Ultrastructure Changes Induced by Stem Nematodes in Hypocotyl Tissue of Alfalfa[1]

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Abstract: Scarified seeds of *Medicago sativa* L. 'Ranger' and 'Lahontan' alfalfa were allowed to imbibe water for 36 hr and then were inoculated with stem nematodes, *Ditylenchus dipsaci* Kühn. Seedlings were grown in sterilized Provo sand at 20 C and hypocotyl sections harvested at 1, 3 and 7 days. Evidence from electron micrographs indicated that cells of noninfected control plants contained normally developing chloroplasts bearing stroma, thylakoids, starch grains and plastoglobuli. The cytoplasm contained a nucleus, endoplasmic reticulum, vacuoles, mitochondria, ribosomes and dictyosomes. No morphological symptoms of nematode infection were observed in infected plants of either Ranger of Lahontan alfalfa 1 day after inoculation. Electron micrographs of tissue from the infected plants, however, indicated more osmiophilic bodies (lipid bodies) per cell than did the noninfected control, with more lipid bodies present in Ranger than in Lahontan. Three and 7 days after planting, swollen hypocotyls could be seen; the degree of swelling was greater in Ranger than in Lahontan. Electron micrographs of infected tissues indicated that both cultivars were undergoing the same kind of damage. Injured organelles were endoplasmic reticulum, chloroplasts and the nucleus. Histochemical staining indicated no changes in the middle lamellae. *Key Words:* fine structure, host-parasite interactions.

Many fields of alfalfa are lost each year to the alfalfa stem nematode, *Ditylenchus dipsaci* Kühn. When the plants are weakened by the nematodes, other organisms then invade. Chemical control of the nematodes is not yet feasible (4, 20); hence, the development of resistant cultivars offers the only control (6, 20). Griffin (5) found no relationship in host response and numbers of nematodes that invaded susceptible and resistant alfalfa. Moreover, Krusberg (11, 12), using light microscopy, did not observe any histochemical differences in cell walls of nematode-infected and noninfected alfalfa plants. Changes, however, may occur at the fine structural level long before visible symptoms are evident. If so, definition of such changes might elucidate subtle, yet important, differences between susceptible and resistant or tolerant alfalfa lines.

This study, conducted at the fine structural level, was designed to determine: (i) whether differences in fine structure exist between Ranger (susceptible) and Lahontan (tolerant) alfalfa lines; (ii) whether changes are induced by stem nematode infection in those lines; (iii) whether fine structural effects vary with numbers of nematodes and, if so, whether these effects are indicative of overall host tissue responses; and (iv) whether the pectin content of the middle lamellae of host cell wall tissue differs before and after infection.

MATERIALS AND METHODS

Seeds of alfalfa, *Medicago sativa* L. 'Ranger' (susceptible line) and 'Lahontan' (a tolerant line) were scarified with 100-grit silicon carbide water sandpaper and germinated for 36 hr at 25 C in petri dishes lined with filter paper moistened with distilled water.

The germinating alfalfa seeds were placed in sterilized Provo sand at about 100% of field capacity. One-milliliter suspensions, prepared according to the method of Thorne (21), containing either 0, 20 or 100 *Ditylenchus dipsaci* Kühn was pipetted directly over the germinating seed. Seeds were covered with 6 mm of sand. Seedlings were grown at 20 C and under a light intensity of ∿32250 lux (3000 ft-c) for 16 hr per day.

Hypocotyl sections (1 mm) of infected and noninfected alfalfa seedlings were harvested 1, 3, and 7 days after inoculation. The fixation of all samples was in Karnovsky's (9) glutaraldehyde-formaldehyde solution buffered

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with cacodylate (pH 7.2) for 4 hr at 25 C and rinsed twice with the same buffer. Secondary fixation of all samples was achieved with 2% osmium tetroxide in cacodylate buffer for 1 hr at 4 C, followed by two changes of the same buffer as a rinse. All samples were dehydrated with increasing concentrations of ethanol, cleared with propylene oxide and embedded in Epon 812 (14).

The method of Albersheim and Killias (1) was utilized for staining pectins. Pectin determinations were made on hypocotyl sections harvested at 7 days and fixed by Karnovsky's procedure (9). The samples were fixed secondarily with 2% osmium tetroxide for 1 hr at 4 C, rinsed with two changes of cacodylate buffer, dehydrated to 60% ethanol and then held in 60% ethanol alkaline hydroxylamine solution for 1 hr at 4 C. They were subsequently washed in 0.1 M HCl for 15 min, put in 2% $FeCl_3$ for 1 hr, dehydrated with acetone, cleared with propylene oxide and embedded in Epon 812 (14).

Thin sections were cut with either a glass or diamond knife on a Sorvall MT-2 ultramicrotome. Sections were stained with saturated aqueous uranyl acetate at 60 C for 12 min (22) followed by lead citrate at 25 C for 2-5 min (15). Sections were examined with a Zeiss EM-9A electron microscope and significant observations recorded photographically.

RESULTS

No morphological symptoms of nematode infection were observed in either Ranger (susceptible) or Lahontan (tolerant) alfalfa under any treatment conditions 1 day after inoculation. Three and 7 days after planting, however, swollen hypocotyls could be seen. The swelling was more pronounced in Ranger than in Lahontan at both ages.

The fine structure of cells of the hypocotyl region of control plants of both Ranger and Lahontan alfalfa are shown in Fig. 1A, 1B. The cytoplasm and the organelles embedded within it were typically distributed around the periphery of the cell. A large central vacuole, which was bounded by a single membrane, the tonoplast, constituted the major portion of the cell. The plasmalemma appeared as a continuous thin line and was closely appressed to the cell wall.

Chloroplasts and a relatively large nucleus were the most conspicuous organelles observed. In the chloroplasts, a well-organized grana-lamellae system was distributed evenly within a homogeneously electron-dense proteinaceous matrix–the stroma. Ribosome-like particles were scattered randomly within the stroma, and a few were attached to the surface of thylakoid membranes (Fig. 1A, double arrows). Starch grains of various number, size and electron density were also present in the stroma of most chloroplasts. Electron-dense plastoglobuli were another component observed in the stroma between the membranes, but never in the grana. The globules were without any membranes and varied in size and number within different chloroplasts. Fibrous structures, which are believed to be a DNA-fibril complex, were observed frequently within the electron-transparent areas in the stroma of many of the chloroplasts. These DNA-containing areas are referred to as nucleoids. A genetic material with similar structure was also observed in some mitochondria (Fig. 1A, double arrows).

Nuclei with the double membrane envelopes were observed. The nuclear envelope was discontinuous and broken at irregular intervals by nuclear pores (Fig. 1A). The nucleolus was embedded in the fibrillar nucleoplasm without a membrane around it. It was usually seen as an aggregate of numerous fibrillar and granular components. Chromatin, the complexes of chromosomal DNA and protein, appeared as densely packed aggregates of ribosome-like particles and were located peripherally in the nucleus (Fig. 1A). The cytoplasm of cells from control plants of both cultivars also contained mitochondria, endoplasmic reticulum, ribosomes and dictyosomes. The endoplasmic reticulum and dictyosomes, while not abundant, were easily detected. The ribosomes appeared as both polysomes and as free forms. The double membranous mitochondria were variable in shape and size, usually globular, ellipsoidal or somewhat elongated. The interior cristae were formed generally by the infolding of the inner membrane, and lay within a moderately electron-dense matrix, presumably proteinaceous in nature. A few small electron-opaque mitochondrial granules were often scattered within the mitochondrial matrix between the cristae (Fig. 1B, arrows). Plasmadesmata were seen frequently on the cell walls (Fig. 1A). Osmiophilic bodies were noted in the cytoplasm (Fig. 1B).

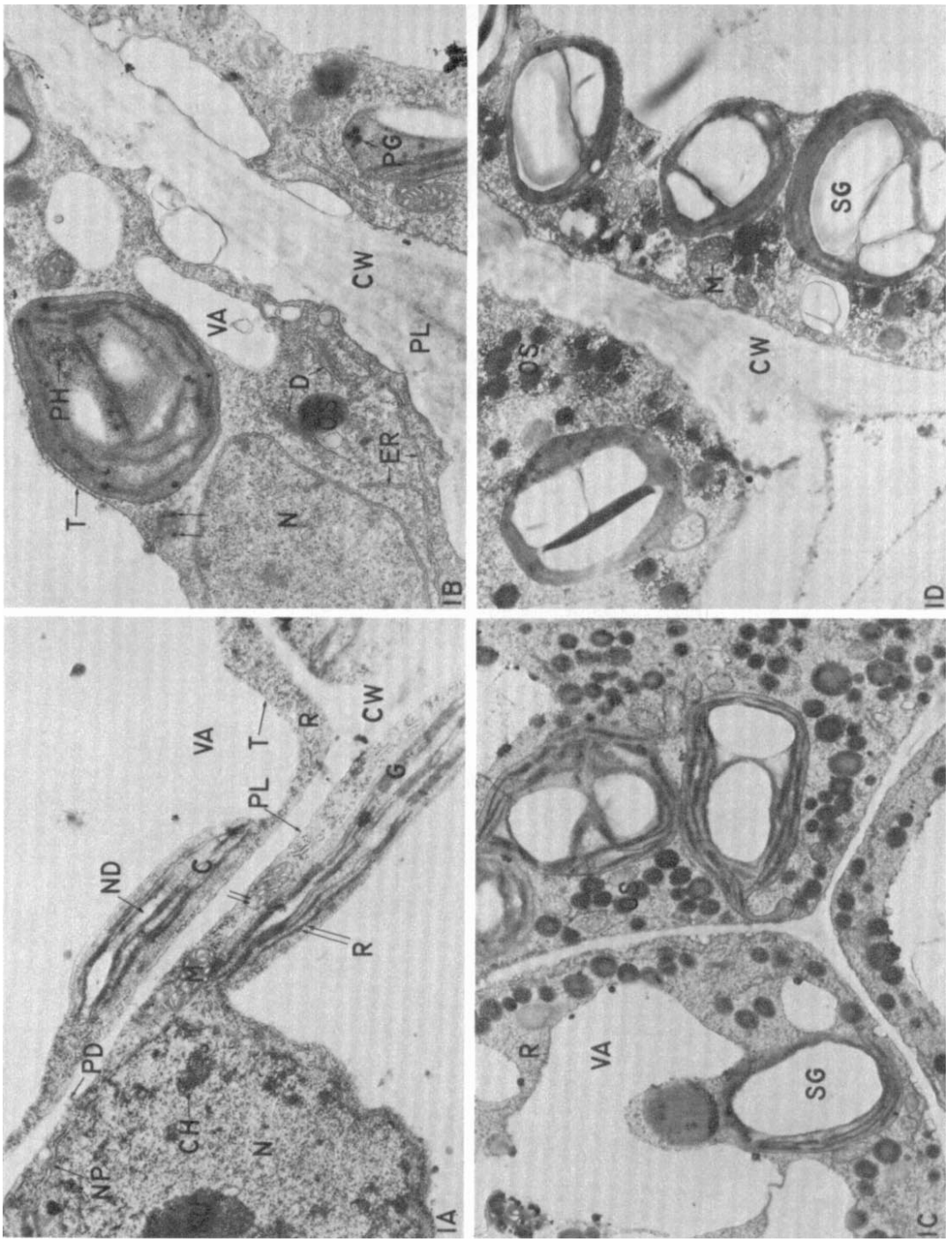

FIG. 1. **A.** Seven-day-old 'Ranger' alfalfa control plant with normal cell content. (× 20,700). **B.** One-day-old Ranger alfalfa control plant with normal cell content. (× 37,620). **C.** Osmiophilic bodies in one-day-old Ranger alfalfa plant infected with nematodes. (× 14,200). **D.** Osmiophilic bodies in one-day-old 'Lahontan' alfalfa plant infected with nematodes. (× 33,100). Symbol legend: C = Chloroplast; CE = Chloroplast Envelope; CH = Chromatin; CW = Cell Wall; CY = Cytoplasm; D = Dictyosome; ER = Endoplasmic Reticulum; EDM = Electron Dense Material; G = Grana; M = Mitochondria; N = Nucleus; ND = DNA-Containing Nucleoid; NE = Nuclear Envelope; NM = Nematode; NP = Nuclear Pore; NU = Nucleolus; OS = Osmiophilic Bodies; PD = Plasmadesma; PG = Plastoglobuli; PH = Phytoferritin; PL = Plasmalemma; R = Ribosome; S = Stroma; SG = Starch Grains; T = Tonoplast; V = Vesicle; VA = Vacuole.

On the first day after inoculation with *D. dipsaci*, infected Ranger and Lahontan plants exhibited more osmiophilic bodies (lipid bodies) per cell than did the controls (Fig. 1C, 1D). The number of osmiophilic bodies, however, was less in Lahontan than in Ranger.

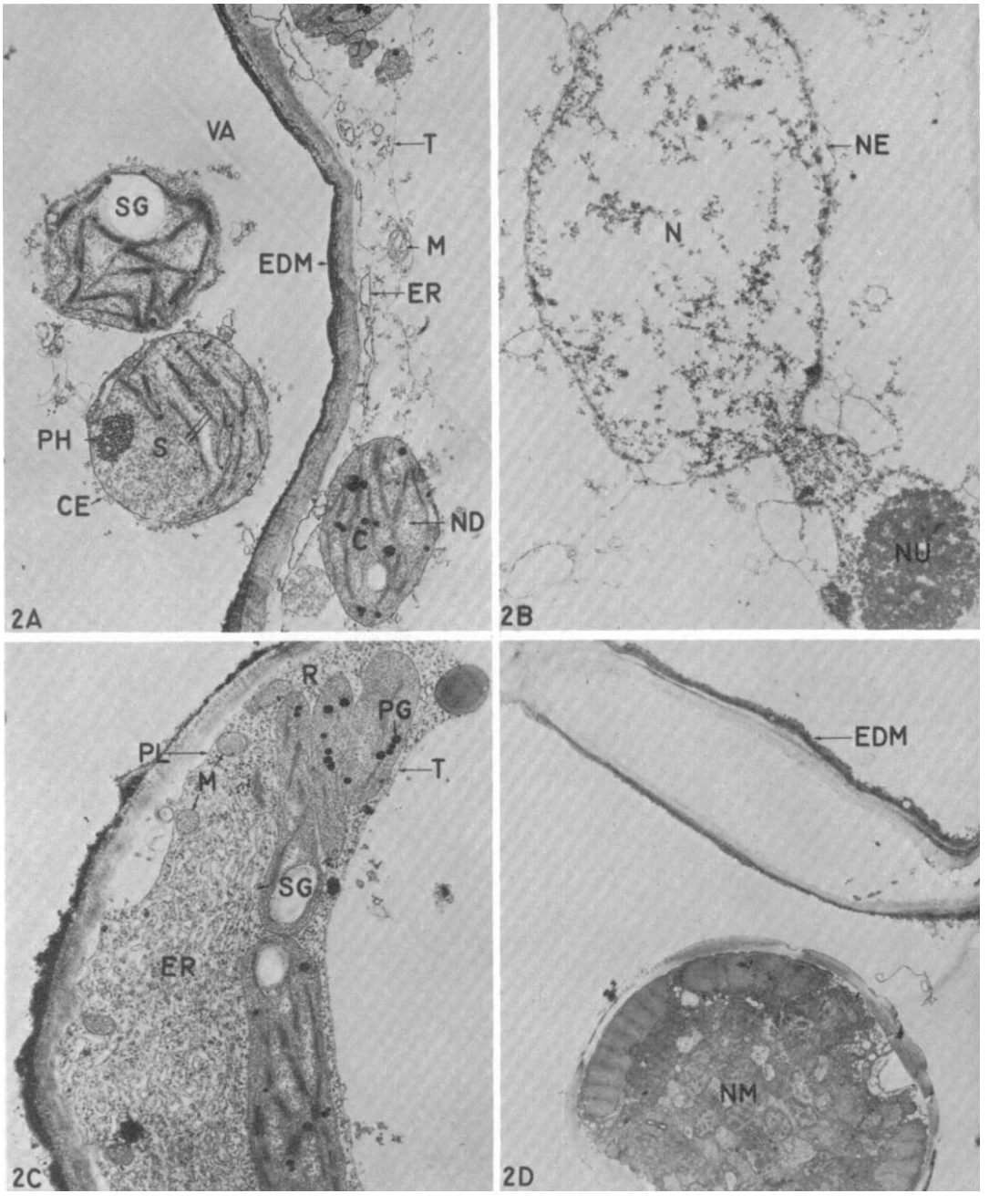

FIG. 2. **A.** Three-day-old 'Ranger' alfalfa plant infected with nematodes. Note the osmiophilic staining of the side of the cell wall, the swollen chloroplasts, the rupture of the outer chloroplast membrane and the swollen thylakoid units (arrows). (×14,510). **B.** Three-day-old Ranger alfalfa plant infected with nematodes. Note swelling and lobing of the nucleus, rupture of outer nuclear membrane, and the leakage of nuclear content. (×21,230). **C.** Three-day-old Ranger alfalfa nematode infected plant with dense cytoplasm and an abundance of ribosomes and numerous cisternae of smooth and rough endoplasmic reticulum. (×13,580). **D.** Three-day-old Ranger alfalfa plant infected with nematodes. Note the response of host tissue beside the nematode. (×5,570). For symbol legend, see Fig. 1 caption.

No other changes in organellar structure were noted at this age.

Three days after planting, however, the chloroplasts in infected cells of Ranger were swollen and the outer chloroplast membrane was ruptured (Fig. 2A). Swelling of the thylakoid units also was noted (arrows). In a few cases, the nucleus was swollen and the outer nuclear membrane was ruptured (Fig. 2B), resulting in leakage of nuclear material. Moreover, compared to the controls, the cytoplasm of infected cells was more dense, with an abundance of ribosomes and numerous cisternae of smooth and rough endoplasmic reticulum (Fig. 2C). Further, one side of the cell wall became more electron-dense (Fig. 2A, 2D). Host tissue at the infection site; i.e., next to the nematode, showed a complete loss of cytoplasm and an osmiophilic cell wall on the side adjacent to the nematode (Fig. 2D).

With few exceptions, 3 days after planting, Lahontan contained normally developing chloroplasts, cytoplasm and nuclei even in the infected cells. In a very few cases, however, when the cell was heavily infected, the infection responses at the fine structural level were the same as those in Ranger; i.e., the endoplasmic reticulum became swollen in

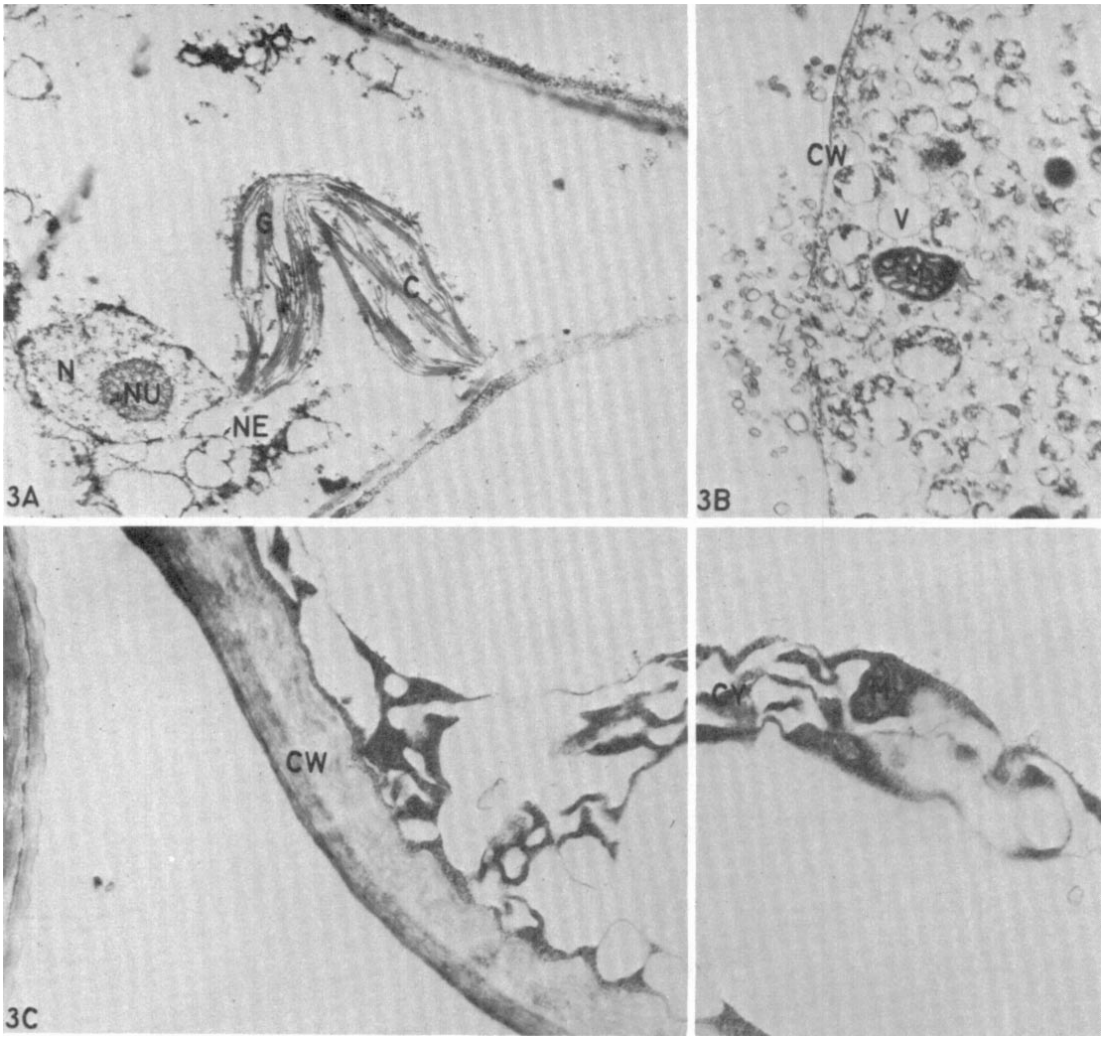

FIG. 3. **A.** Seven-day-old'Lahontan'alfalfa plant heavily infected with nematodes. Note the disrupted nucleus and chloroplast. (×20,970). **B.** Seven-day-old'Ranger'alfalfa plant infected with nematodes. Note the ruptured cell wall and mixing of the cytoplasm of the two cells. Note also the numerous vesicles. (×22,300). **C.** Seven-day-old Ranger alfalfa plant inoculated with 100 nematodes per plant. Note the plasmalyzed cytoplasm. (×22,600). For symbol legend, see Fig. 1 caption.

sausage-like chains (Fig. 2A); numerous vesicles formed; chloroplasts swelled; and the outer membranes of the chloroplasts ruptured. Swelling of thylakoid units of the grana was also noted. Even at the infection site, however, the cell wall remained unchanged in Lahontan seedlings.

In Ranger, 7 days after infection, partial to complete disruption of chloroplasts was evident (Fig. 3A). Also, abundant "vesicles" were

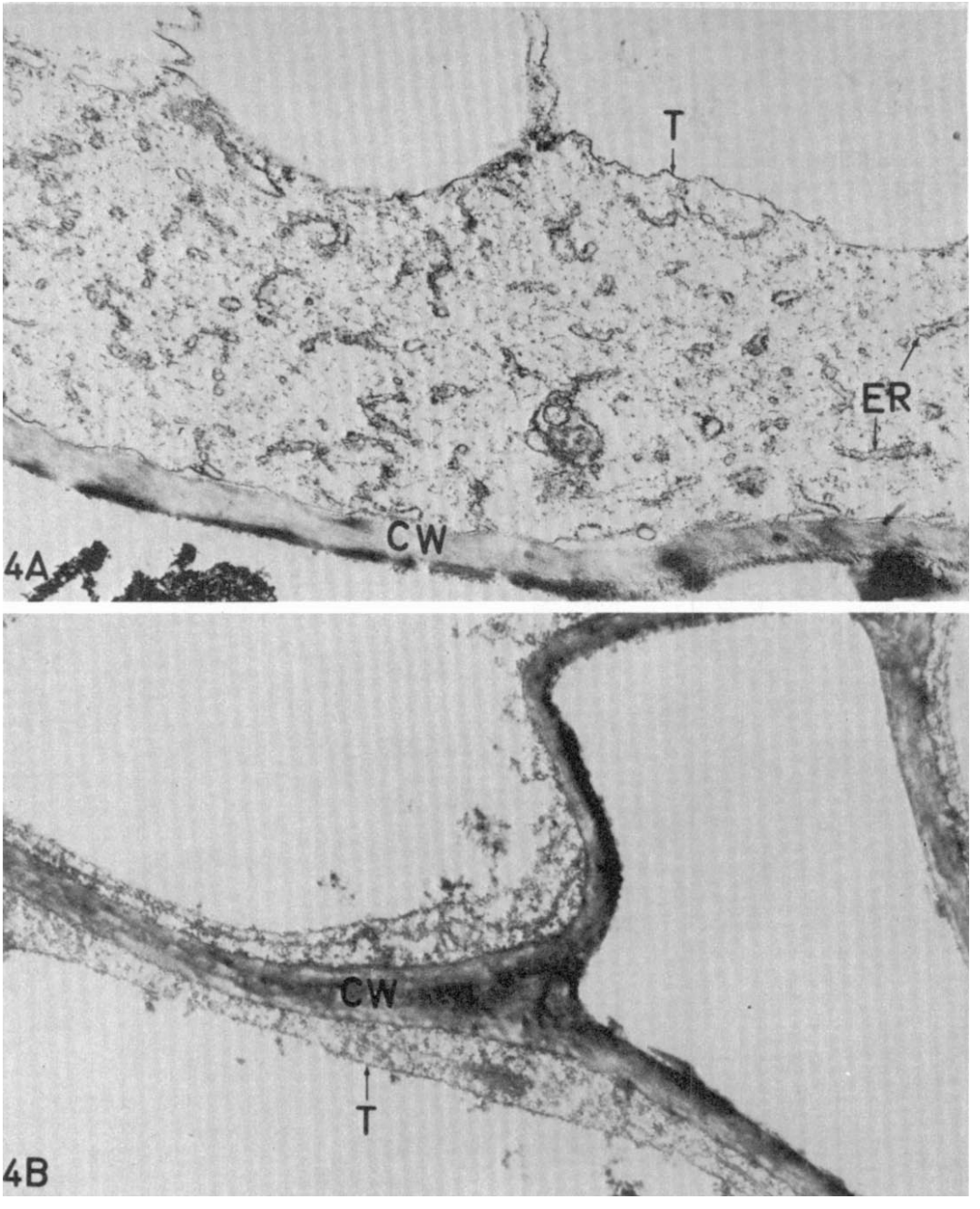

FIG. 4. **A.** Seven-day-old 'Lahontan' alfalfa plant inoculated with 100 nematodes per plant. Note the osmiophilic substance on the cell wall and swelling of the endoplasmic reticulum. (×37,550). **B.** Pectin in the cell wall of seven-day-old Ranger alfalfa plant infected with nematodes. (×37,550). For symbol legend, see Fig. 1 caption.

found in the cytoplasm and cell walls were broken down, which allowed the cytoplasm of adjacent cells to intermingle (Fig. 3B). By contrast, in Lahontan, 7 days after infection, most chloroplasts were normal, although heavily infected areas showed characteristic symptoms such as the leaking of nuclear contents, broken chloroplasts and swollen endoplasmic reticulum as was observed in Ranger. Cell walls, however, still remained normal in Lahontan seedlings.

For a comparison of the host varietal responses to fixed numbers of nematodes, each seedling in the following experiment was inoculated with 100 nematodes. Damage to host tissues when 100 nematodes per plant was used was no greater than damage to those inoculated with only 20 nematodes. The cytoplasm at heavily infected sites was plasmolyzed in Ranger seedlings subjected to inoculation with 100 nematodes (Fig. 3C). In Lahontan, slightly infected cells showed slight swelling of the endoplasmic reticulum only. In heavily infected cells of both cultivars, osmiophilic cell walls, broken and swollen endoplasmic reticulum and numerous vesicles were observed (Fig. 4A).

Other features noted in both Ranger and Lahontan were crystalline and paracrystalline arrays of electron dense granules in the chloroplasts of control and infected plants at all ages (Fig. 1B, 2A). The granules were parallel to one another and formed a three-dimensional lattice. Myelin figures were found in some cells, with no apparent correlation with time after nematode infection. Infected and noninfected seedlings of both cultivars were sometimes seen to have chloroplasts containing numerous small vesicles along their periphery. No obvious difference was noted in the pectin content of cell walls in nematode-infected or noninfected plants at all ages in either Ranger or Lahontan (Fig. 4B).

DISCUSSION

It has been established that the first visible symptom of infection by the stem nematode *Ditylenchus dipsaci* is the formation of a gall or a swelling of the stem. Moreover, Griffin (5) has reported that the hypocotyl, cotyledonary petioles, and to a lesser extent the epicotyl were invaded first. These observations were confirmed in this study.

The frequency with which morphological symptoms appear, however, seems to be a function of cultivar response. For example, Krusberg (10) noted that in young alfalfa seedlings, cavities developed in the cotyledonary cortex, the epidermal cells enlarged and cell reaction to dyes changed within 12 hr after inoculation. Moreover, within 36 hr of inoculation, the conspicous galling of young alfalfa stems, caused by cell hypertrophy, was morphologically visible. The earliest swelling observed in this study, however, appeared 72 hr after inoculation.

The increased number of lipid bodies observed in nematode-infected tissue over that of the controls may represent an accumulation from membrane breakdown products, and may imply senescence on the part of the infected tissue (18). Moreover, the susceptible Ranger had a greater number of these lipid bodies than the tolerant Lahontan. The permeability of the membranes obviously was altered by the nematode infection, probably through enzymatic reactions. In this respect, these data agree with those of Sheetz and Crittenden (19), who histochemically demonstrated a greater abundance of lipids in the giant cells of root-knot nematode-susceptible than in root-knot nematode-resistant soybean plants. The difference in number of lipid bodies per cell between susceptible and resistant or tolerant lines may provide some clue to differences in responses to nematodes between Lahontan (tolerant) and Ranger (susceptible) alfalfa. Correlative biochemical studies, however, are needed to confirm that these differences are real.

Light and electron microscopic studies have shown that pectin substances are concentrated within the middle lamella and primary wall of normal alfalfa seedlings. The method whereby pectic substances have been demonstrated with the electron microscope involves the heavy metal, iron (1). The reaction in question forms pectic hydroxamic acids by substituting hydroxylamine for the methoxyl groups of pectin. The latter yield insoluble iron complexes when treated with ferric iron. If the nematodes were indeed secreting substances that dissolve the middle lamellae, then a reaction involving the chemicals mentioned above would occur only in noninfected plants. The reactions in nematode-infected and noninfected plants were the same, however. These findings agree with the statement made by Krusberg (11, 12) that there is no histochemical evidence for pectin removal, and

that the degradation of pectic compounds in the middle lamellae of alfalfa by nematode pectinase is not a factor in pathogenesis.

The granular complex observed in some of the chloroplasts have been referred to as phytoferritin (7, 8, 16, 17), and hypothesized as an iron-protein complex that allowed the plant to store iron in a nontoxic form. The reports most commonly identified the complex in differentiating plastids, and often in meristematic cells. The phytoferritin may provide iron for the development of young cells, including the elaboration of photosynthetic material. Robards and Robinson (17) reported that phytoferritin was completely unaffected by the treatments of deoxyribonuclease or ribonuclease, thus excluding the possibility that the inclusions were viral particles.

The myelin figures occasionally seen in cells from infected and noninfected tissue may represent fixation artifacts. Curgy (3) indicated that myelin figures were seen only after aldehyde fixation or aldehyde-OsO_4 double fixation. Fixation with OsO_4, with $KMnO_4$, or with a combined glutaraldehyde-OsO_4 mixture did not reveal such structures. Myelin figures may also indicate an injury response.

Laetsch (13) suggested that the presence of the tubules of the peripheral reticulum of dicotyledonous chloroplasts possess the C_4-dicarboxylic acid pathway of photosynthetic CO_2 fixation. According to J. H. Hillard (*personal communication*) the presence (C_4) or absence (C_3) of a parenchymatous bundle sheath on a leaf cross-section is the most reliable structural criterion for C_3 and C_4 plants. On this basis, then alfalfa should be classified as a C_3 plant. The presence of tubules of peripheral reticulum in the alfalfa chloroplasts, however, suggests that peripheral reticulum or vesicles may be unrelated to the type of photosynthetic pathways.

These data also suggest that, in alfalfa, there is no relationship between severity of host response and numbers of invading nematodes. Since one organism can do as much ultimate damage to a plant as several, the resistance apparently has to be absolute to be effective. In this respect, these data agree with those of Griffin (5). He speculated that such responses were due to the ability of *D. dipsaci* to invade resistant as well as susceptible alfalfa plants. For unknown reasons, however, the nematode population gradually declined in resistant alfalfa (2, 5, 6). Electron micrographs of infected tissue indicated the types of damage were the same between Lahontan and Ranger. Only the infection rate and degree of damage were different between the two lines. Results of this study indicate that more extensive experiments are needed at both the biochemical and electron microscopical levels.

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