# Stem Nematode Infection of Resistant and Susceptible Cultivars of Alfalfa

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## ABSTRACT

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Chromatin content of granular cells surrounding nematode-induced cavities was increased in both resistant and susceptible cultivars of alfalfa. Increases were consistent in 8- and 12-day infections of Buffalo, a susceptible cultivar, but chromatin content of Washoe, a resistant cultivar, decreased after 8 days. Cellular disruption was progressive in both cultivars. After 16–18 days of infection, cells of Washoe were not functioning, whereas cells of Buffalo were disrupted but still functioning. Mechanical disruption of cells with a fine glass rod did not cause galling. Ligninlike reactions around wounds were stronger in Washoe than in Buffalo.

*Additional key words: Ditylenchus dipsaci, Medicago sativa,* photometry, resistance mechanism, ultrastructure.

Nematodes enter young tissues in early stages of infection and congregate at the stem apex near developing leaves (16). According to Krusberg (11), histologic damage to alfalfa plants is evident 6 hr after inoculation with *Ditylenchus dipsaci* (Kühn) Filipjev. Nematodes are found in portions of the terminal bud, in the cotyledons slightly above the axils, in the embryonic leaves, and in the shoot apex. Affected cells fail to stain normally. After 24 hr, many cell and tissue changes have occurred. Cortical cells become irregular in shape and often separate from one another. The cytoplasm is withdrawn and walls collapse, causing large cavities to form. In resistant cultivars of alfalfa, *D. dipsaci* causes cells near the nematode to enlarge and separate from one another (4). These effects are evident before actual contact between the cell and the nematode (12).

No subcellular differences were noted between tolerant (Lahontan) and susceptible (Ranger) cultivars in 1-, 3-, and 7-day infections (2). Some cells surrounding the cavities contained dense cytoplasm similar to nutritive cells described by Goodey (6). However, Goodey found only slight structural differences between the resistant cultivar Washoe and the susceptible cultivar Buffalo. In Washoe, more heavily lignified walls developed in the vicinity of the nematode, interfering with nematode movement, but cells died more quickly than in Buffalo and the damage did not become as widespread. DNA content of granular cortical cells surrounding the nematodes in galled areas never increased as much in Washoe as in Buffalo and decreased from the eighth through the twelfth day.

Development of alfalfa (*Medicago sativa* L.) lines resistant to the stem nematode would be facilitated if young plants could be tested for resistance. The location of an anatomic or ultrastructural marker would aid in identifying resistant plants and thus speed up the testing process.

Our study was designed to compare the structural differences as seen by light and electron microscopy between the resistant cultivar Washoe and the susceptible cultivar Buffalo infected with *D. dipsaci*. In addition, light microscopic examination was done on plants of both cultivars that were mechanically injured to simulate the damage caused by nematode movement.

## MATERIALS AND METHODS

Seeds of Washoe (Nevada Foundation Seed Stock Inc. DW966C, 1969) (7) and Buffalo were germinated in aerated water. The seedlings were planted in fine sand in 30-ml individual clear plastic condiment cups vented at the base to provide drainage. The containers were placed in rectangular metal casings, and each container was capped with another clear nonvented container to provide high humidity. Seedlings were grown for 2 days in a controlled-environment chamber under fluorescent lights of 10,760 lux intensity, 14-hr days and 10-hr nights, both at 20 C.

The nematodes used for inoculation were extracted from plants collected from infested fields near Stillwater and Coyle, OK. A modified Christie-Perry technique was used in which the plants were immersed in water for 15 min and the supernatant water was poured through a 1.19-mm strainer and a 44-$\mu$m screen. Material remaining on the screen was rinsed onto a double thickness of facial tissue supported by a screen in a nonvented plastic pot filled with water. The nematode suspension was concentrated by serial

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decanting, then poured into a counting dish.

Plants were inoculated by handpicking nematodes into a drop of water placed between the cotyledons. Ten nematodes, including adult males, adult females, and larvae, were transferred to each plant, except the control seedlings. These were spaced alternately in the metal trays, and a drop of water without nematodes was placed between the cotyledons.

Plants of both cultivars were grown in a controlled-environment chamber for 10 days after treatment or inoculation. To insure that infestation had occurred, only galled plants were used in tests for nematode damage.

To evaluate mechanical damage effects, a 0.01-mm glass rod was inserted to approximately one-third of the stem diameter, then removed. Damaged stem segments were fixed after 10 days.

**Photometry.** Segments of hypocotyls near the cotyledonary node were killed and fixed in modified Bouin's fixative (1), dehydrated, infiltrated, and embedded. Longitudinal sections (10 μm) were cut with a rotary microtome and mounted in non-Formalin gelatin adhesive (1). DNA was stained by the Feulgen technique, using a 20-min hydrolysis in 5 *N* HCl, a 2-hr staining time, and three bleaches. Maximum absorption was at 550 nm. The plug method of visible wavelength microspectrophotometry was used with a Farrand monochromator and a Zeiss bright field microscope (1). A 150-W xenon arc lamp was used for illumination. For most treatments, 100 nuclear measurements were performed. Nuclear radii were measured with an ocular micrometer.

**Histology and enzyme localization.** Tests for saturated and unsaturated lipids, pectins, cellulose, and lignin were done 10 days after treatment. Hypocotyl segments were quick-frozen and sectioned at −18 C to a thickness of 16 μm using a cryostat (Model CTD International Harris Cryostat). Meyer's albumen adhesive and Farrant's mounting medium were used in all histochemical procedures. Results were recorded photographically (8,9). Lipids were examined by staining with Sudan black B (3). Ruthenium red was used for pectins, phloroglucinol and hydrochloric acid for lignin, and iodine and sulfuric acid for cellulose (8). The enzyme phenolase (2.20.3.1 *o*-diphenol:oxygen oxydoreductase) (3) was tested because it is often involved in wounding or cutting responses in plants.

**Histology.** Ten days after inoculation, hypocotyl sections of both cultivars were fixed in Formalin-propionic acid-ethanol (1:1:18 v/v) fixative for 24 hr. Tissues were embedded in Paraplast (Sherwood Medical Industries, St. Louis, MO) and stained with Johansen's quadruple stain (9).

**Ultrastructure.** Plants were harvested 8, 12, 16, and 18 days after inoculation. Hypocotyl sections of infected alfalfa seedlings and controls were fixed in cacodylate-buffered glutaraldehyde for 2 hr at 6 C, rinsed four times during 1 hr in buffer, and postfixed in 1% osmium tetroxide (14). The sections were dehydrated with ethanol, infiltrated with Spurr's epoxy resin of the regular formulation, and polymerized at 70 C for 8 hr (15). Thin sections (silver gray) were mounted on 300-mesh copper grids, stained with uranyl acetate and lead citrate, and viewed with a Phillips 200 electron microscope.

TABLE 1. DNA content of cell nuclei from granular cortical cells of *Ditylenchus dipsaci*-infested and control cortical cells of alfalfa

| Treatment | Plug optical density | Standard deviation | Absorption units/ nucleus | Increase (%) | Nuclear size (μm) | Tested (no.) |
|---|---|---|---|---|---|---|
| Day 8 | | | | | | |
| Buffalo | | | | | | |
| Control | 0.13 | ±0.05 | 0.32 | ··· | 6.0 | 111 |
| Nematode-infested | 0.23 | ±0.09 | 0.56 | 75 | 11.0 | 100 |
| Washoe | | | | | | |
| Control | 0.17 | ±0.05 | 0.44 | ··· | 6.0 | 100 |
| Nematode-infested | 0.27 | ±0.07 | 0.71 | 61 | 10.5 | 100 |
| Day 12 | | | | | | |
| Buffalo | | | | | | |
| Control | 0.13 | ±0.04 | 0.32 | ··· | 6.0 | 100 |
| Nematode-infested | 0.33 | ±0.11 | 0.81 | 153 | 12.0 | 88 |
| Washoe | | | | | | |
| Control | 0.17 | ±0.04 | 0.44 | ··· | 6.0 | 100 |
| Nematode-infested | 0.24 | ±0.06 | 0.63 | 43 | 9.5 | 38 |

## RESULTS

Nematode infection of both the resistant cultivar Washoe and the susceptible cultivar Buffalo was followed by gall formation within 7–10 days. Galls usually were in the hypocotyl area of the stem slightly above the cotyledonary node. Leaves became distorted and reduced in size, and the apex was stunted.

**Photometry.** The amount of DNA per cell in granular cells of plants varied with treatment. At 8 days, both Washoe and Buffalo showed increases in DNA content. At 12 days, however, Buffalo had DNA levels nearly three times the control levels, whereas the levels in Washoe were less than at 8 days (Table 1).

**Histology of nematode-infested plants.** Sections of galls from Buffalo contained portions of nematodes and eggs. The galls consisted of large cortical cavities with darkly stained and granular cells on the peripheries. Some of the cavities contained cell walls that showed heavy ligninlike staining with safranin (Fig. 1B). Some hypertrophy of cells surrounding cavities was evident. Nematodes were in all parts of the cortex from just inside the epidermis to near the vascular cylinder (Fig. 1C). Similar galls developed in Washoe, but cellular destruction and cavity formation were less extensive than in Buffalo (Fig. 1A). Cell walls surrounding the cavities showed very heavy ligninlike reactions. Washoe had fewer enlarged cortical cells with granular cytoplasm than Buffalo. The entry point of the nematode was heavily stained with safranin.

**Histology of mechanically injured plants.** Galls did not develop on mechanically injured plants of either cultivar. Cells in the path of the glass rod were destroyed in both cultivars, but the surrounding cortical cells were normal. Lignification was light to moderate in Buffalo and heavy in Washoe.

**Histochemistry of nematode-infested plants.** Results of the Sudan black B test for lipids appeared similar in control and infested plants of both cultivars, as did results of the tests for pectins and cellulose. Phloroglucinol-HCl reactions were greatest around the gall and the entrance point of the nematode in infested plants; response in noninfested plants was slight. The amount of lignification around galled portions and entry points was greater in Washoe than in Buffalo.

**Histochemistry of mechanically injured plants.** Pectin, cellulose, and lipid reactions in wounded plants of both cultivars were similar to those in control plants, but staining for lignin was increased. The degree of staining in secondary walls was greater in Washoe than in Buffalo.

**Enzyme localization.** Control and experimental plants of both cultivars showed no change in phenolase activity. Both the nematode-infested and the mechanically injured plants responded in the same manner as the controls. Activity in galled or injured portions of a stem was not higher than that in other portions of the same stem section.

**Ultrastructure.** In Washoe 8 days after inoculation, cortical cells showed an increase in free ribosomes, swollen endoplasmic reticulum, some broken membranes, and collapsed walls. In Buffalo 8 days after inoculation, cells showed massed endoplasmic reticulum, an increase in free ribosomes, and invagination of membranes. At 12 days, separation of chloroplast lamellae, swollen endoplasmic reticulum, broken nuclear membranes, an increase in free ribosomes, and an electron-dense tonoplast were observed in Washoe (Fig. 1D) and broken membranes, swollen endoplasmic reticulum, and an increase in free ribosomes in Buffalo. At 16 days, disorganized endoplasmic reticulum, large vacuoles, disrupted nuclear membranes, separated chloroplast lamellae, and mitochondria lacking internal structure were seen in Washoe, and deposits on the walls of cavities near the nematode, no endoplasmic reticulum, disrupted nuclear membranes, separated chloroplast lamellae, and mitochondria lacking internal structure were observed in Buffalo (Fig. 1E). At 18 days, broken membranes,

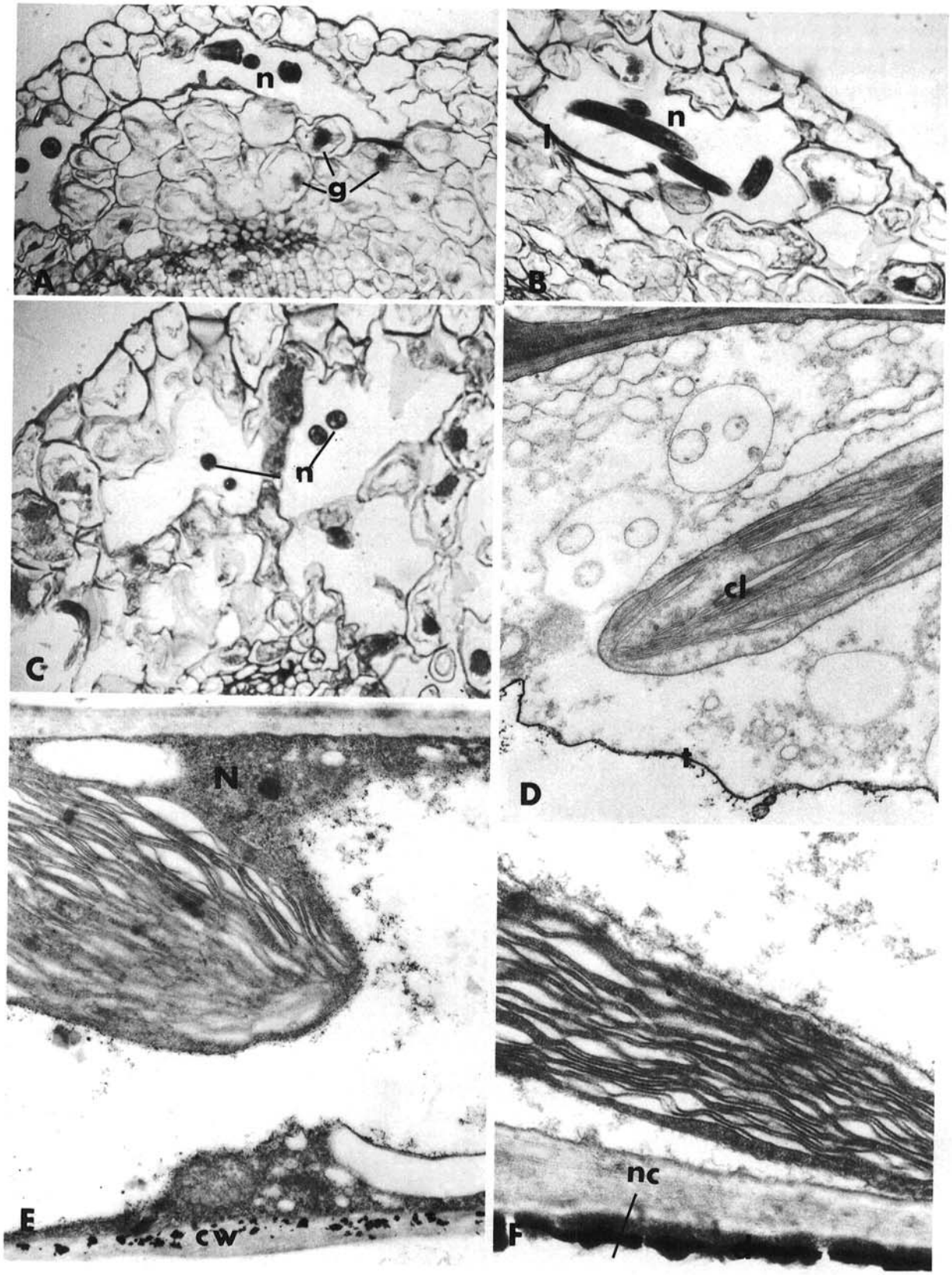


**Fig. 1. A–C,** Infection of alfalfa with *Ditylenchus dipsaci* at 10 days (×100): **A,** Washoe cultivar showing cavity with nematodes (n) and a small number of granular cells (g). **B,** Buffalo cultivar with nematode (n) and lignified cavity wall (l). **C,** Buffalo cultivar showing distribution of nematodes (n) within a gall. **D–F,** Ultrastructural degeneration resulting from *D. dipsaci:* **D,** Washoe cultivar at day 12 showing swollen chloroplast lamella (cl), inflated endoplasmic reticulum, and dark-staining tonoplast (t) (×39,600). **E,** Buffalo cultivar at day 16 showing dark deposits in wall (cw), lamellar structure of chloroplast, and disintegration of nucleus (N) and cytoplasm (×13,500). **F,** Washoe cultivar at day 18 showing heavy deposits (d) on wall near nematode cavity (nc), degenerating tonoplast and organelles (×49,500).

separated chloroplast lamellae, few ribosomes, and no endoplasmic reticulum were noted in Washoe; some wall outgrowths as well as a deposit on the wall facing the nematode cavity also were seen (Fig. 1F). At day 18, separated chloroplast lamellae, increased free ribosomes, mitochondria lacking internal structure, and little endoplasmic reticulum were observed in Buffalo. Control specimens contained normal chloroplasts, intact cytoplasmic membranes, and normal mitochondria; rough endoplasmic reticulum was widespread in cell cytoplasm.

## DISCUSSION

The alfalfa cultivars Buffalo and Washoe differed in histologic response to nematode attack. Cavities within the galls of Washoe plants were smaller and more heavily stained with phloroglucinol-HCl than those of Buffalo plants. Possibly, lignification limited the size of the cavity and confined the nematode. Also, fewer cells with dark granular cytoplasm were observed in Washoe than in Buffalo. Goodey (6) suggested that these cells are a food source; lack of nutritive cells should be a factor in limiting the growth of the nematode population.

Granular cells surrounding the nematode cavities appear to be polyploid in chromatin content. Endo (5) found that *Heterodera glycines* caused an increase in size and DNA content of nuclei in soybean roots. Increased chromatin amounts may be correlated with an increase in synthetic power in the cell that translates to increased cell products. In Washoe, chromatin content increased slightly during the early stages, but enough to support the nematode and its reproduction may not have been produced. Chromatin content continued to increase in Buffalo, and cell products, possibly those necessary for nematode survival, were produced.

Mechanical injury did not cause galling. Mechanical damage alone is not important in gall formation, and other factors are probably responsible for stimulating gall production. Lignification responses followed injury by either the glass rod or the nematode. Wall thickening was greater in the resistant cultivar and may be of value during testing for resistance. Nematodes walled off by heavy lignification would be unable to feed, and the area they could infest and their ability to reproduce would be limited.

Compounds other than lipids, pectins, and cellulose may be involved in the plant's response to nematodes, or control may reside at a different level. Lack of change in pectins agrees with findings by other workers unable to show that pectinases are important factors in gall production, although pectinases are found in nematode extracts (10,13).

Cellular disruption was progressive in nematode-infested areas of alfalfa stems. The first effects were cell membrane degradation and loss of ribosomes from the endoplasmic reticulum. Later, the nucleus, chloroplasts, and mitochondria were disrupted. Effects on the resistant cultivar Washoe and the susceptible cultivar Buffalo were not distinctly different. Washoe stems showed a large amount of cellular damage through the 10th day, whereas Buffalo sustained greatest destruction at 18 days. Washoe had early cellular disruption and reached a totally nonfunctioning state by 16–18 days. These long-term results are different than those for plants 1–7 days old studied by Chang et al (2). Early loss of cell function around the nematode may be tied to resistance factors in Washoe. Histochemical analysis at the cellular level might be useful in elucidating the compounds involved in Washoe resistance.

The diseased state is more fully understood when its development is followed at both light and electron microscopic levels. Addition of nuclear studies and histochemical tests provides more information, and in our study the state of the nucleus appeared to be of major significance. The lessened amount of resistant as compared with susceptible cultivar nuclear DNA over a period of 4 days indicates that nuclear control may be important in restricting the nematode within the plant. Over the same period, cellular decline is also evident to a greater extent in Washoe than in Buffalo.

A number of interrelated factors are involved in general resistance, and nuclear phenomena will be important in understanding the significance of these factors.

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