Effects of Temperature on the Fine-Structural Responses in The Hypocotyl Region of Alfalfa Lines to Ditylenchus dipsaci[1]

DORIS C. N. CHANG, W. F. CAMPBELL, and G. D. GRIFFIN[2]

Abstract: Fine-structural analyses were made of the response of host tissue, *Medicago sativa* L. 'Ranger' and 'Lahontan', to infection by the stem nematode, *Ditylenchus dipsaci* (Kühn) Filipjev. Seedlings were grown at 15 and 25 C, and hypocotyl regions were sampled 1, 3, or 7 days after inoculation. Electron micrographs of infected alfalfa tissue indicated that the same types of damage were inflicted on Lahontan (stem-nematode-tolerant) and Ranger (stem-nematode-susceptible). Only the infection rate and degree of damage differed between lines and temperatures, with the greater injury occurring at the higher temperatures. After 3 and 7 days of infection, the symptoms observed were: swollen and broken endoplasmic reticulum (ER), distended and broken chloroplasts, loss of nuclear material, and bulging and rupturing of nuclear envelopes. Cells with infected cytoplasm contained more ER, ribosomes, vesicles, and Golgi apparatuses, suggesting increased metabolic activities. Lobing nuclei were observed in all samples. Lipid contents varied with temperature in 1-day-old seedlings. At 15 and 25 C, electron-dense substrances were commonly found along the tonoplast, and on the cell wall. Also, some cells with enlarged ER were noted in the noninfected controls at these temperatures. *Key Words:* electron microscopy, host-parasite interactions.

The degree of mobility of, and the damage caused by, plant-parasitic nematodes are functions of temperature at the time of infectivity (2, 6, 9, 10, 11, 18, 20, 23, 24). Hanna and Hawn (11) found fewer nematodes invading tolerant Lahontan alfalfa than the susceptible cultivar Grimm at 15.5 C. Griffin (9) reported *D. dipsaci* (Kühn) Filipjev penetrated resistant and susceptible alfalfa at temperatures between 5 and 30 C, with maximum infection at 20 C. Ranger alfalfa was susceptible at temperatures from 10 to 30 C, but the susceptibility of Lahontan alfalfa increased slightly as the temperature increased from 5 to 20, and increased sharply between 25 and 30 C. Moreover, he noted no relationship between numbers of invading nematodes and host response, whether the alfalfa was susceptible or resistant. Grundbacher and Stanford (10) also noted differing degrees of susceptibility as the temperature increased. They observed that Lahontan alfalfa was less resistant to *D. dipsaci* at 15.5 and 21 C, than at 11 C.

Received for publication 30 August 1974.

[1]This investigation is part of a thesis submitted by the senior author in partial fulfillment of the requirements for a Ph.D. degree in Plant Science, Utah State University, Logan. Contribution from the Plant Science Department and Agricultural Research Service, United States Department of Agriculture, Utah Agricultural Experiment Station, Logan. Published with the approval of the Director of the Utah Agricultural Experiment Station as Journal Series Article 1773.

[2]Respectively, former Graduate Assistant, now Department of Horticulture, National Taiwan University, Taipei, Taiwan, China; Associate Professor Plant Science, Utah State University; and Nematologist, Agricultural Research Service, United States Department of Agriculture, Crops Research Laboratory, Utah State University, Logan 84322. Research supported in part by a National Defense Education Act Type IV Fellowship awarded to the senior author.

Using the electron microscope, Chang et al. (7) observed that at 20 C Lahontan and Ranger alfalfas sustained the same kind of damage after stem nematode infection. Organelles showing injury were ER, chloroplast, and nucleus. Increased ER and proliferation of cytoplasm in host tissue of tomato (14), broad bean (12), soybean (8) and red clover (3) also have been observed after nematode infection. Physiological relationships between the nematodes and the observed alterations in fine structure are not well understood. Moreover, little information is available concerning the fine structural changes that occur in alfalfa infected with *D. dipsaci*. The present study was undertaken in an attempt to determine if there are detectable differences in the responses of Lahontan (stem-nematode-tolerant) and Ranger (stem-nematode-susceptible) alfalfas to temperature; if temperature affects the expression of nematode-induced host responses; and whether changes can be detected in Lahontan that might account for its lessened tolerance to stem nematode attacks with increased temperature.

MATERIALS AND METHODS

Alfalfa, *Medicago sativa* L., seedlings, 'Lahontan' (stem-nematode-tolerant) and 'Ranger' (stem-nematode-susceptible), inoculated with 0 or 20 stem nematodes, *Ditylenchus dipsaci* (Kühn) Filipjev, were grown as described in detail earlier (7). Seedlings were placed in growth chambers at 15 or 25 C, with 16 h of light at about 32,290 lux. Samples were harvested at 1-, 3-, or 7-day

intervals, and were prepared for electron microscopy as described previously (7).

OBSERVATIONS

Alfalfa seedling growth was directly influenced by temperature (Fig. 1-A, 1-B, 1-C). No visible symptoms of nematode infection, however, were observed at either temperature in Ranger or Lahontan 1 day after inoculation. Three days after planting, swollen hypocotyls were found at 25 C in both cultivars (Fig. 1-B). Visible symptoms were observed in 7-day-old seedlings at both 15 and 25 C (Fig. 1-C). After 7 days at 15 and 25 C, the infection rate of the hypocotyl was much higher in Ranger, 92% and 100%, than in Lahontan, 18% and 72%, respectively. In the hypocotyl region, the nematode infection was found only in the cortical region (Fig. 2-A, 2-B).

15 C: The number of lipid bodies in 1-day-old noninfected plants was higher than those in infected plants of both Ranger and Lahontan. Also at 15 C, more electron-dense substances (EDS) were present continuously along the tonoplasts (Fig. 3-A), in a semi-continuous manner on the side of the cell wall facing the intercellular space (Fig. 3-B), or became separate spots distributed along the cell walls (Fig. 3-C) than were observed at the higher temperature. EDS were found in all samples. Swollen ER was observed in both lines at all ages (Fig. 3-D).

In general, the degree of damage in both Ranger and Lahontan to stem nematode infection at 15 C was less than that observed at the higher temperature. Chloroplasts, cell-walls, mitochondria, and nuclei appeared to be normal in 1-day-old tissues. Some Golgi apparatuses could be observed in all samples.

In 3-day-old seedlings, ER in the infected areas was slightly swollen and numerous vesicles were present in the cytoplasm (Fig. 3-E). At the infection sites, the chloroplasts and nuclei showed similar responses, such as broken chloroplasts, as well as bulging and rupturing of nuclear envelopes in both cultivars (Fig. 3-F). At this stage, usually, most chloroplasts near the infection sites were free of damage (Fig. 4-A). Cell walls and mitochondria were normal in all treatments. Fewer lipid bodies were observed in 3-day-old samples than in 1-day-old samples. No change in Golgi apparatus could be found.

Seven days after inoculation, actively secreting Golgi apparatuses and Golgi vesicles were found in infected cytoplasm (Fig. 4-B). Swelling of ER was found in both control and treated plants (Fig. 4-C, 4-D). Few swollen chloroplasts were noted in infected tissues (Fig. 4-D). The infected nuclei were less dense than the control (Fig. 4-C, 4-D). Cell walls and mitochondria were normal in all treatments. Also, the phytoferritin complexes contained in the chloroplasts were found not

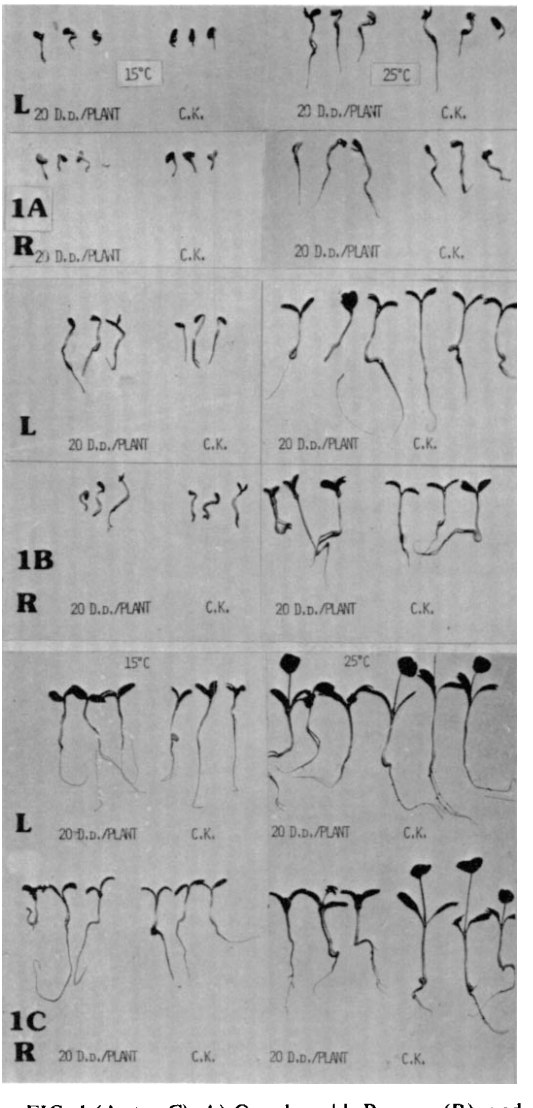


FIG. 1-(A to C). A) One-day-old Ranger (R) and Lahontan (L) alfalfa seedlings exposed to 15 and 25 C and nematodes. B) Three-day-old seedlings after similar treatment. Note the slight swelling in the cotyledonary node region. C) Seven-day-old seedlings after similar treatment. Visible swelling of the nodal tissue is noted in both cultivars at both temperatures.

ABBREVIATIONS ON FIGURES

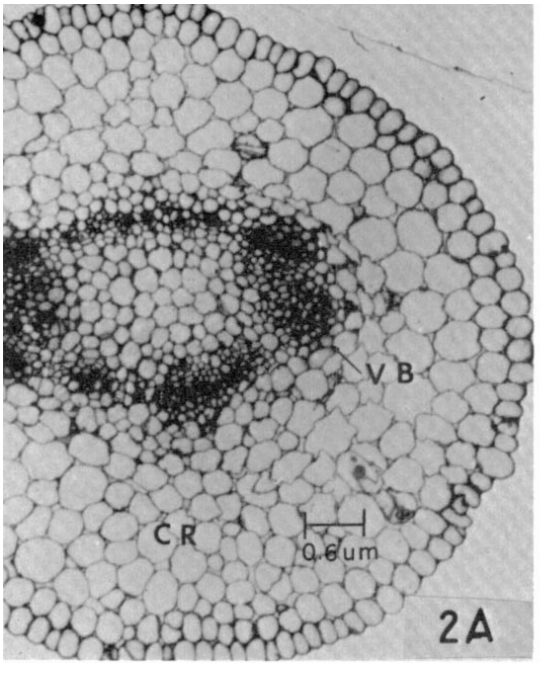
C	=	Chloroplast	GV	=	Golgi vesicles	PD	=	Plasmodesma
CE	=	Chloroplast envelope	ICS	=	Intercellular space	PG	=	Plastoglobuli
CR	=	Cortical region	LB	=	Lipid bodies	PH	=	Phytoferritin
CW	=	Cell wall	M	=	Mitochondria	S	=	Stroma
D	=	Dictyosomes	N	=	Nucleus	SG	=	Starch grain
EDS	=	Electron-dense substances	NE	=	Nuclear envelope	T	=	Tonoplast
ER	=	Endoplasmic reticulum	NM	=	Nematode	VA	=	Vacuole
G	=	Grana	NU	=	Nucleolus	VB	=	Vascular bundle
GA	=	Golgi apparatus						

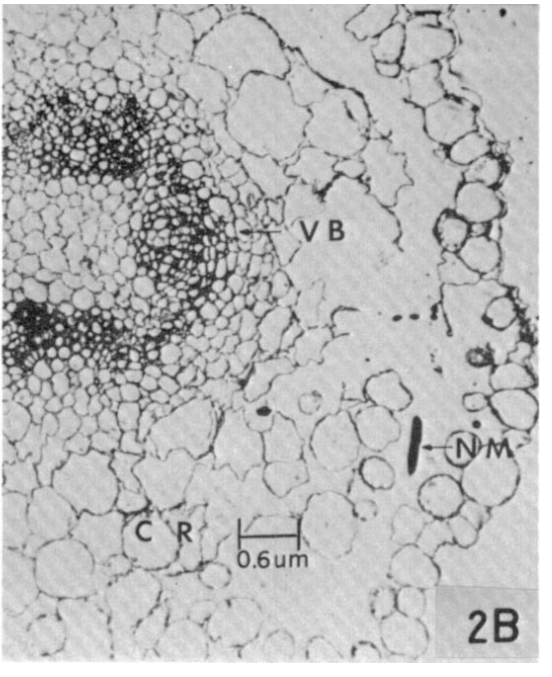
FIG. 2-(A, B). **A)** Cross-section of hypocotyl region of control Ranger alfalfa seedling grown at 25 C. **B)** Cross-section of hypocotyl region of nematode infected Ranger alfalfa seedling grown at 25 C. Note the infection is only in the cortical region.

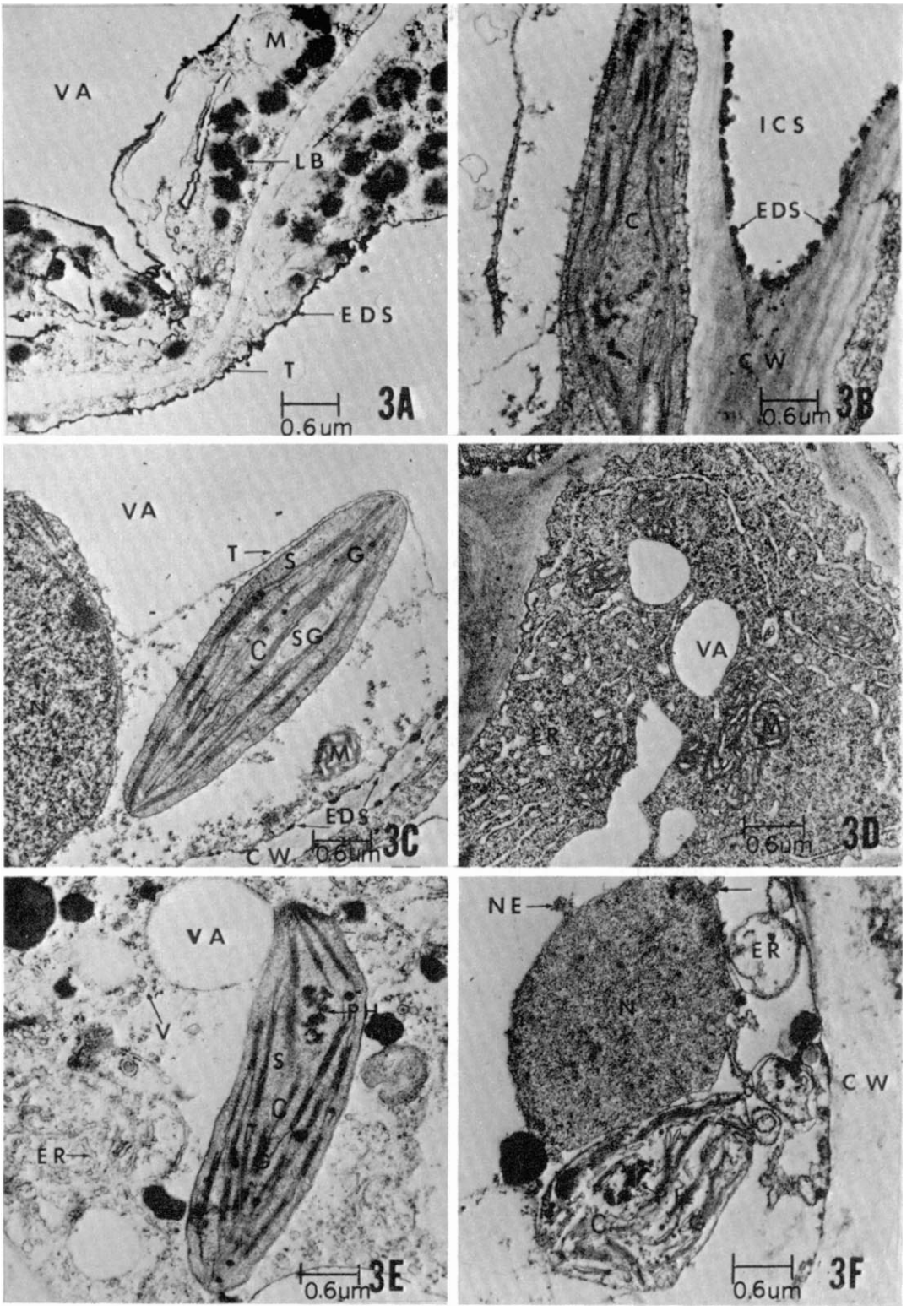
FIG. 3-(A to F). **A)** Continuous electron-dense substances (EDS) along the tonoplast in 1-day-old Ranger alfalfa seedlings grown at 15 C; **B)** Semi-continuous EDS on one side of the cell wall facing the intercellular space in 7-day-old Ranger alfalfa seedlings grown at 15 C; **C)** Separate EDS distributed along the cell wall in 7-day-old Ranger alfalfa seedlings grown at 15 C; **D)** Seven-day-old Lahontan alfalfa control plant at 15 C. Typical swollen endoplasmic reticulum (ER) in the cytoplasm could be found in all alfalfa seedlings grown at 15 C; **E)** Cell adjacent to nematode infection site in 1-day-old Lahontan alfalfa plant at 15 C. Note the 'normal' chloroplast with phytoferritin, the swollen and broken ER, and the numerous vesicles in the cytoplasm; **F)** Three-day-old heavily infected cell of Ranger alfalfa plant at 15 C. Note damaged chloroplasts, swollen ER, and a nucleus with bulging nuclear envelope.

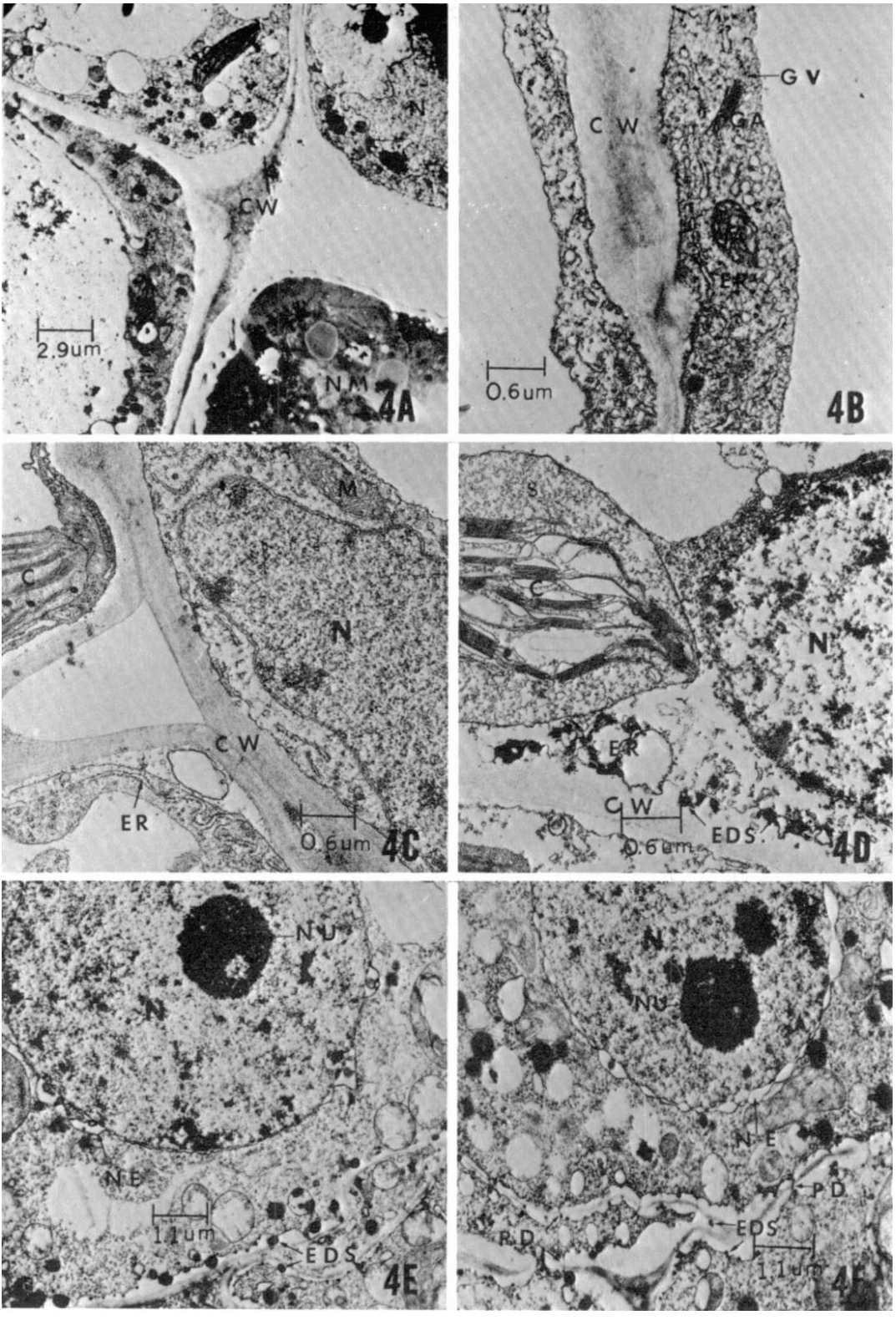
FIG. 4-(A to F). **A)** Three-day-old nematode infected Lahontan alfalfa plant at 15 C, showing nematode and host tissue which contained normal chloroplasts and cell wall; **B)** Seven-day-old nematode infected Lahontan alfalfa plant at 15 C. Note the Golgi apparatus and numerous Golgi vesicles; **C)** Seven-day-old control Lahontan alfalfa plant at 15 C with slight dilation of the ER and normal organellar structure; **D)** Seven-day-old Lahontan alfalfa plant heavily infected with nematodes at 15 C; **E)** One-day-old Ranger alfalfa infected plant at 25 C, with partially swollen nuclear envelope. Note also the EDS along the cell wall and plasmalemma interface; **F)** One-day-old severely infected Ranger alfalfa plant at 25 C. Note the intermittent dilation of the entire nuclear envelope.

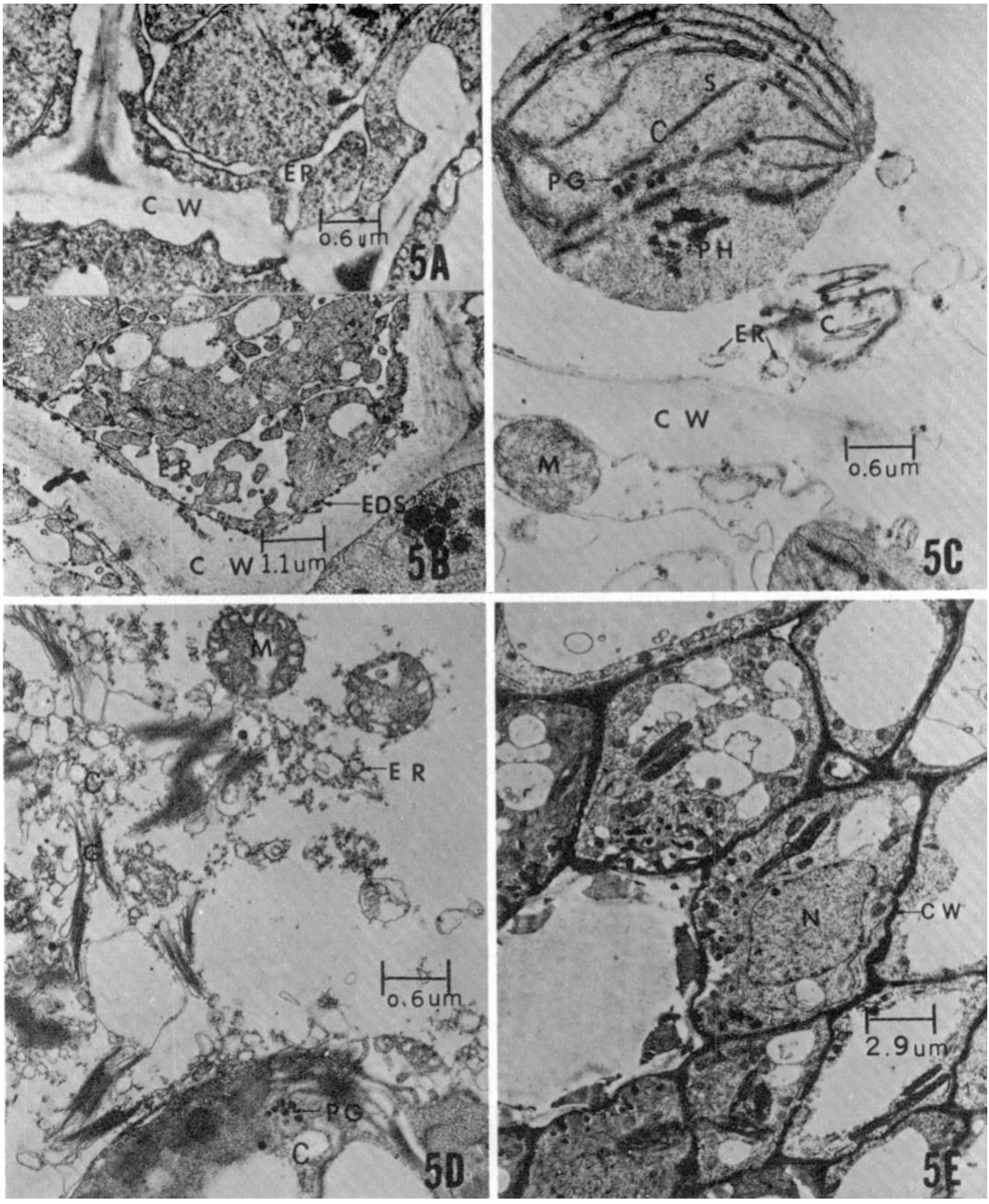
FIG. 5-(A to E). **A)** One-day-old Ranger alfalfa plant infected with nematodes at 25 C. Note the swollen endoplasmic reticulum; **B)** One-day-old control Ranger alfalfa plant at 25 C; **C)** Three-day-old severely infected Ranger alfalfa plant at 25 C with swollen and disrupted chloroplasts and vesiculation; **D)** Seven-day-old Ranger alfalfa plant heavily infected with nematodes at 25 C. Note damaged and swollen mitochondria and completely disrupted chloroplasts; **E)** Cells from the vascular region of a 7-day-old Ranger plant at 25 C.






M
VA
LB
EDS
T
0.6um
3A
ICS
EDS
C
C W
0.6um
3B
VA
T
S
G
C
SG
N
M
EDS
C W
0.6um
3C
VA
ER
M
0.6um
3D
V
PH
S
C
ER
0.6um
3E
NE
ER
N
C W
C
0.6um
3F


N
CW
2.9um
NM
4A
GV
CW
GA
ER
0.6um
4B
M
C
N
CW
ER
0.6um
4C
S
C
N
ER
CW
0.6um
EDS
4D
NU
N
NE
1.1um
EDS
4E
N
NU
NE
PD
EDS
RD
1.1um
4F



to be affected by nematode infection (Fig. 3-E, 3-F). Few lipid bodies were seen in any 7-day-old samples.

25 C: With few exceptions, the damage responses were similar in the two lines. One striking effect of the higher temperature was the partial to all-round heavily swollen nuclear envelope (NE) in 1-day-old nematode-infected Ranger seedlings (Fig. 4-E, 4-F). Also numerous plasmodesmata were present (Fig. 4-F). These symptoms were not observed in infected Lahontan plants at this temperature.

Swollen ER in sausage-like chains was observed in many cells of infected 1-day-old Ranger seedlings, and in some cells on non-infected control plants (Fig. 5-A, 5-B). Configuration of the swollen ER was similar

to that of nuclear envelopes. In 1-day-old Lahontan, swollen ER was also noted.

Separate and continuous EDS, similar to those found at 15 C, were also observed on the cell walls and tonoplasts in all treatment groups at 25 C (Fig. 4-E, 4-F, 5-B).

More Golgi apparatuses and Golgi vesicles were noted in all treatments at 25 C than at 15 C. Lipid content was higher in Lahontan than Ranger in 1-day-old samples at 25 C. Chloroplasts, cell walls, and mitochondria, however, were normal.

Three days after planting, infected tissues exhibited some responses of swollen chloroplasts in both lines. At this stage, mitochondria, cell wall, and even nuclear envelopes, appeared to be normal. Also, only a few lipid bodies were noted.

Seven days after inoculation, round and partially broken or completely disrupted chloroplasts and swollen ER were noted in the infected group at 25 C (Fig. 5-C, 5-D). Other organelles were about the same as those observed at 15 C. Vascular bundle cells were not affected by the nematode infection at either temperature (Fig. 5-E). These cells were much smaller than the cortical cells.

DISCUSSION

This study illustrates the response of alfalfa hypocotyl tissue to stem nematode invasion at two different temperatures. These data indicated an increase in stem nematode infectivity with an increase in temperature, namely from 18 to 72% in Lahontan and 92 to 100% in Ranger at 15 and 25 C, respectively. In this respect, these data agree with earlier studies (3, 9, 10, 15). These workers indicated that nematodes invaded and then left resistant plants, whereas they tended to remain in susceptible ones. The reduction in infectivity may also be related to the hypersensitivity of resistance of tolerant plants; i.e., cell walls collapse and wall-off the invading organism (3, 16, 17).

These observations in infected seedlings also indicated that the degree of damage sustained by both Ranger and Lahontan because of *D. dipsaci* infections was less at 15 C than at 25 C. Lahontan alfalfa has been regarded as tolerant to nematode infection, therefore, fewer damage responses were expected. 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., ER became swollen in sausage-like chains, numerous vesicles formed, chloroplasts swelled, and the outer membranes of the chloroplasts ruptured. One important feature about Lahontan, even at the infection site, was that the cell wall remained unchanged at both temperatures. It has been speculated by Bingefors (3, 4, 5), and Seinhorst (21) that differences in the chemical composition of the middle lamellae may account for the resistance. Chang et al. (7), however, using cytochemical techniques could detect no differences in the middle lamellae of infected and noninfected plants. Also, Albersheim et al. (1) considered pathogenesis by bacteria or fungi to be an interaction between the pathogen and the carbohydrates of the host. Such observations suggest a differential chemical composition in host cell walls, which may be the functional system in nematode-plant host relationships.

Moreover, in this study, electron-dense substances (EDS) were found to be present quite often on the cell walls and tonoplasts in all treatments. Chang et al. (7) observed similar EDS in only a few instances when alfalfa plants were grown at 20 C. Also, few, if any changes were observed in cell walls upon nematode infection. Further studies on the nature of EDS, and the differences in chemical composition of cell walls of Lahontan and Ranger, and how these may relate to nematode infections at different temperatures should be conducted.

At 25 C, heavily swollen NE in 1-day-old infected Ranger was observed. These symptoms were also observed in areas far removed from the invading nematodes, suggesting that a secretion was emitted by the invading nematodes.

In 3-day-old samples, no swollen NE were found in either lines. Whether the swollen NE recover from the damage, or break into pieces such as swollen ER is not known.

Swollen ER was observed in both Ranger and Lahontan at all ages and all treatments in plants grown at 15 C and 25 C. Whether the swollen ER was caused by an unfavorable osmolarity of the fixing solution, the cool (15 C) or high (25 C) temperature, or nematode infection needs further clarification. Any abnormal environmental condition might be able to cause the swelling of ER.

Based on the electron micrographs only, it

seems the number of lipid bodies varied with temperature and within the alfalfa lines. Since the number of lipid bodies was reported to be associated with the resistance of soybeans (22) to nematode infection, further investigations are being made in this area.

These results also show that chloroplasts in both infected and noninfected plants of all ages contained phytoferritin complexes; also the apparent normal mitochondria in all treatments indicated that the nematode infection had no effect on them.

It has been shown that cells from infected plants grown at 25 C contained more Golgi apparatuses and Golgi vesicles than those grown at 15 C. Nematode-infected cells are metabolically active (14). Morphologically, the hypocotyls of 25 C treated seedlings were much bigger than the controls and those treated at 15 or 20 C (7). Thus, it is logical to find more actively secreting Golgi apparatus and vesicles, because this organelle is known to participate in cell-wall formation (13, 19).

While the degree of damage was lower in Lahontan than in Ranger, the type of damage was similar in both lines. Moreover, less damage was observed in both lines at the lower temperature. In this study, swollen ER and lobing nuclei were observed in all samples. Also, the cell walls did not show any change in either treatment group. Electron-dense substances are found more often at these two temperatures than those reported in 20 C (7). Although this study did not answer the question why Lahontan is less tolerant to stem nematode attacks with increased temperatures, the results indicate the need for correlative biochemical studies.

LITERATURE CITED

1. ALBERSHEIM, P., T. M. JONES, and P. D. ENGLISH. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7:171-194.
2. BARKER, K. R., and J. N. SASSER. 1959. Biology and control of the stem nematode, Ditylenchus dipsaci. Phytopathology 49:664-670.
3. BINGEFORS, S. 1951. The nature of resistance to the stem nematode, Ditylenchus dipsaci (Kühn) Filipjev, in red clover, Trifolium pratense L. Acta Agric. Scand. 1:180-189.
4. BINGEFORS, S. 1961. Stem nematode in Lucerne in Sweden. II. Resistance in Lucerne against stem nematode. K. Lantbrukshogsk. Ann. 27:385-398.
5. BINGEFORS, S. 1962. On resistance to stem nematode in Lucerne. Nematologica 7:17.
6. BLAKE, C. D. 1962. Some observations of the orientation of Ditylenchus dipsaci and invasion of oat seedlings. Nematologica 8:177-192.
7. CHANG, D. C. N., W. F. CAMPBELL, and G. D. GRIFFIN. 1973. Electron microscopy of stem nematode (Ditylenchus dipsaci Kühn) induced changes in susceptible and tolerant Medicago sativa L. J. Nematol. 5:165-173.
8. GIPSON, I., K. S. KIM, and R. D. RIGGS. 1971. An ultrastructural study of syncytium development in soybean roots infected with Heterodera glycines. Phytopathology 61:347-353.
9. GRIFFIN, G. D. 1968. The pathogenicity of Ditylenchus dipsaci to alfalfa and the relationship of temperature to plant infection and susceptibility. Phytopathology 58:929-932.
10. GRUNDBACHER, F. J., and E. H. STANFORD. 1962. Effect of temperature on resistance of alfalfa to the stem nematode (Ditylenchus dipsaci). Phytopathology 52:791-794.
11. HANNA, M. R., and E. J. HAWN. 1965. Seedling inoculation studies with the alfalfa stem nematode. Can. J. Plant Sci. 45:357-363.
12. HUANG, C. S., and A. R. MAGGENTI. 1969. Mitotic aberrations and nuclear changes of developing giant cells in Vicia faba caused by root knot nematode, Meloidogyne javanica. Phytopathology 59:447-455.
13. LARSON, D. A. 1965. Fine structural changes in the cytoplasm of germinating pollen. Am. J. Bot. 52:139-154.
14. PAULSON, R. E., and J. M. WEBSTER. 1970. Giant cell formation in tomato roots caused by Meloidogyne incognita and Meloidogyne hapla (nematode) infection. Can. J. Bot. 48:271-276.
15. REYNOLDS, H. W., W. W. CARTER, and J. H. O'BANNON. 1970. Symptomless resistance of alfalfa to Meloidogyne incognita acrita. J. Nematol. 2:131-134.
16. RIGGS, R. D., and N. N. WINSTEAD. 1959. Studies on resistance in tomato to root-knot nematodes and on the occurrence of pathogenic biotypes. Phytopathology 49:716-724.
17. ROHDE, R. A. 1965. The nature of resistance in plants to nematodes. Phytopathology 55:1159-1162.
18. ROHDE, R. A., and W. R. JENKINS. 1957. Effect of temperature on the life cycle of stubby-root nematodes. Phytopathology 47:29.
19. ROSEN, W. G., S. G. GAWLIK, W. V. DASHEK, and K. A. SIEGESMUND. 1964. Fine structure and cytochemistry of Lilium pollen tubes. Am. J. Bot. 51:61-71.
20. SAYRE, R. M., and W. B. MOUNTAIN. 1962. The bulb and stem nematode (Ditylenchus dipsaci) on onion in southwestern Ontario. Phytopathology 52:510-516.
21. SEINHORST, J. W. 1956. Population studies on stem eelworms. Nematologica 1:59-164.
22. SHEETZ, R. W., and H. W. CRITTENDEN. 1966. Histochemistry of soybean varieties resistant or susceptible to Meloidogyne incognita acrita. Phytopathology 56:586 (Abstr.).
23. TYLER, J. 1933. Development of the root-knot nematode as affected by temperature. Hilgardia 7:392-415.
24. WALLACE, H. R. 1958. Movement of eelworms. 2. A comparative study of the movement in soil of Heterodera schachtii Schmidt and of Ditylenchus dipsaci (Kühn) Filipjev. Ann. Appl. Biol. 46:86-94.