Lysosomal Distribution and Acid Phosphatase Activity in White Clover Infected with Clover Yellow Mosaic Virus

J. C. Tu

Biological Sciences Electron Microscope Laboratory, University of Alberta, Edmonton, Alberta, Canada, T6G 2E1

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ABSTRACT

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More lysosomes were observed in thin sections of leaves and root nodules of white clover *(Trifolium repens)* infected with clover yellow mosaic virus (CYMV) than in sections from virus-free tissues. In the leaf, lysosomes were found in vacuoles or in the cytoplasm near the vacuole. In the root nodule, they were generally seen near the plasma membrane and in the vicinity of virus aggregates. Biochemical assays and histochemical localization tests showed more acid phosphatase (a lysosomal enzyme) activity in the sap of leaves and root nodules of CYMV-infected than in the healthy plants. The specific activity of acid phosphatase in leaves and root nodules of the CYMV-infected plants increased approximately 100% over the healthy controls. The increased lysosomes and acid phosphatase activity in relation to early senescence of the leaves and early degeneration of the root nodules in the virus pathogenesis are discussed.

Additional key words: lysosome, acid phosphatase, clover yellow mosaic virus, *Trifolium repens,* root nodule.

A lysosome is bound by a unit membrane, contains many hydrolytic enzymes, and is formed by the Golgi apparatus (4). The constituent enzymes are known to be synthesized in the rough endoplasmic reticulum and are transported to the Golgi apparatus (4, 22).

Although lysosomes contain many hydrolytic enzymes, one of the enzymes which is unique to the lysosome is acid phosphatase (4). The lysosome is responsible for the cellular digestion of materials contained in the pinocytotic and phagocytotic vesicles, and is also responsible for the autophagic activities in the senescent tissues.

Even though the importance of lysosomes in the animal pathological processes has been extensively studied and documented (4, 22), the role of lysosomes in the plant pathological processes has not yet been well explored (22). There are clear indications that lysosomes and lysosomal enzymes exist in higher plants (1, 2, 3, 6, 8, 9, 10, 11, 15, 16). For example, higher lysosomal enzymatic activity has been noted in the mechanically injured (15, 23) or pollutant-injured (5) cells as well as in senescent cells (1, 2, 3).

Virus-infected plants often show some degree of early senescence; particularly those cells in the yellowing, mottling, and mosaic areas of the leaves. Furthermore, virus-infected legumes are known to produce smaller and less efficient root nodules (20, 21). The smaller nodules may result from the early degeneration of the meristematic cells. Degeneration of root nodules is known to start in the center of the root nodule, proceed outwards to meristematic cells, and finally stop the nodule growth (14). The phenomenon of early senescence in a virus-infected plant points to the possibility that the lysosome is involved in virus pathogenesis.

It is, therefore, desirable to investigate the lysosomes and their enzymatic activity in the healthy and virus-infected plant. This paper is to report the findings obtained from the ultrastructural observation of lysosomes, and from biochemical assay of acid phosphatase, an enzyme in leaves and root nodules of healthy and clover yellow mosaic virus (CYMV)-infected white clover (*Trifolium repens* L.), found uniquely in the lysosomes.

MATERIALS AND METHODS

Seeds of white clover (*Trifolium repens* L.) were germinated on a wet filter paper in a petri plate for 3 days. The seedlings were transplanted to 15-cm diameter clay pots, one plant per pot. Immediately after transplanting, the soil was inoculated with rhizobia (*Rhizobium trifolium* H.K.C.) at the concentration of 10^4 rhizobia/cm^3 soil. These plants were maintained in a 21 C controlled environment, and were programmed at 14 hours dark and 10 hours light, with an intensity of 26,900 lux at the bench level.

Two weeks after transplanting, the potted plants were divided into two groups. One group was mechanically inoculated with CYMV, and the other was kept as a healthy control. The method of inoculation was described previously (20). Samples of leaves and root nodules for the experiments were taken 8 weeks after CYMV inoculation. In sampling leaves, the third fully expanded young leaves were used.

Ultrastructural investigation.—Both leaves and root nodules of healthy and CYMV-infected plants were studied. Leaves were cut into 1 mm^2 pieces. In the case of infected leaves, the samples were cut off from yellow mosaic patches. Root nodules of 1 × 1.5 - 2.0 mm in size were halved longitudinally. All samples were fixed separately in 4% glutaraldehyde in phosphate buffer, pH 7.0 for 1 hour, post-fixed in 2% OsO_4 in the same buffer

for 2 hours, and processed for routine dehydration and embedding according to the method previously described (18). Sections were cut, picked up with a Formvar-coated grid, stained with 2% aqueous uranyl acetate and 0.2% lead citrate, and examined in a Philips EM-300 electron microscope at 80 kv.

Biochemical assay.—Acid phosphatase activity was assayed by using *p*-nitrophenyl phosphate (purchased from Sigma Chemical Co., St. Louis, Missouri) as substrate. The basis for the test is that when acid phosphatase acts on *p*-nitrophenyl phosphate in an acid medium, phosphate ions and *p*-nitrophenol are produced. *p*-Nitrophenol is colorless in an acid medium, but gives yellow color in an alkaline solution with maximum absorption at 400-420 nm wave length. Thus, the yield of *p*-nitrophenol is directly proportional to the acid phosphatase activity.

Preparation of crude sap.—Leaf samples were taken from healthy and CYMV-diseased plants. Only third fully expanded young leaves were sampled. At this stage, the diseased leaves showed pronounced mosaic symptoms. Root nodules from healthy and diseased plants were also excised from roots and were placed in separate petri dishes moistened with wet filter papers. Thus, a total of four samples of crude sap were available for each assay. Each sample of crude sap was prepared by grinding 1 g of tissue with 2 ml of water with mortar and pestle precooled at 4 C, diluting 10-fold with water, and then straining through two layers of cheesecloth. The crude sap was assayed for total protein by the method described by Lowry et al. (12).

Enzymatic assay.—Each sample of crude sap was assayed in a series of eight test tubes. Seven of these tubes contained 1 ml of incubation medium which was made up of 0.5 ml of 12 mM of *p*-nitrophenyl phosphate and 0.5 ml of pH 4.8 citric acid. The seven test tubes were placed in a 37 C water bath for a few minutes to equilibrate their temperature. Then a crude sap was added to these tubes in quantities of: 0, 0.01, 0.025, 0.05, 0.1, 0.2, and 0.4 ml per tube. Water was added to adjust the differences in volumes resulting from the use of different amounts of crude sap in each tube. Thus, the tube containing no crude sap was a blank control for the test. The eighth tube, a crude sap control, contained 0.4 ml of crude sap which had been fixed immediately after addition by adding 5.8 ml of 0.1 N NaOH.

After 30 minutes of incubation at 37 C, reactions were stopped by addition of 4.8 ml of 0.1 N NaOH to each test tube. The solution in each test tube was diluted 20 times with water before reading the optical density (O.D.) in a Spectro-20 colorimeter. The optical densities obtained from various dilutions of the crude sap were converted into μg of *p*-nitrophenol produced by using pure (99.8% purity) *p*-nitrophenol as standard.

Partial purification of lysosomes.—Twenty-five grams of healthy and CYMV-diseased leaves of white clover were harvested. The leaves were homogenized with a mortar and pestle with small amounts of cold isolation medium (0.44 M sucrose, 0.003 M EDTA, 0.05 M Tris-HCl and 0.1% bovine serum albumin, pH 7.20). A total of 35 ml of isolation medium was added during the homogenizing process. The homogenate was strained through four layers of cheesecloth and the filtrate was centrifuged for 10 minutes at 150 *g*. The supernatant was again centrifuged at 750 *g* for an additional 10 minutes. Then the supernatant was pelleted at 8,500 *g* for 5 minutes. The pellet was kept at 4 C for later use.

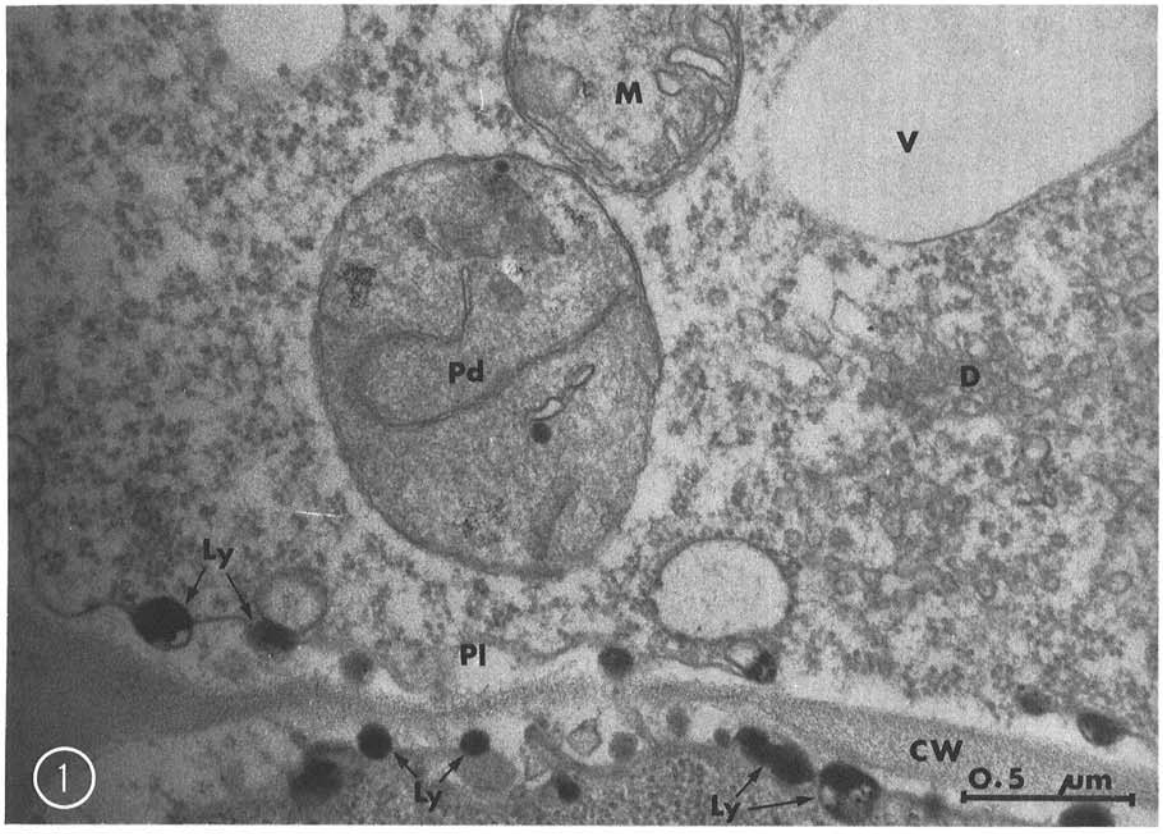
Histochemical localization of acid phosphatase.—The partially purified pellets of both healthy and CYMV-diseased leaves were suspended in 0.5 ml of isolation medium and thoroughly mixed. The suspensions were mixed with 2% Bacto agar (1:1, v/v) at 45 C. The mixture was allowed to solidify at room temperature. The solidified agar blocks were cut into 1 mm^3 pieces, fixed briefly (10 minutes) in 1% glutaraldehyde in 0.1 M cacodylate nitrate buffer, pH 7.0. The blocks were washed twice in the same buffer, postfixed in 1% buffered OsO_4 for 5 minutes, and washed with three changes of Tyrode's solution, which consisted of NaCl, 0.15 M; KCl, 3mM; $CaCl_2$, 2mM; $MgCl_2$, 1mM; glucose, 6 mM; $NaHCO_3$, 12 mM; and NaH_2PO_4, 0.4 mM for 1 hour. Then, each sample was divided into two groups. One was incubated in Gomori's acid phosphatase substrate (7) overnight (14 hours) at 37 C in a water bath shaker. The other group (control) was incubated in Gomori's substrate plus 0.01 M sodium fluoride (an acid phosphatase inhibitor). Later, the tissues were rinsed in Tyrode's solution washed for 2 minutes in 2% acetic acid, and treated in 2% ammonium sulfite for 1 minute as described by Sheldon et al. (17). The ammonium sulfite treatment was used to induce the formation of lead sulfide, an electron dense product. The tissues were washed again and dehydrated in a graded ethanol-propylene oxide and embedded in Araldite. Sections were cut and observed in a Philip EM-300 electron microscope with or without further staining. Staining was done with uranyl acetate and lead citrate.

Semi-quantitation of number of lysosomes.—Since the lysosome-rich pellets of healthy and CYMV-diseased leaves were prepared with the same procedures and care was given in each step of handling, the pellets could be used for semi-quantitation of the number of lysosomes in healthy and CYMV-diseased leaf tissues. Thin sections of pellets subjected to histochemical treatment were examined in an electron microscope and a total of 100 micrographs each for healthy and CYMV-diseased material were taken at random at the magnification of × 29,000. The number of lysosomes were counted from the negatives.

RESULTS

Ultrastructural observations.– Thin sections of healthy and CYMV-infected leaves and root nodules were examined (Fig. 1, 2, 3, 4). In the diseased plants, virus aggregates were found in the cytoplasm of leaf cells and root nodule parenchymatous cells, In leaf cells, the virus aggregates were usually located in the cytoplasm between the chloroplasts. The osmiophilic bodies and the starch grains in the chloroplasts of the diseased plants were larger and higher in number. Since these observations were in close agreement with previous documentation for various virus-infected plants (13), no further elaboration is presented. In the root nodule tissue cells, virus aggregates were found not only in the root nodule cortical tissue cells, but also in the bacteroidal cells. Particularly in the root nodule cortical cells, a virus aggregate could attain one half of the size of a parenchymatous cell.

Aside from the above mentioned differences, the most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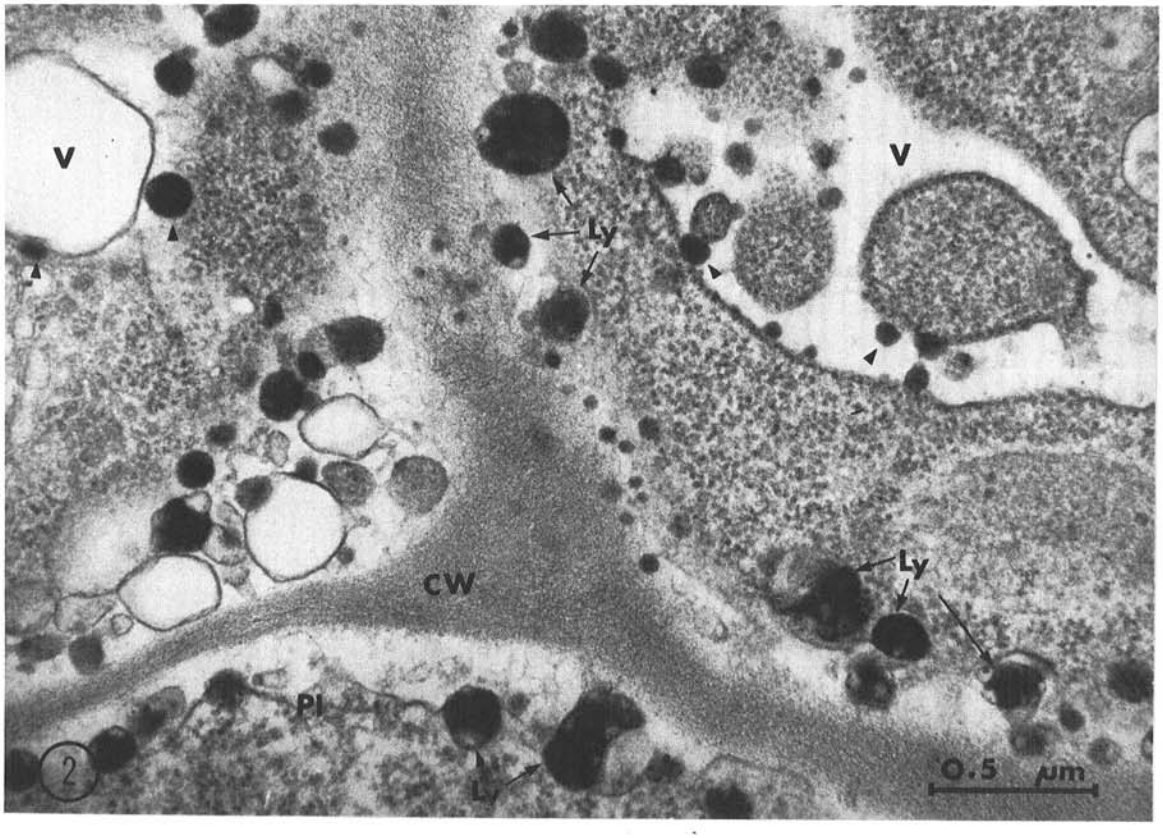
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prominent deviation between the leaf cells and the root nodule cells in the healthy and the diseased plants was in the number, size, and distribution of their lysosomes.

In both the healthy and CYMV-infected leaf cells, lysosomes were randomly distributed in the cytoplasm (Fig. 3, 4). However, lysosomes were more numerous in CYMV-infected leaf cells.

In both the healthy and CYMV-infected root nodule cells, the majority of lysosomes was found near either the plasma membrane or the vacuoles (Fig. 1, 2). As in the leaf cells, CYMV-infected root nodule cells contained more lysosomes (Fig. 2) than their healthy controls. Also, lysosomes were larger in the CYMV-infected root nodule cells.

It must be noted that the unit membrane of a lysosome can only be visualized in some but not all lysosomes. This is because lysosomes generally have a high electron density which tends to make the surrounding unit membrane indiscernible.

Biochemical study.—An assay of crude saps of leaves and root nodules of healthy and CYMV-infected plants showed that the total protein content in the fresh leaves (15.2 mg/g) and root nodules (18.8 mg/g) of CYMV-diseased plants was less than those in the leaves (16.5 mg/g) and root nodules (26.0 mg/g) of healthy plants.

The results of assay of acid phosphatase activity in crude sap samples show that CYMV-diseased leaves and root nodules had specific acid phosphatase activities about double those of the comparable healthy controls (Table 1). Regardless of leaves or nodules, the difference in the specific acid phosphatase activity between CYMV-diseased and healthy plants is highly significant. The difference between CYMV-diseased leaves and healthy leaves is between the 1 and 2% levels of probability ($0.01 < P < 0.02$; and the difference between root nodules of CYMV-diseased and healthy plants is at the 0.1% level ($P < 0.001$). However, the difference in the specific activity of acid phosphatase in leaves and in root nodules of the same CYMV-diseased plant, or of the same healthy plant, was not significant. The former had a P-value between 0.5 and 0.6, and the latter had a P-value between 0.8 and 0.9 (Table 1).

Histochemical study.—It must be noted that the ultrastructure of the isolated organelles was not preserved as in regularly fixed material because of the short fixation in glutaraldehyde in order to preserve acid phosphatase activity.

Thin sections of histochemically tested preparations

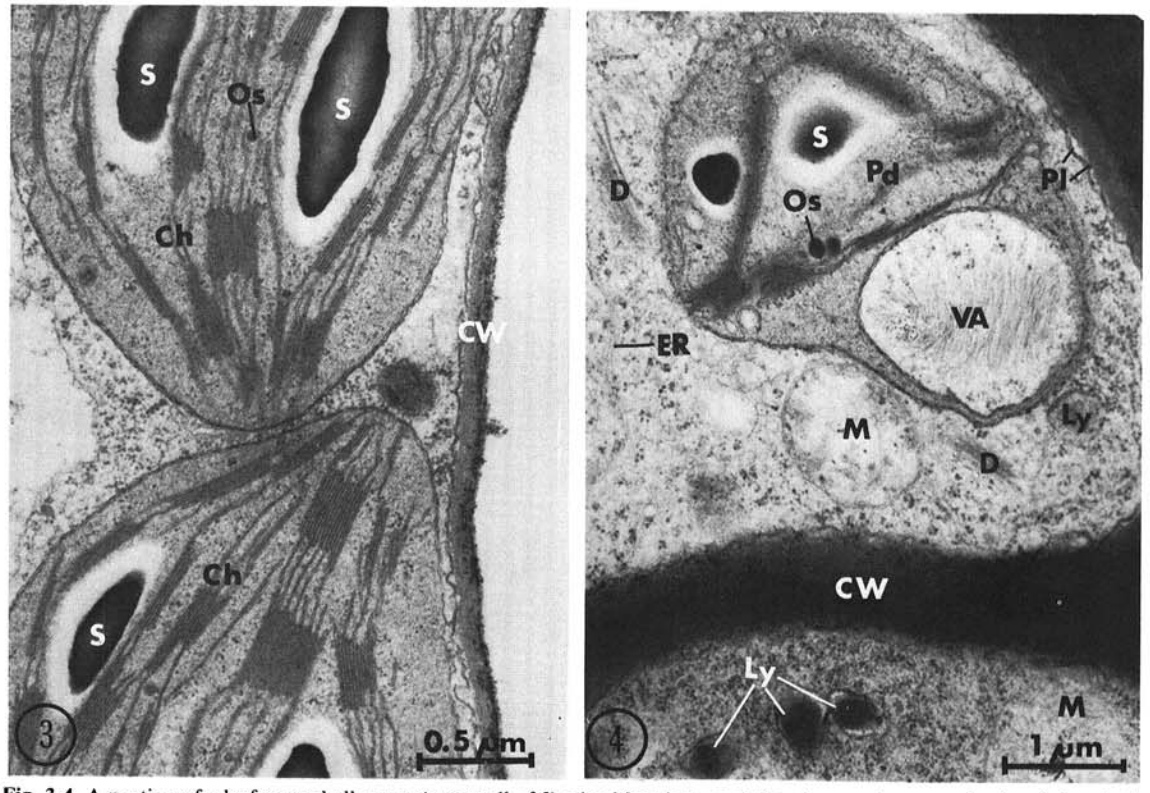

Fig. 3-4. A portion of a leaf mesophyll parenchyma cell of **3)** a healthy plant, and **4)** a clover yellow mosaic virus-infected plant showing the presence of several lysosomes in the cytoplasm. *Legend:* Ch, chloroplast; CW, cell wall; D, dictyosome; ER, endoplasmic reticulum; Ly, lysosome; M, mitochondrion; Os, osmiophilic body; Pd, plastid; Pl, plasma membrane; S, starch; and VA, virus aggregate.

Fig. 1-2. Electron micrographs showing thin sections of root nodule cortical cells. **1)** Section of a healthy clover root nodule showing the presence of a few lysosomes in the proximity of the plasma membrane (arrows). Note: Vacuoles of the healthy plant are free of lysosomes. **2)** Section of a CYMV-infected clover root nodule showing numerous lysosomes present in the proximity of the plasma membrane (arrows) and vacuoles (arrowheads). *Legend:* CW, cell wall; D, dictyosome; Ly, lysosome; M, mitochondrion; Pd, plastid; Pl, plasma membrane; and V, vacuole.

are usually examined without additional staining to avoid introduction of artifacts. The present results, however, show that the additional uranyl acetate and lead citrate staining enhanced the overall contrast and aided in the identification of cellular organelles without altering any essential feature of the preparations (19). Thus, stained preparations were preferred.

The electron-dense reaction products were found only on some unit membrane-bound structures. Thus, these structures were identified as lysosomes. Other unit membrane-bound structures which did not have the reaction products were presumably microbodies. Since the lysosome-rich pellets were only partially purified preparations, the presence of mitochondria and debris from cell wall material was expected. However, the mitochondria and the debris from the cell wall were free of electron-dense reaction products (Fig. 5, 6).

The fact that reaction products were present in sections of pellets derived from both healthy and CYMV-diseased leaves suggests that lysosomes were present in both preparations. However, both control preparations were free of dense reaction products (Fig. 7).

The lysosomes were counted in one hundred micrographs, each taken at random with 35-mm negatives at × 29,000. The counts in CYMV-diseased material averaged 13 lysosomes per micrograph, healthy material had an average of seven lysosomes. Thus, the CYMV-diseased preparation had approximately 46% more lysosomes than the healthy one. The difference between healthy and CYMV-diseased preparation is at the 1% level of probability ($P < 0.01$). Thus, the results of the histochemical test are consistent with the results of biochemical assay.

DISCUSSION

The higher specific activity of acid phosphatase obtained in the biochemical assay, and the observation of lysosomes in the thin sections of CYMV-infected leaves

TABLE 1. Statistical analysis of replicated tests of acid phosphatase specific activity in terms of the rate of conversion of *p*-nitrophenyl phosphate into *p*-nitrophenol (μg *p*-nitrophenol per minute per milligram protein)

Preparation	Replication	Healthy	Diseased
Leaf	I	283	746
	II	384	789
	III	414	684
	IV	329	576
		Ave. 352±47 (A)[a]	699±69 (B)
Root nodule	I	320	674
	II	378	638
	III	368	684
	IV	385	658
		Ave. 363±21 (C)	664±16 (D)

[a] *P*-values of significance between A and B, C and D, A and C, and B and D are $0.01 < P < 0.02$, $P < 0.001$, $0.8 < P < 0.9$, and $0.5 < P < 0.6$, respectively.

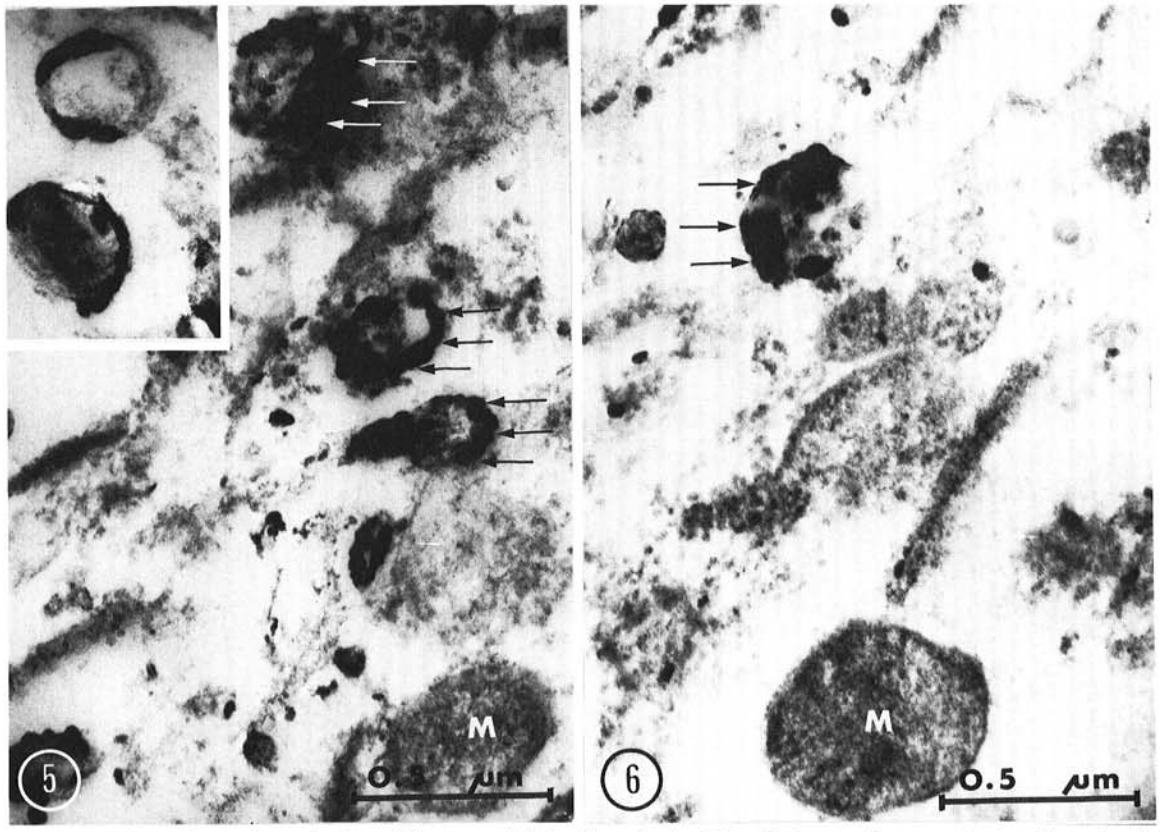

Fig. 5-6. Stained thin sections of pellets of lysosome-rich fractions obtained from **5)** clover yellow mosaic virus-diseased and **6)** healthy clover leaves showing electron-dense products on lysosomes (arrows). The pellets were incubated in Gomori's acid phosphatase medium. *Legend:* M, mitochondrion.

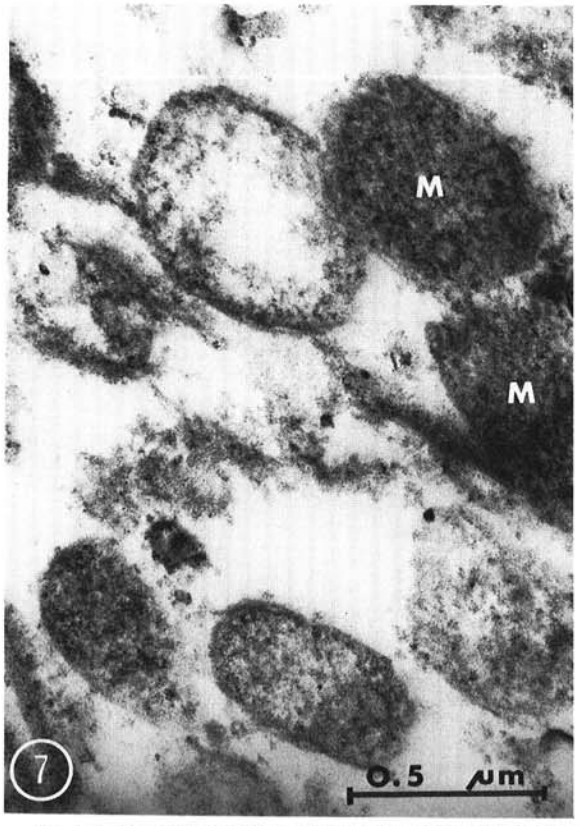

Fig. 7. A thin section of a lysosome-rich pellet obtained from clover leaves incubated in Gomori's acid phosphatase medium that contained sodium fluoride. This is a control for the material illustrated in Fig. 5 and 6. Note the absence of electron-dense deposits. *Legend:* M, mitochondrion.

and root nodules indicate that lysosomes play a role in the virus pathogenesis. The increased acid phosphatase activity in the CYMV-infected plants may reflect an increased autophagic activity.

It has been found that virus-infected legumes have relatively small nodules (21). The meristematic tissue cells of these infected root nodules exhibit early degeneration and early cessation of growth. Such phenomena could be accounted for, at least in part, by the high autophagic activity caused by high lysosomal activity.

Since lysosomal activity seems to be different in a virus-infected host, and since lysosomes contain many hydrolytic enzymes other than acid phosphatase, it is reasonable to assume that other enzymes may likewise have their roles in the virus pathogenesis, and contribute to the physiological alterations in a virus-diseased plant. Thus, studying the activity of other enzymes in the virus pathogenesis should further the understanding of the virus pathophysiology.

This study demonstrated the presence of higher specific acid phosphatase activity and higher numbers of lysosomes in CYMV-diseased plants. This provides positive indication that early senescence due to increased lysosomal activity could have contributed in part to the reduction of forage yield.

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