Ultrastructural Localization of Polyphenoloxidase Activity in Leaves of Healthy and Diseased Waterhyacinth

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ABSTRACT

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The ultrastructural localization of polyphenoloxidase (PPO) was studied in healthy and *Acremonium zonatum*-infected waterhyacinth leaves. The deposition of an electron-dense reaction product in leaf tissue incubated in D-3, 4-dihydroxyphenylalanine (DOPA) was considered to be a positive test for PPO activity. In healthy leaves, reaction product was observed in the thylakoid spaces and fret channels of chloroplasts in only three types of cells; vascular parenchyma, bundle sheath, and phenol-storing cells. Sections from diseased leaves had reaction product in all cells containing chloroplasts. This observation was consistent throughout the leaf whether in areas immediately surrounding a lesion or in green tissue up to 5 cm distant. PPO activity was not observed in any organelle other than chloroplasts in either healthy or diseased leaves. The increase of this enzyme activity after infection is highly suggestive of an active role for PPO during pathogenesis.

Additional key words: biological control, *Eichhornia crassipes,* disease resistance.

The importance of phenol-oxidizing enzymes in normal plant metabolism has been well documented (3,7,19). In addition, much evidence suggests their involvement in disease resistance mechanisms. Cook and Taubenhaus (4) were perhaps the first to suggest that tannin fungitoxicity was due to the action of an oxidase which formed a germicidal fluid. Since their report in 1911, the results of numerous studies have correlated increased activites of phenol-oxidizing enzymes with disease resistance (2,8,24,25). Over the years, these correlations have led to the supposition that phenolic substances and their oxidizing enzymes contribute to the resistance of plants against pathogens. Although there is considerable circumstantial evidence for this, there is still very little hard evidence supporting an active role of phenols in disease resistance.

Polyphenoloxidase (PPO) is a key enzyme associated with the oxidation of plant phenolics. PPO activity long has been thought to reside within the chloroplasts of plant cells (1), but until recently cytochemical localization had not been demonstrated. Using techniques developed by Novikoff et al (20) and Okun et al (21) for the localization of tyrosinase in animal tissue, Czaninski and Catesson (5,6) localized PPO in plant cell chloroplasts. Since 1972, several investigators (9–11,18,22,23) have shown that PPO activity is localized within the thylakoids of plastids in several plant species.

PPO activity usually does not appear to be restricted to any particular cell type or location. However, Mueller and Beckman (18) recently demonstrated a restricted localization of PPO. They showed that PPO in cotton root endodermis was limited to the plastids, whereas in hypocotyl tissue it was present in cells of the epidermis, cortex, endodermis, and scattered cells of the stele.

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To our knowledge, all studies to date on the ultrastructural localization of PPO in plants have been conducted on healthy tissue. Knowledge of any changes in the location of PPO which might occur upon infection might help resolve some of the controversy surrounding phenol-oxidizing enzymes and disease resistance. The present study was therefore initiated to compare the localization of PPO in healthy and leaf-spot diseased waterhyacinth leaves. A preliminary report of these findings has appeared (15).

MATERIALS AND METHODS

Plant material and inoculation. Waterhyacinths (*Eichhornia crassipes* [Mart.] Solms) were collected from natural infestations in south Florida and maintained under greenhouse conditions in Gainesville. Three to five leaves on each of 10 plants selected at random were inoculated with *Acremonium zonatum* (Sawada) Gams as previously described (13,14). The inoculated plants were then placed in 37.85-L (10-gal) glass aquaria half-filled with tap water and fitted with plastic covers to maintain relative humidity near saturation. Two weeks postinoculation, leaf segments from areas immediately surrounding the lesions and from areas 3–5 cm distant from any lesion were removed and prepared for electron microscopy as described below. Healthy control plants were maintained separately under similar conditions and after 2 wk, segments were removed from areas corresponding approximately to the same locations as those from diseased leaves and similarly prepared for electron microscopy.

Enzyme localization and electron microscopy. The procedure for localization of PPO follows closely that described by Czaninski and Catesson (6). The leaf segments were placed in a buffered (0.2 M sodium cacodylate, pH 7.2) solution of 5.0% glutaraldehyde, cut

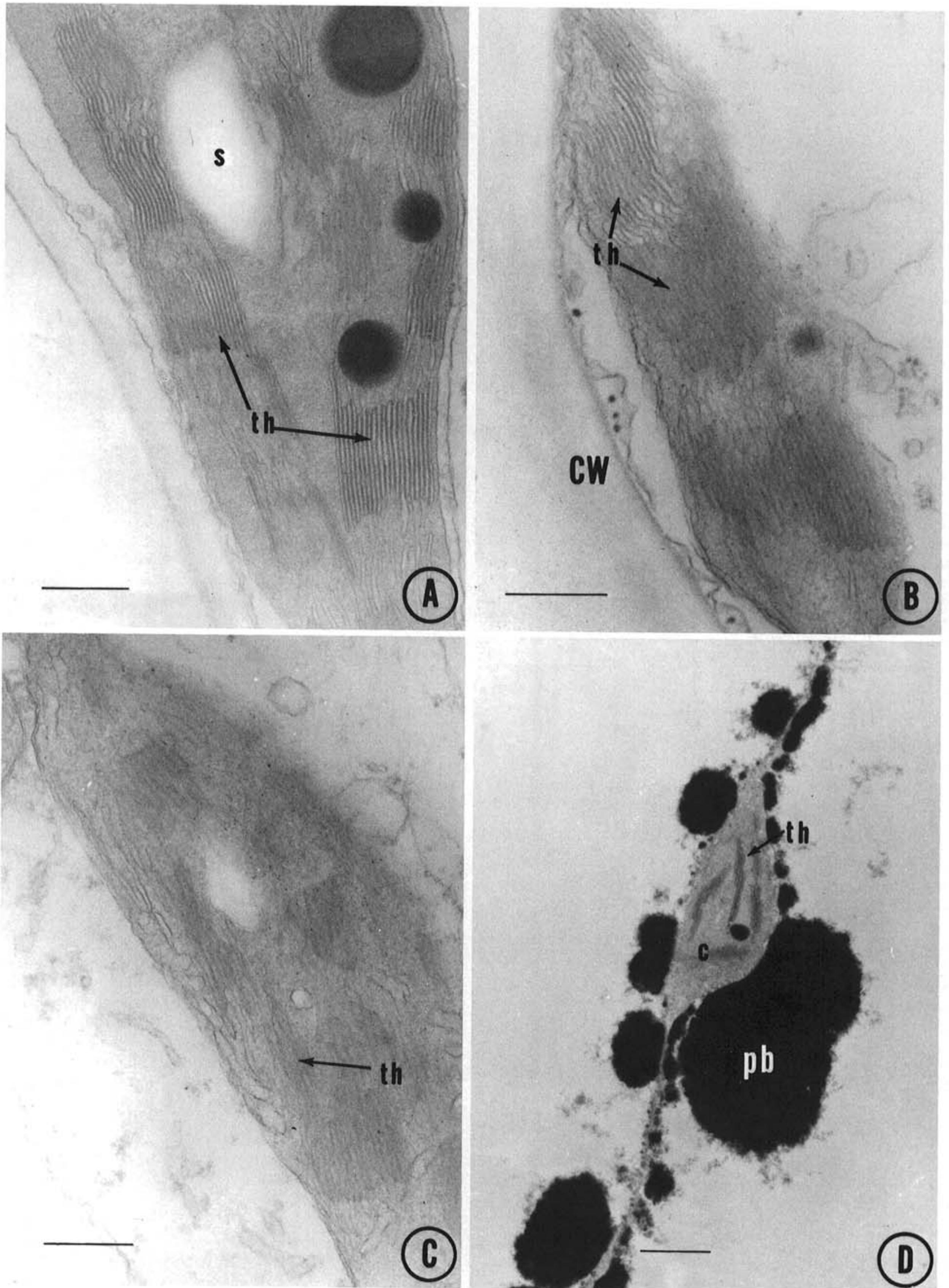

Fig. 1. Healthy waterhyacinth leaf tissue incubated in buffer without DOPA and without poststaining. The thylakoid spaces and fret channels are free of any naturally occurring electron-dense DOPA reaction product. s = starch, th = thylakoids, cw = cell wall, c = chloroplast, pb = phenol body. Scale bars = 0.5μm. **A,** Palisade cell chloroplast. **B,** Bundle sheath cell chloroplast. **C,** Vascular parenchyma cell chloroplast. **D,** Phenol-storing cell chloroplast.

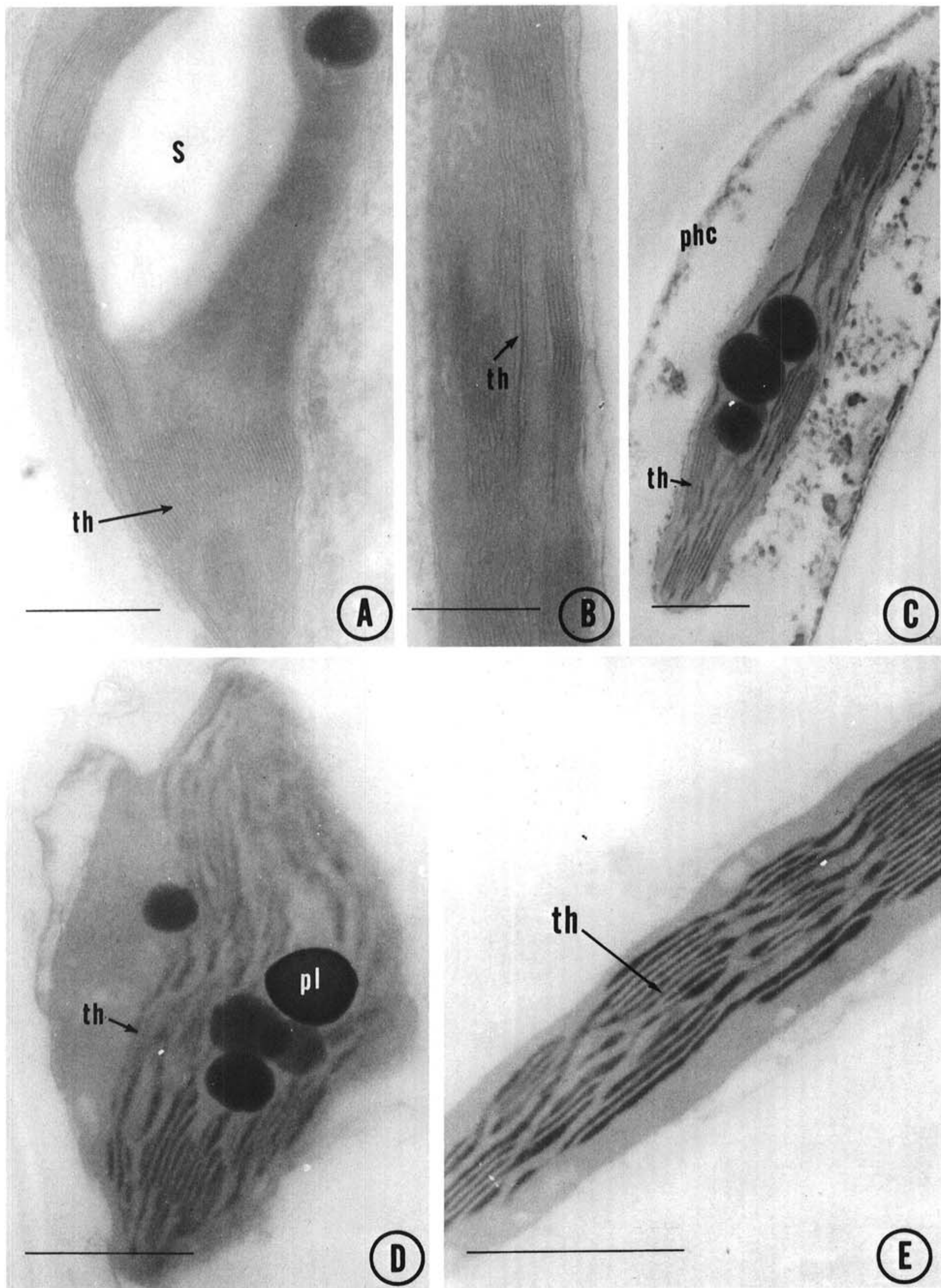

Fig. 2. Healthy waterhyacinth leaf tissue incubated with DOPA and without poststaining showing the restricted localization of PPO within the chloroplasts of certain cell types. Chloroplasts in **A**, palisade, and **B**, spongy mesophyll cells are PPO negative. Electron-dense deposition of the DOPA reaction product occurs only within chloroplasts of **C**, phenol-storing cells; **D**, vascular parenchyma cells; and **E**, bundle sheath cells. th = thylakoids, s = starch, phc = phenol-storing cells, pl = plastoglobuli. Scale bars = 0.5 μm.

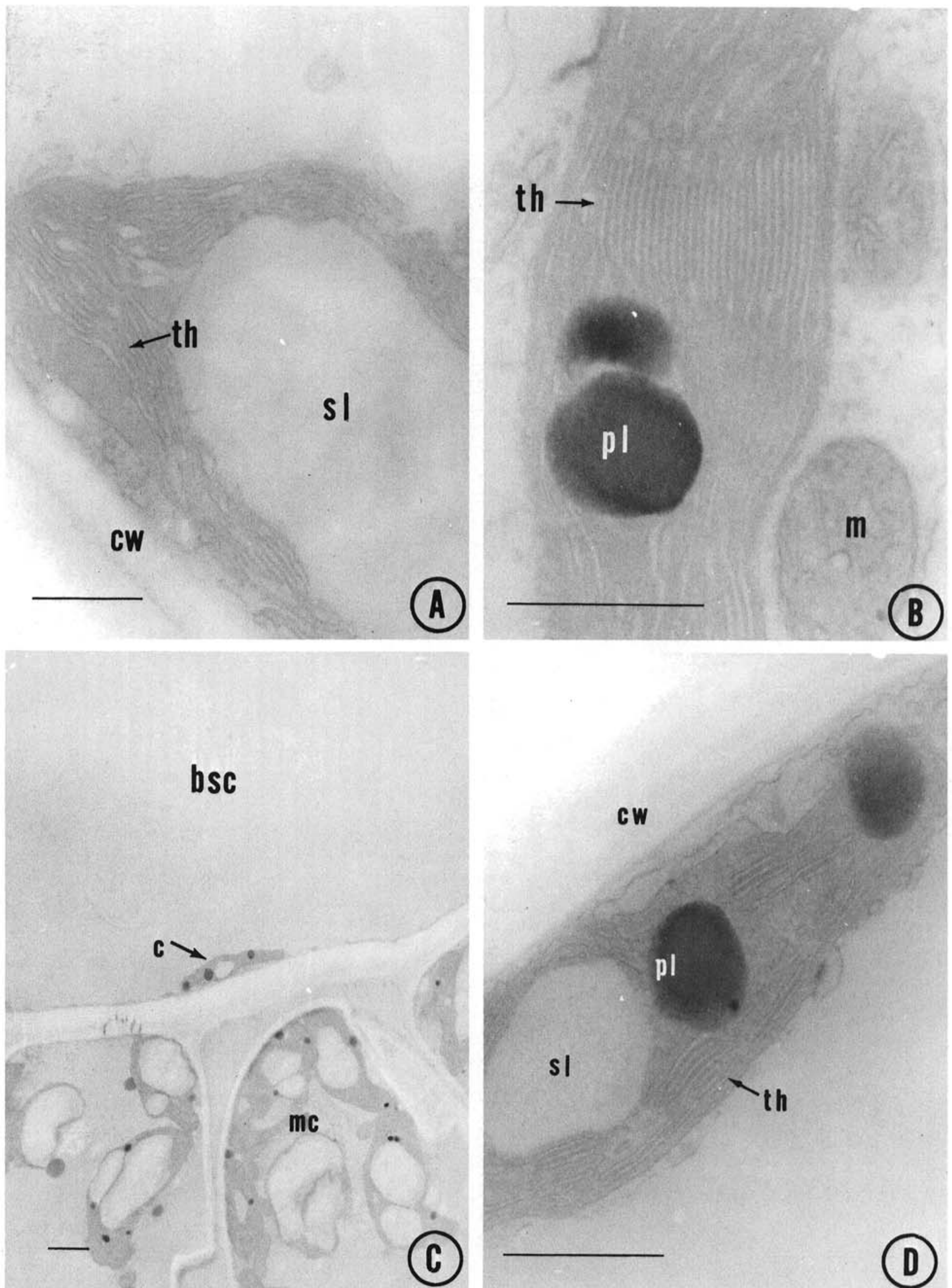

Fig. 3. Healthy waterhyacinth leaf tissue boiled for 10 min prior to incubation in DOPA. Chloroplasts in all cell types are negative for PPO activity. All figures are without poststaining. th = thylakoids, cw = cell wall, sl = starch lacuna, pl = plastoglobuli, m = mitochondrion, bsc = bundle sheath cell, mc = mesophyll cell. Scale bars = 0.5 μm in A, B, and D; 1.0 μm in C. **A,** Spongy mesophyll cell chloroplast. **B,** Vascular parenchyma cell chloroplast. **C,** Bundle sheath cell chloroplast (arrow) and adjacent mesophyll cells. **D,** Enlargement of bundle sheath cell chloroplast from Fig. 3C.

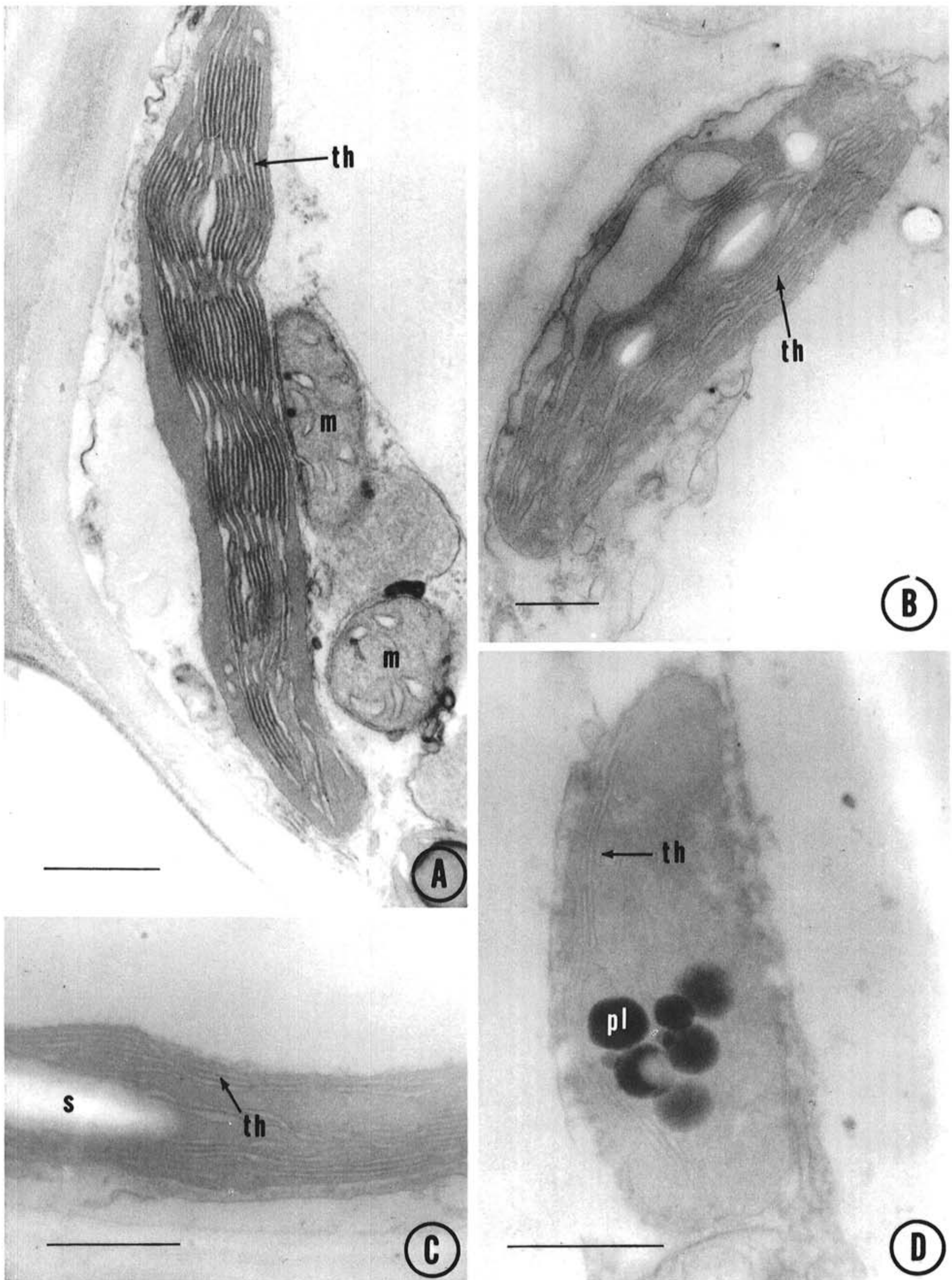

Fig. 4. Healthy waterhyacinth leaf tissue incubated in DIECA prior to incubation in DOPA. There is no detectable DOPA reaction product in any of the thylakoids, indicating an inhibition of PPO activity. Fig. A was poststained, B, C, and D were not. Scale bars = 0.5 μm. th = thylakoids, m = mitochondrion, s = starch, pl = plastoglobuli. **A,** Palisade cell chloroplast. **B,** Vascular parenchyma cell chloroplast. **C,** Bundle sheath cell chloroplast. **D,** Phenol-storing cell chloroplast.

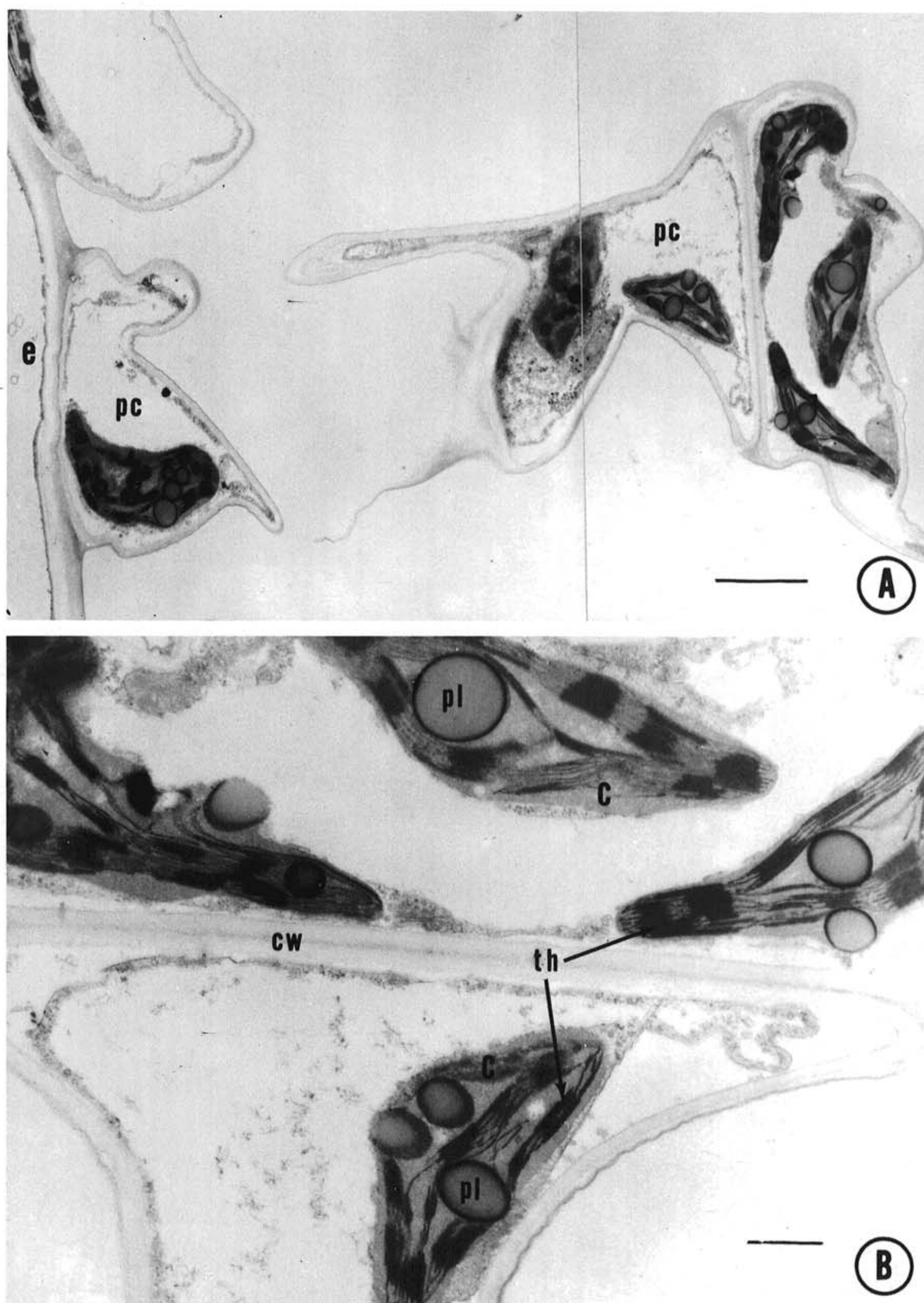

Fig. 5. Localization of PPO activity in chloroplasts of palisade cells of *Acremonium zonatum*-infected waterhyacinth leaves. Thylakoids contain electron-dense reaction product, positive for PPO activity. Both figures have been poststained. pc = palisade cell, e = epidermis, c = chloroplast, cw = cell wall, pl = plastoglobuli, th = thylakoids. **A,** Scale bar = 2.0μm. **B,** Englargement of chloroplasts in A. Scale bar = 0.5 μm.

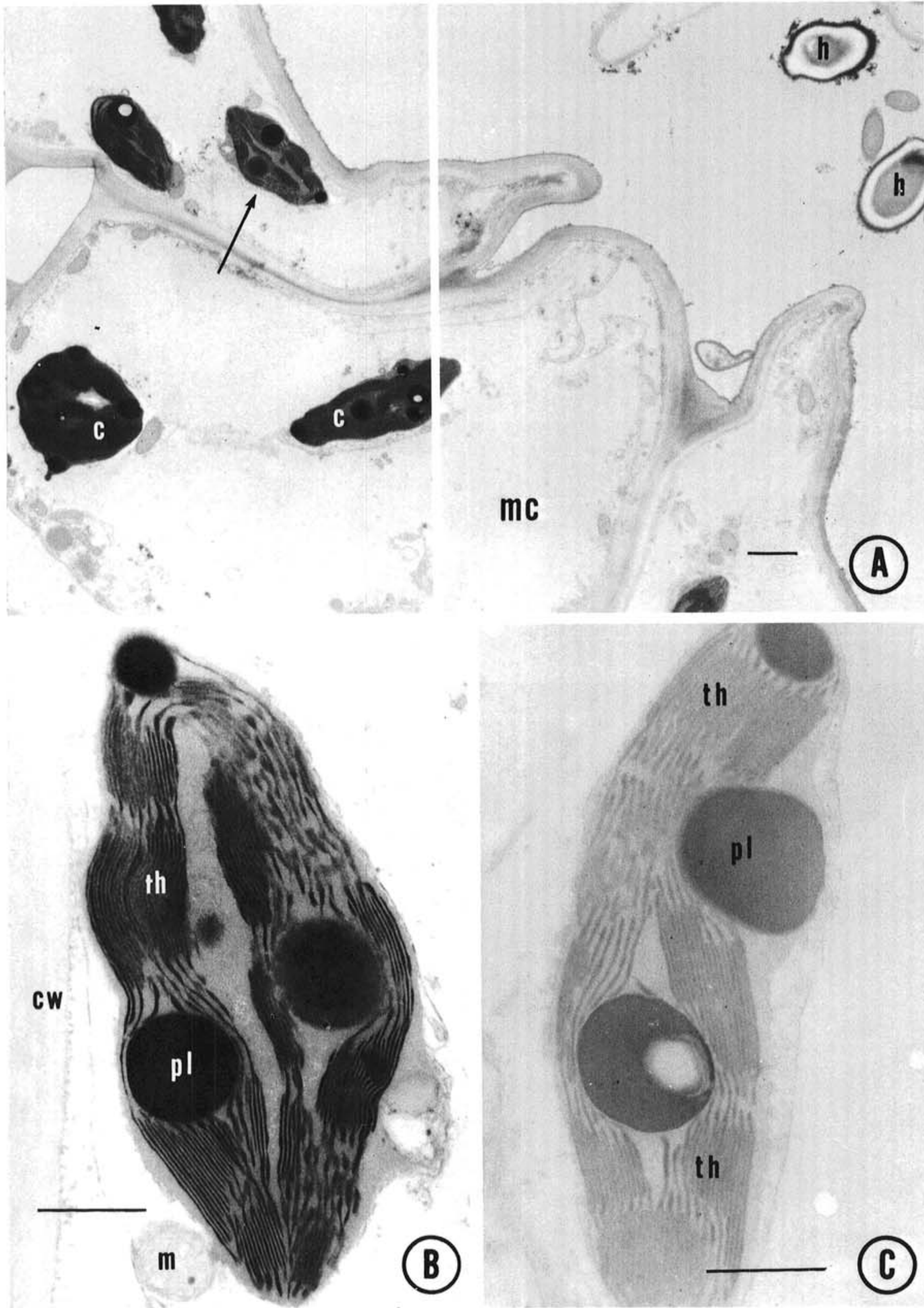

Fig. 6. Localization of PPO activity in chloroplasts of spongy mesophyll cells of *Acremonium zonatum*-infected waterhyacinth leaves. c = chloroplast, mc = mesophyll cell, h = fungal hyphae, cw = cell wall, pl = plastoglobuli, th = thylakoids, m = mitochondrion. **A,** Chloroplasts contain a considerable amount of electron-dense reaction product, positive for PPO activity. Arrow points to chloroplasts enlarged in Fig. 6B. Scale bar = 1.0μm. **B,** Enlargement of chloroplast from infected mesophyll cell showing an intense deposition of DOPA reaction product within the thylakoids. Note the absence of reaction product in the mitochondrion. Scale bar = 0.5μm. Both A and B have been poststained. **C,** Mesophyll cell chloroplast from infected leaf tissue without poststaining. Positive PPO reaction can be detected, but clarity and intensity is less than with poststaining. Scale bar = 0.5μm.

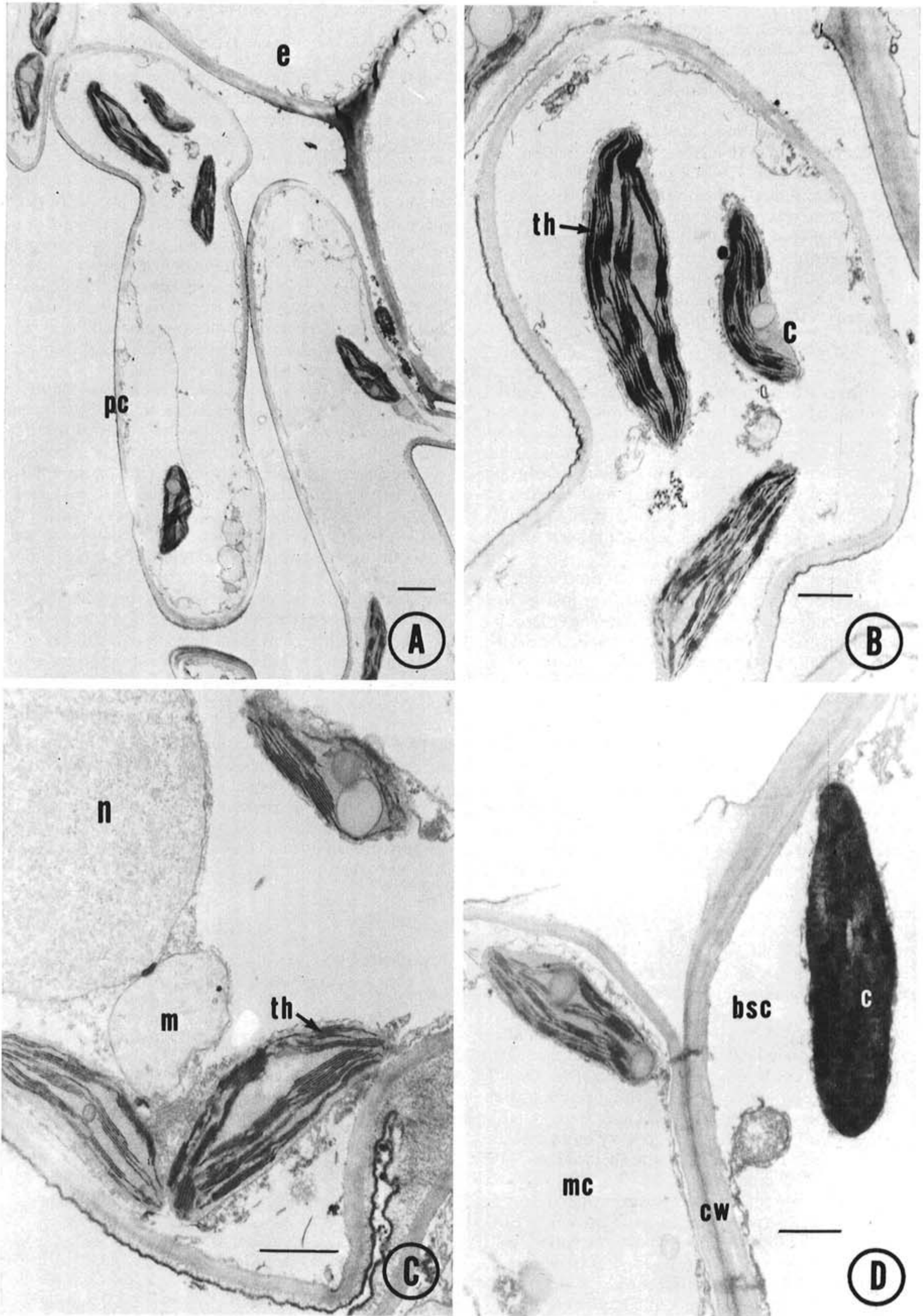

Fig. 7. Localization of PPO activity of *Acremonium zonatum*-infected leaves in cells several centimeters away from a visible lesion. pc = palisade cell, e = epidermis, c = chloroplast, th = thylakoids, n = nucleus, m = mitochondrion, mc = mesophyll cell, cw = cell wall, bsc = bundle sheath cell. All figures have been poststained. **A,** Palisade cells. Scale bar = 1.0 μm. **B,** Enlargement of upper portion of palisade cell in A showing electron-dense reaction product within the thylakoids. Scale bar = 1.0 μm. **C,** Spongy mesophyll cell. Chloroplasts are PPO positive, the mitochondria and nucleus are negative. Scale bar = 0.5 μm. **D,** Bundle sheath cell chloroplast with extremely dense reaction product. Scale bar = 0.5 μm.

into three to five pieces, and separated into three groups. Each group then was treated by one of the following methods: (i) boiled in distilled water for 10 min; (ii) incubated in 0.02 M sodium diethyldithiocarbamate (DIECA) for 20 min at 22 C and then washed five times in 0.2 M sodium cacodylate buffer, pH 7.2; and (iii) no treatment. After receiving the treatments, each group was preincubated in a DOPA solution (50 mg D-3,4-dihydroxyphenylalanine in 10 ml of 0.067 M phosphate buffer, pH 7.0) at 4 C overnight. After preincubation, the segments were incubated in fresh DOPA for 1 hr (fresh solution added after 30 min) at 37 C, followed by five washings in 0.5 M sucrose. After incubation, the leaf segments were postfixed in 2% OsO_4 for 2 hr at 22 C, passed through a graded ethanol series, and embedded in a low-viscosity epoxy resin (26). The resin was polymerized overnight at 60 C, and thin sections were cut with a diamond knife in a Sorvall MT-2 ultramicrotome. The sections were placed on single-hole, Formvar-coated copper grids, some were counterstained with 0.5% uranyl acetate and 1.0% lead citrate, and all sections were examined with a Hitachi HU-11E electron microscope.

RESULTS

The principle of the reaction for the cytochemical localization of PPO involves obtaining an insoluble, electron-dense reaction product (dopaquinone) from the synthetic substrate at the point where enzyme oxidation has occurred (6). The depostion results from a complexing of the DOPA with the osmium tetroxide during postfixation. Counterstaining the sections with lead citrate and uranyl acetate did not interfere with the reaction product, but it enhanced the intensity and clarity of the reaction and surrounding material.

Examination of healthy leaf tissue incubated in buffer without DOPA showed that in all cells examined, chloroplasts had distinctly clear thylakoid spaces free of electron-dense deposits (Fig. 1A–D), which suggested a lack of substrate and/or enzyme activity in those cells. On the other hand, sections incubated in buffer with DOPA showed the presence of reaction product within the thylakoids of chloroplasts of three specific cell types; phenol-storing cells, (Fig. 2C–E). In each case, the thylakoid spaces and fret channels were the only areas positive for PPO activity. The reaction product appeared to have diffused little, if any, from the thylakoids and no other organelles were observed to be positively stained. Chloroplasts in the remaining cell types, the palisade and spongy mesophyll cells, had no reaction product within the thylakoids, which indicated an absence of any detectable PPO activity (Fig. 2A–B). These observations were consistent throughout in all leaf sections examined.

Leaf segments that had been boiled prior to incubation in DOPA did not show a positive PPO reaction within the chloroplasts, suggesting heat inactivation of the enzyme (Fig. 3A–D). The thylakoids were distorted after the tissue was boiled (Fig. 3A) and starch granules became swollen and formed large lacunae (Fig. 3A and 3C).

Healthy leaf segments incubated in DIECA (a specific inhibitor of PPO) prior to incubation in DOPA, showed reaction product in none of the chloroplasts in all cell types examined (Fig. 4A–D). After poststaining, thylakoid spaces and fret channels contrasted sharply with the stroma and only the partitions were notably electron-dense (Fig. 4A). Thus, the electron density of lead citrate used in poststaining shoud not be confused with the electron-dense product of the positive PPO reaction.

In contrast to the chloroplasts in healthy waterhyacinth leaves, those in all cell types in diseased leaves were consistently PPO positive, including palisade (Fig. 5A–B) and spongy mesophyll cells (Fig. 6A–C). In essence, there was an increase in PPO activity in the remaining cell types of the leaves upon infection.

This change in palisade and spongy mesophyll cell chloroplasts from PPO negative to PPO positive was quite pronounced in sections from areas immediately surrounding the lesions. Sections from green tissue several centimeters away from any lesion showed that even chloroplasts in cells up to 5 cm away from the center of infection were PPO positive, including palisade cells (Fig. 7A–B) and spongy mesophyll cells (Fig. 7C–D). In cells normally positive for PPO activity, such as bundle sheath cells, there appeared to be increased activity, as those chloroplasts became very electron-dense after infection (Fig. 7D). As in healthy tissue, chloroplasts were the only PPO-positive organelles seen in diseased tissue.

DISCUSSION

The present study describes for the first time a restricted localization of PPO in chloroplasts of certain cells of healthy leaves and a subsequent increase in all other cells after infection. The absence of any detectable reaction product in palisade and spongy mesophyll cell chloroplasts in healthy plants suggests several possibilities: (i) the enzyme is not present in these particular chloroplasts but is synthesized de novo during the infection process; (ii) the enzyme is present in healthy cells but in concentrations too low to be detected by this procedure; (iii) the enzyme is present in adequate concentrations but in an inactive form; or (iv) the enzyme is present in sufficient concentrations and in its active form but the substrate does not reach it, perhaps because of differences in membrane permeability. The present data, unfortunuately, do not distinguish among the above possibilities for the restricted localization.

PPO activity in vitro increases over 300% in infected waterhyacinth leaves compared to that in healthy leaves (13,15). The present data suggest that at least part of this increase comes from PPO activity in additional cells upon infection.

The exact role of PPO in plants remains speculative. One role may be the oxidization of monophenols to polyphenols (16) and subsequently to quinones or semiquinines which are antimicrobial (8). Although this implies a rather simplistic mechanism, in actuality such a role is probably much more complex.

Increased PPO activites and the subsequent increased phenol levels may be stimulated by several factors other than pathogenesis; eg, adverse environmental conditions and wounding (12). However, their increase in infected tissue should not be discounted. In waterhyacinth, increased PPO activity, increased concentrations of phenols, and an increased concentration of subepidermal phenol cells have been correlated with a limited leaf spot disease caused by *A. zonatum* (13–15).

We concur with Mueller and Beckman (17,18) and Wardrop and Cronshaw (27) that phenol synthesis most probably occurs within the thylakoids of plastids and is correlated with the presence of PPO. In addition, we suspect that the restricted localization of PPO activity in healthy waterhyacinth leaves and the unrestricted occurrences in infected leaves suggest an active and specific role for this enzyme during pathogenesis.

LITERATURE CITED

1. ARNON, D. 1949. Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol. 24:1-15.
2. BELL, A. A. 1974. Biochemical basis of resistance of plants to pathogens. Pages 403–462 in: F. G. Maxwell and F. A. Harris, eds. Proc. of the Summer Institute of Biological Control of Plants, Insects and Diseases. Mississippi University Press, Jackson, MS.
3. CONN, E. E. 1964. Enzymology of phenolic biosynthesis. Pages 399–435 in J. B. Harborne, ed. Biochemistry of Phenolic Compounds. Academic Press, New York. 618 pp.
4. COOK, M T., and J. J. TAUBENHAUS. 1911. The relation of parasitic fungi to contents of the cells of host plants. I. The toxicity of tannin. Del. Agric. Exp. Stn. Bull. 91. 77 pp.
5. CZANINSKI, Y., and A. M. CATESSON. 1972. Localisation ultra structurale d'activités polyphénoloxydosiques dan les chloroplastes de *Nicotiana glutinosa*. J. Microscopie 15:409-414.
6. CZANINSKI, Y., and A. M. CATESSON. 1974. Polyphenoloxidases (Plants). Pages 66–78 in: M. A. Hayat, ed. Electron Microscopy of Enzymes. Vol. 2. Van Nostrand Reinhold Co., New York. 158 pp.
7. DAWSON, C. R., and R. J. MAGEE. 1955. Plant tyrosinase (Polyphenoloxidase.) Meth. Enzymol. 2:817-831.
8. FARKAS, G. L., and Z. KIRÁLY. 1962. Role of phenolic compounds in the physiology of plant disease and disease resistance. Phytopathol. Z. 44:105-150.
9. HENRY, E. W. 1974. Ultrastructural localization of polyphenoloxidase activity in chloroplasts of *Nicotiana tabacum*. (Abstr.) Page 63 in: Plant Physiol. Annu. Suppl.

10. HENRY, E. W. 1975. Polyphenoloxidase activity in thylakoids and membrane-bound granular components of *Nicotiana tabacum* chloroplasts. J. Microscopie 22:109-116.
11. HENRY, E. W. 1976. Ultrastructure localization of polyphenoloxidase in chloroplasts of *Brassica napus* cv. Zephyr. Z. Pflanzenphysiol. 78:446-452.
12. KOSUGE, T. 1969. The role of phenolics in host response to infection. Annu. Rev. Phytopathol. 7:195-222.
13. MARTYN, R. D. 1977. Disease resistance mechanisms in waterhyacinths and their significance in biocontrol programs with phytopathogens. Ph.D. Dissertation, University of Florida, Gainesville. 204 pp.
14. MARTYN, R. D., and T. E. FREEMAN. 1978. Evaluation of *Acremonium zonatum* as a potential biocontrol agent of waterhyacinth. Plant Dis. Rep. 62:604-608.
15. MARTYN, R. D., D. A. SAMUELSON, and T. E. FREEMAN. 1976. Phenolic compounds as a possible means of disease resistance in waterhyacinths and their significance in biocontrol programs with phytopathogens. (Abstr.) Proc. Am. Phytopathol. Soc. 3:340.
16. MASON, H. S. 1955. Comparative biochemistry of the phenolase complex. Adv. Enzymol. 16:105-184.
17. MUELLER, W. C., and C. H. BECKMAN. 1976. Ultrastructure and development of phenolic-storing cells in cotton roots. Can. J. Bot. 54:2074-2082.
18. MUELLER, W. C., and C. H. BECKMAN. 1978. Ultrastructural localization of polyphenoloxidase and peroxidase in roots and hypocotyls of cotton seedlings. Can. J. Bot. 56:1579-1587.
19. NEISH, A. C. 1960. Biosynthetic pathways of aromatic compounds. Ann. Rev. Plant Physiol. 11:55-80.
20. NOVIKOFF, A. B., A. ALBALA, and L. BIEMPICA. 1968. Ultrastructural and cytochemical observations on B-16 and Harding-Passey mouse melanomas. The origin of premelanosomes and compound melanosomes. J. Histochem. Cytochem. 16:299-319.
21. OKUN, M. R., L. M. EDELSTEIN, N. OR, G. HAMADA, B. DONNELLAN, and W. F. LEVER. 1970. Histochemical differentiation of peroxidase-mediated from tyrosinase-mediated melanin formation in mammalian tissue. Histochemie 23:295-309.
22. PARISH, R. W. 1972. The intracellular location of phenoloxidases, peroxidases and phosphatases in stems and leaves of spinach beet (*Beta vulgaris* L. spp. *vulgaris*). Eur. J. Biochem. 31:446-455.
23. PARISH, R. W. 1972. The intracellular location of phenoloxidase and peroxidase in stems of spinach beet (*Beta vulgaris* L. spp. *vulgaris*). Z. Pflanzenphysiol. 66:176-186.
24. PRIDHAM, J. B. 1960. Phenolics in Plants in Health and Disease. Pergamon Press, New York. 131 pp.
25. RUBIN, B. A., and E. V. ARTISIKHOVSKAYA. 1964. Biochemistry of the pathological darkening of plant tissues. Annu. Rev. Phytopathol. 2:157-178.
26. SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
27. WARDROP, A. B., and J. CRONSHAW. 1962. Formation of phenolic substances in the ray parenchyma of angiosperms. Nature 193:90-92.