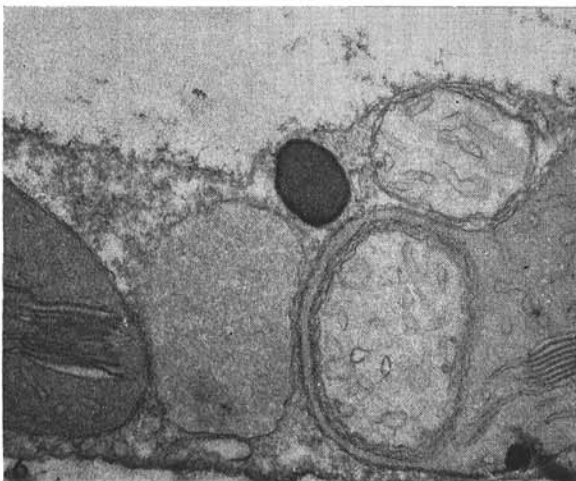
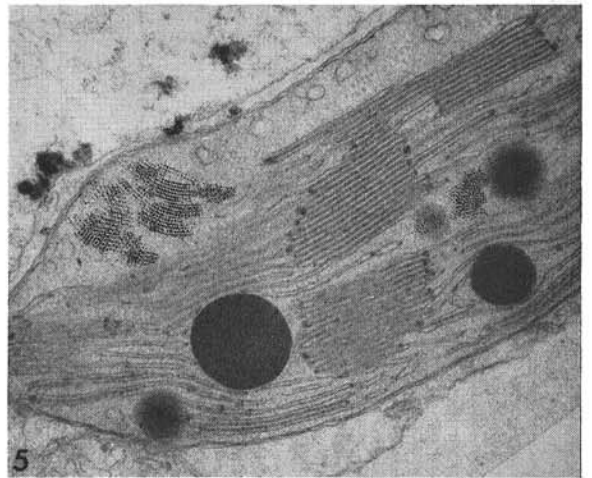
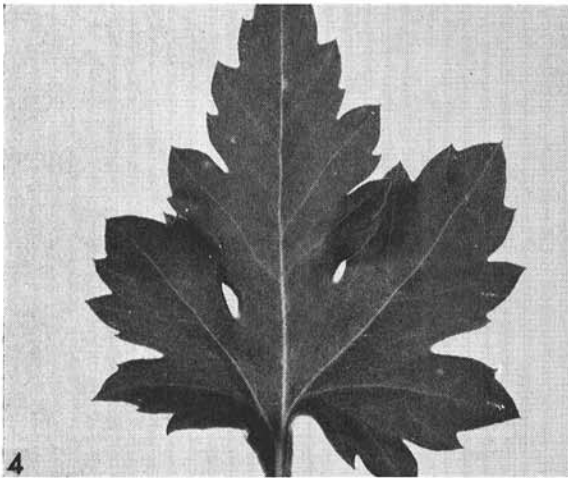
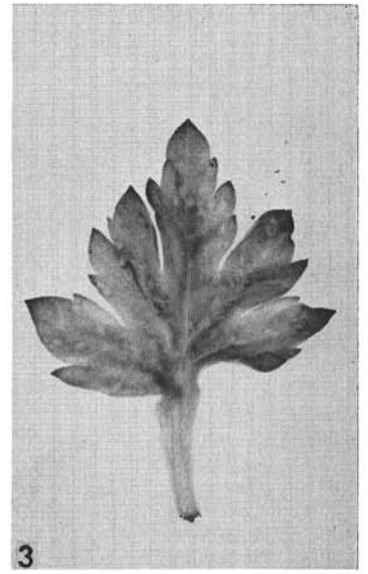
Chlorotic spots were present on the unexpanded leaves of CSV-free American Mistletoe grown in 1,200 ft-c of illumination at temperatures from 18-23 C in fall and winter (Fig. 1). Many of the spots disappeared from fully expanded leaves under the same light intensity. Spots on fully expanded leaves disappeared completely if plants were placed in a light intensity of 500 ft-c or less. English Mistletoe did not usually show spotting under these conditions. Occasionally, a few spots were observed in winter on immature leaves that were only partially expanded.

A high incidence of spotting occurred on the immature leaves of CSV-free American Mistletoe in the greenhouse with light intensities to 2,500 ft-c and temperatures from 23-32 C in summer (Fig. 2). Many of the spots persisted on mature leaves. A few spots were observed on the unexpanded leaves of English Mistletoe grown in the same light and temperature. Fully expanded leaves were free of spots.

No spotting was observed on the mature leaves of CSV-free American or English Mistletoe grown with 500 ft-c fluorescent illumination (18 hr light, 6 hr dark) at either 21 or 27 C.

Chlorotic spots up to 4 mm in diam were present on the leaves of CSV-infected American and English Mistletoe in summer in the greenhouse. Spots on leaves of diseased plants ranged from about 0.5-2 mm in diam in winter. Some leaves showed no spots under low light in winter which was often less than 500 ft-c.

Failure of supplemental iron to modify symptoms.—Supplemental iron chelate was added to rooted cuttings



of CSV-free and CSV-infected American Mistletoe, grown in perlite and watered 3 times a week with Hoagland No. 1 nutrient solution (5). Groups of diseased and healthy plants were watered with a complete solution minus iron. Two other groups were watered with the complete solution, plus 6.5 $\mu\text{g}/\text{ml}$ and 26.0 $\mu\text{g}/\text{ml}$ iron, supplied as Sequestrene NaFe containing 17% Fe-monosodium iron 3 + EDTA. Experiments were conducted in both summer and winter in the greenhouse, and the influence of iron nutrition on spotting was observed. Samples were taken from the leaves of treated and untreated plants for electron-microscopic observation after 40 days.

The addition of supplemental iron chelate did not modify the expression of chlorotic spotting on the leaves of diseased or healthy American Mistletoe at either concentration in summer or winter. Spots remained visible, although the entire leaf of the CSV-free and CSV-infected iron deficient plants showed severe chlorosis (Fig. 3).

Grafting tests.—To eliminate the possibility that the chlorotic spotting observed on CSV-free American Mistletoe was induced by an undetected virus, we grafted CSV-free English Mistletoe onto CSV-free American Mistletoe and grew the grafted plants in the greenhouse for more than 1 year. English Mistletoe scions were observed for increased spotting during the year.

The English selection was also grafted onto CSV-infected American Mistletoe to determine whether stunt symptoms observed on American Mistletoe could be induced on the English selection.

No increase in chlorotic spotting was observed on English Mistletoe scions grafted into CSV-free American Mistletoe (Fig. 4). Therefore, there is no evidence from these transmission tests that there is another graft-transmissible agent in the American understock. English scions grafted onto CSV-infected American Mistletoe understock showed a reaction similar to the spotting observed on the stunt-infected American understock.

Ultrastructure of green and chlorotic tissue.—Mistletoe plants grown in the greenhouse in late summer, fall, and winter were sampled for electron microscopy. Samples 1-2 mm² were cut from the green areas of CSV-infected Mistletoe leaves. The green tissue was fixed for 2.5-3 hr under vacuum in cold 0.04 M Na₂HPO₄-KH₂PO₄, pH 7.0, buffered 1.5% acrolein, and 2% glutaraldehyde. The tissues were rinsed 3 times in PO₄ buffer for 1.5 hr and postfixed in 0.04 M, PO₄ buffered 1% osmium tetroxide. The samples were dehydrated in ethanol and embedded in Epon 812. Sections were cut with a diamond knife, collected on 200-mesh grids coated with Formvar, and stained for 30 min

with saturated uranyl acetate, followed by lead citrate for 8 min. Chlorotic tissue samples were processed by the same method, except that 0.5% glutaraldehyde was mixed with 1.5% acrolein.

Cross sections of green tissue from leaves of CSV-infected Mistletoe grown in the greenhouse in light intensities up to 2,500 ft-c and temperatures from 23-32 C showed elongated palisade cells and mesophyll cells with large surrounding intercellular spaces. In yellow tissue samples, the intercellular space was reduced and the cells were small and closely packed. Chloroplasts were large and numerous under these conditions, and the plastids contained large starch granules.

No viruslike particles were observed in epidermal, palisade, or mesophyll cells from green or yellow leaf samples of CSV-infected American or English Mistletoe.

Electron-dense inclusions were observed in chloroplasts from chlorotic spots of both CSV-infected and CSV-free American Mistletoe. The inclusions were most numerous in tissue from chlorotic leaf spots of plants grown in the greenhouse during mid- to late summer. Inclusions were also observed in the adjacent green tissue in CSV-infected plants sampled in mid- to late summer. Fewer inclusions were observed in chloroplasts from chlorotic spotted leaf tissue of plants grown under supplemental light in winter. Inclusions were rarely found in chloroplasts of chlorotic spotted tissue from stunted leaves grown without supplemental illumination during the winter. Fewer plastids were observed in the leaves of plants grown in the low light intensities of fall and winter. No inclusions were observed in chloroplasts of palisade and mesophyll parenchyma cells from green tissue of CSV-free American Mistletoe sampled in late summer, fall, and winter. Inclusions were sometimes found in chloroplasts of cells of the vascular bundle in the CSV-free samples.

Inclusions often occurred at the end of the plastid, but they were also present between the stroma lamellae (Fig. 5). They are composed of electron-dense dots that may represent the core of the iron-protein complex. The dots in the inclusion are dense without uranyl acetate or lead staining. Individual dots within an inclusion are about 55-70 A in diam. The size of the inclusions varies greatly. Some are about 0.3 μ in the greatest dimension. Continuity of structure within the inclusion is often difficult to establish. We traced one inclusion through 15 serial sections. Although adjacent sections showed some related structure, the structural pattern was dissimilar four sections away.

Inclusions were not observed in chloroplasts of CSV-

Fig. 1-7. 1) Chlorotic spotting on a leaf of the American selection of chrysanthemum stunt virus (CSV)-free *Chrysanthemum morifolium* 'Mistletoe' grown under 1,200 ft-c of fluorescent light and 18-23 C in winter. 2) A leaf from CSV-free American Mistletoe grown in light intensities up to 2,500 ft-c and temperatures from 23-32 C in summer. 3) Chrysanthemum stunt virus-infected Mistletoe grown on an iron-deficient medium. Outlines of chlorotic spots remain visible on the leaf. 4) English Mistletoe grafted onto CSV-free American Mistletoe rootstock. No increase in spotting was observed on the leaves of the English scion. 5) Chloroplast with phytoferritin inclusions near the end of the plastid and between the stroma lamellae. Sample from chlorotic tissue of CSV-infected American Mistletoe. ($\times 43,200$) 6) A mitochondrion enclosed by a chloroplast membrane from an iron-deficient CSV-infected Mistletoe leaf. ($\times 41,200$) 7) Mitochondria in an invagination of the chloroplast membrane from the leaf of an iron-deficient American Mistletoe plant infected with CSV. ($\times 18,800$)

free and CSV-infected American Mistletoe plants grown with Hoagland's solution minus iron, regardless of light intensity. However, modifications in normal plastid structure occurred. Chloroplasts from spots on yellowed leaves contained grana with abnormal structure. Mitochondria present in some plastids of CSV-infected plants were engulfed by an invagination of the chloroplast membrane (Fig. 6). Occasionally, more than one mitochondrion was surrounded by the plastid membrane (Fig. 7).

No increase in electron-dense inclusions in chloroplasts of yellow tissue from CSV-infected and CSV-free plants grown with supplemental iron chelate was consistently observed when samples were compared with those from plants grown in soil.

DISCUSSION.—The American source of Mistletoe chrysanthemum may be used in a program of stunt-indexing only in those geographical areas where low light and temperature conditions reduce the amount of nonspecific chlorotic spotting on CSV-free plants.

Only a few chlorotic spots were present on the English source of CSV-free Mistletoe grown in the greenhouse in summer. The absence of additional spotting on CSV-free English scions grafted onto CSV-free American Mistletoe indicates that the plants differ genetically in their predisposition to spotting, or that the English Mistletoe is resistant to a spot-inducing agent present in the American Mistletoe. Flowering tests showed that the English and American selections are genetically different. Electron microscopy gives no evidence that CSV-free American Mistletoe is infected with another virus. Our results show that the English source of Mistletoe is a more reliable indicator than the American selection for stunt virus testing.

Electron-dense inclusions similar to those we describe were first reported from pea embryos and young bean seedlings grown in the light or dark (6). The authors suggested that the inclusions were a storage form of iron in immature tissues. Phytoferritin was also observed in mature plastids of *Phaseolus vulgaris* leaves of plants treated with an iron chelate (10). Inclusions were observed only when iron was supplied following a period of iron starvation.

Electron-dense inclusions were found in the chloroplasts of *Beta vulgaris* leaves infected with beet yellows virus. Although the authors at first thought the inclusions were composed of virus particles (2), the association between the inclusions and phytoferritin was later recognized (3). Studies of similar inclusions from the cambial zone of willow did not confirm the earlier report that the chloroplast particles were virus (8). The

particles were insensitive to ribonuclease treatment (9). Ferritinlike inclusions were also observed in cells of pokeweed infected with pokeweed mosaic virus (7). Phytoferritin was also described from three legume species where the accumulation of iron was correlated with plants growing under "suboptimal" conditions (1).

Our report is the first to correlate phytoferritin accumulation with high light and temperature conditions in healthy and diseased plants. Inclusions were most numerous in chloroplasts from large chlorotic spots of CSV-infected leaves of plants grown in the greenhouse in late summer. Inclusions were fewer in the chloroplasts from chlorotic spots on leaves of CSV-infected plants grown under low light and temperature in the winter.

We suggest that the effect of light on phytoferritin accumulation in Mistletoe chrysanthemum is related to the conversion of green tissue to chlorotic tissue. It seems likely that the formation of phytoferritin prevents a toxic accumulation of iron that could result when chlorotic tissue is formed. The presence of inclusions in chloroplasts from green tissue of stunt-infected leaves provides evidence that a virus-induced accumulation of phytoferritin occurs independently of tissue chlorosis.

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