A STRUCTURAL STUDY OF HOST-PARASITE RELATIONSHIPS OF *DITYLENCHUS DIPSACI* (KUHN) FILIPJEV AND *MEDICAGO SATIVA* L.

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A STRUCTURAL STUDY OF HOST-PARASITE RELATIONSHIPS

OF DITYLENCHUS DIPSACI (KUHN) FILIPJEV

AND MEDICAGO SATIVA L.

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CHAPTER I

INTRODUCTION

The plant parasitic nematode, *Ditylenchus dipsaci* (Kuhn, 1857) Filipjev, 1936, is an important pathogen of alfalfa (*Medicago sativa* L.) in Oklahoma and throughout the world's alfalfa growing areas. Biological races of *D. dipsaci* have developed in response to selection pressures. The race which infests alfalfa exhibits a high degree of host specificity. Other races of *D. dipsaci* attack other crop and ornamental plants and are especially important in bulb producing countries of the world (Thorne, 1961).

Existing control measures include either crop rotations with three to five year intervals between alfalfa crops or sanitary procedures to keep the pest from infesting clean fields. These procedures have been found to be expensive and at best marginally effective. Chemical control procedures employing systemic nematicides have been developed but due to the high rates of application needed for control these procedures are not permitted under present pesticide regulations.

Development of resistant varieties has become the most promising avenue to the control of this plant pathogen. The resistance of the plants to nematode pathogens is based on the amount of nematode reproduction which takes place in plants and is not dependent on plant survival. The host plant may have total resistance, in which case the nematode does not reproduce at all, or reproduction of the nematode may

be depressed only slightly. Susceptible plants are classified as those which do not hinder nematode reproduction (Rhode, 1960). The reaction of resistant plants may vary from local necrosis to the death of the entire plant with the nematode reproduction rate varying accordingly.

Fast, reliable procedures for screening resistant and susceptible plants would greatly facilitate the development of resistant varieties. Possible histological or ultrastructural markers are present which would aid in this goal. This study was designed to compare the reactions of two varieties of alfalfa, susceptible Buffalo variety and resistant Washoe variety. Various histochemical tests and histological observations, ultrastructural studies and spectral studies on DNA content and attractiveness to nematodes were part of the work. Tests were conducted in which both resistant and susceptible varieties of plants were mechanically injured and their reactions to identical histochemical procedures and histological observations were recorded. These tests were an attempt to simulate mechanical damage caused by nematode feeding and movement. Plant response to injury could then be contrasted with the plant response to both mechanical and enzymatic damage caused by the nematode. Ultrastructural studies of normal and galled plants and photometric determination of DNA content in cells surrounding sites of infestation were used to follow up the histological observations. Attractiveness of the varieties to nematodes could play an important part in the initial infection process so this subject was explored also.

CHAPTER II

LITERATURE REVIEW

General

Since the mid-nineteenth century _D. dipsaci_ has become important to alfalfa growers in temperate climates throughout the world. Cool, moist conditions favor the reproduction of the nematode. Nematodes cause a loss of three to five percent of the U.S. alfalfa crop each year (Hunt and Peaden, 1972). The first yield decline is usually in three years and fields are unproductive in five to six years. In irrigated fields with recycled water, stands may be unproductive in two to three years (Hunt and Peaden, 1972). _D. dipsaci_ is the most wide-spread and economically important nematode affecting alfalfa in the U.S. (Graham, _et al._, 1972). Control methods include rotation and use of clean seed. Chemical methods of control are not practical at this time due to the cost and the lack of safe chemicals. Resistant varieties are effective in areas for which they are adapted.

Many descriptions of stem nematode infection have been published including those of Barker and Sasser (1959); Seinhorst (1959); Thorne (1961); Krusberg (1962); Griffin (1968); Dropkin (1969); Muse and Williams (1969); and Evans (1971). Symptoms include short internodes, blistering, petiole and leaf vein curvature (Hawn, 1969) and white flagging (Evans, 1971; Campbell and Griffin, 1973). Besides alfalfa, races of

D. dipsaci also parasitize clover, onion, bulbs, phlox and strawberry (Thorne, 1961). Many biological races have been discovered which will not reproduce on other than their preferred hosts. In the alfalfa race of D. dipsaci reproduction is presently known only on alfalfa and a related forage legume, sainfoin, Onobrychis viciaefolia Scop. (Griffin, et al., 1975). The alfalfa race also caused symptoms but did not reproduce on sweet clover, onion, tomato, sugar beet and wheat and may be responsible for decreased yields of these crops following alfalfa. D. dipsaci was most pathogenic on alfalfa and onion and least pathogenic on wheat (Griffin, 1975). A number of studies have dealt with various soil and environmental factors and their effect on the pathogenicity and migration of D. dipsaci. Lewis and Mai (1960) found that the nematode overwinters as fourth stage (pre-adult) larvae or adults and may persist for two years in the soil in the absence of a host crop. Nematodes invade host plants at or above the soil surface and can survive drying in 50% relative humidity for 34 days (Wallace, 1962). Movement of nematodes in the soil is affected by the amount of soil water, aeration and the soil temperature (Hunt et al., 1972). Wallace (1962) found that the nematodes move farther in sandy loam than in clay soil but there was no difference in infection rate between the two soils. Elgin, et al. (1975) found fine sandy loam to be superior to fine sand for penetration of nematodes and better penetration was achieved if the soil was not watered immediately after planting and inoculation. Reduced resistance was noticed by Griffin (1968) at temperatures from 25-30 C but both resistant and susceptible varieties were infected over a range of 5-30 C. Optimum temperature for invasion and reproduction were found to be 15-20 C (Barker and Sasser,

1959) and the life cycle may be completed in 19-23 days in susceptible varieties at this temperature (Graham, et al., 1972). Attraction of D. dipsaci to plants and to chemical substances has also been investigated. Griffin (1969) found that after seven days nematodes introduced 12.5 mm from the plant root had infected all seedlings tested, both resistant and susceptible, but there were 2.59 times more nematodes in the susceptible seedlings. Twenty-five millimeters away, inoculation showed nearly equal infection rates and 50 mm found eight percent of the susceptible infected and none of the resistant. Klingler (1965) found attraction in the immediate vicinity of roots but not more than two cm away. He also found carbon dioxide and a number of amino acids attractive to D. dipsaci. Attraction by temperature at 10° C and a preference for small particle size and high moisture were noted by Wallace (1961).

Disease Description

In early stages of infection, nematodes enter young buds at the soil line and congregate near developing leaves and at the stem apex. The base of an infected stem becomes enlarged, discolored and ridged and necrosis may follow invasion (Thorne, 1961). Krusberg (1960a) found damage six hours after inoculation. Nematodes were found in the cotyledons, in the terminal bud, and at the axils of the cotyledons. After twenty-four hours many cell and tissue changes had taken place. Cortical cells were separated and irregular in shape. The cytoplasm was withdrawn and walls had collapsed causing large cavities to form. Leaflets contained masses of dense granular cells (Krusberg, 1961). J. B. Goodey (1939) described the dense cells as nutritive cells. He

proposed that these cells were later fed upon by the nematodes. Nematodes initiate galls only in very young, active, growing and differentiating tissue, and galls do not form in older tissue (Krusberg, 1960). In studying the location of nematodes in developing infections, Palo (1962) noted that plants inoculated one month prior to examination contained nematodes in all parts of the plant while those inspected two to three months after inoculation had nematodes only in the basal and apical regions.

Krusberg (1963) set down several possible mechanisms for gall initiation by stem nematodes. A direct mechanism included release of foreign materials into the plant and an indirect triggering of an internal plant mechanism was also postulated. Galling will only occur in young tissues. A single nematode can initiate a gall and changes induced in cells are similar to changes induced by plant growth regulators such as IAA and IBA. In other cases plant growth regulators have been found to inhibit gall formation (Krusberg, 1963).

Auxin Effects

Plant growth regulators have been implicated in gall formation by several authors (Sandstedt and Schuster, 1966; Krusberg, 1963; Viglierchio, 1971; and Viglierchio and Yu, 1965, 1968; Balasubramean and Rangaswamu, 1962; and Mountain, 1960). Although the precise action of indole-3-acetic acid is not known at this time, some effects are well known. Fan and Machlachlan (1967) noted synthesis of ribonucleic acid and cellulase in pea epicotyls treated with IAA. IAA also induced swelling of parenchyma cells and cell division. Three days after treatment the amounts of protein and DNA in the cells were double, RNA

tripled and cellulase was twelve to sixteen times the level of the controls. They concluded that RNA synthesis induced by IAA is required for cellulase synthesis and lateral cell expansion regardless of whether cell division occurred.

The relationship between nematodes and plant growth regulators was investigated by Mountain (1960). He felt that proteolytic activity by nematode enzymes would release some amino acids which could be converted to IAA or that bound IAA could be released by the enzymes. By responding to the IAA, the plant would provide the nematode with a suitable site for feeding and reproduction. Studies with Meloidogyne javanica indicated the presence of IAA and indole butyric acid in galled roots but not healthy ones (Balasubramean and Rangaswamu, 1962). Viglierchio and Yu (1968) tested roots infested with three species of Meloidogyne and found that the growth regulators present differed with the species of nematode. Heterodera sp. did not change the auxin level to any noticeable extent in infected plants. Sandstedt and Schuster (1966a) found that IAA increased growth of callus in stem segments, promoted root initiation and inhibited bud initiation while Meloidogyne incognita did not have these effects. They concluded that auxins were not freed by the nematodes nor secreted by the nematodes but rather the nematodes enabled the tissues to retain auxin that normally would be basally transported. They also noted that nematodes did not secrete detectable amounts of auxin or cytokinin but did promote growth in some manner. Nematodes required both auxin and cytokinin for successful development (Sandstedt and Schuster, 1966). The production of an auxin inactivator was postulated by Viglierchio and Yu (1965) who found less IAA in D. dipsaci infected stem tips than in controls. Reduced growth

in seedlings was also initiated by extracts of the nematode. Boiled extracts did not reduce growth.

Galling Response

Dropkin (1955) described the responses of plant tissues to nematodes as hyperplasia, necrosis, dissolution of parts of cell walls and alterations in the differentiation of tissues. Pathogenic effects of *Ditylenchus dipsaci* were attributed to a disturbance of auxin balance in the host by Viglierchio and Yu (1965). They felt that the nematode produces an auxin inactivator that enables it to stunt stem apices and change galling potential due to decreased amounts of IAA found in infected stem tips. Reduced growth in seedlings infiltrated with nematode extracts compared to boiled extracts indicated an enzyme factor. This view is similar to that of Krusberg (1963) who described the mechanism of galling. Factors involved in galling are that only young tissues gall, plant growth regulators inhibit gall formation and cellular changes induced are similar to growth regulator induced changes.

Studies of enzyme activity in galls caused by *D. dipsaci* are mostly limited to pectinases. Normally plant cells die rapidly when attacked by pectinases but do not die next to galled portions of a stem (Wood, 1960). Tracy (1958) and Krusberg (1960) did not detect pectinases in homogenates of *D. dipsaci*. Krusberg (1963) determined that pectinases were not important in the disease development of the plant and (1967) that pectinases were localized and used only in stylet penetration. Muse and Williams (1969) reported pectinase and cellulase activity for two populations of *D. dipsaci*. Using nematode

extracts they determined that the gall-forming nematodes decrease the viscosity of pectin. Riedel and Mai (1971) found that pectolytic enzymes are associated with nematodes in callus culture but could not determine the source of the enzymes. Tracey (1958) found chitinase and cellulase in extracts of *D. dipsaci* and two other species. Goffert and Heiling (1962) reported amylase, invertase and pectinase in salivary secretions of *D. dipsaci*.

Structure

Healthy plants of both resistant and susceptible varieties of alfalfa show no apparent anatomical differences. Infested tissues of Buffalo variety show hypertrophy, necrosis and development of densely staining cells surrounding the nematode. Washoe variety shows less necrosis and fewer densely staining cells. Histochemical tests of the two varieties have shown increased lignification around the nematode entry point in affected tissues in Washoe as compared to Buffalo (Reed, 1974). Dropkin (1955) found hypertrophy and hyperplasia resulting from *D. dipsaci* infection and noted the location of the nematode indicated a preference for intercellular spaces of cortical and parenchyma tissues. Bingefores (1962) and Krusberg (1963) noted cell enlargement, a loss of chloroplasts and larger intercellular spaces due to infection. Effects often are noticeable before direct contact is made with the nematode. Giant cell formation by root knot nematodes has been studied extensively (Paulson and Webster, 1970; Bird, 1961). Bird (1961) described giant cells as resembling meristematic cells in their numerous organelles and metabolically active state. Changes in cell walls, vacuoles, cytoplasm and organelles of giant cells were noted by

Paulson and Webster (1970). Similar studies of alfalfa indicate increased endoplasmic reticulum, ribosomes, golgi apparatus and vesicles as well as nuclear changes in the cells surrounding the galls formed by *D. dipsaci*. Ultrastructural studies of Lahontan and Ranger varieties of alfalfa showed no indication of subcellular differences between these resistant and susceptible varieties. The infection rate and degree of damage were different for the two lines at different temperatures (Chang, 1971). Huang and Maggenti (1969) noted mitotic aberrations in giant cells of *Vicia faba* with *Meloidogyne javanica* infestations. Irregular thickening of cell walls of giant cells caused by *Meloidogyne* sp. were noted by Jones and Northcote (1972, 1972a) and Paulson and Webster (1970). These thickenings were described as being characteristic of transfer cells by Jones and Northcote (1972) who felt that they developed in response to the flow of solutes out of the cell and into the nematode. Increased metabolic activity was determined to be necessary for cell life due to the massive withdrawal of nutrients by the nematode. Death of the nematode will cause a breakdown of the giant cell. The formation of dense cytoplasm in cells may also be a nematode induced factor (Bird, 1962). Rebois *et al*. (1975) studying soybean roots infected with *Rotylenchulus reniformus* found that resistant plants had initial infection stages similar to susceptible plants. Later the resistant plant had thickened cell walls and more boundary formations. Uncontrolled lysis and the lack of an anabolic stage were also important in resistant plants. Susceptible plants reached an anabolic stage in which there was interruption of wall lysis and secondary wall thickening occurred. This stage was seen as necessary to provide a feeding site for the nematode.

Resistance

Resistance to the stem nematode is the most acceptable control at present. Breeding programs for resistant varieties of alfalfa have been developed throughout the world. One difficulty which must be overcome is that races of *D. dipsaci* which attack alfalfa vary from one region to another. Alfalfa varieties which are resistant in Argentina or Sweden or California are not in other regions (Grundbacher and Stanford, 1962). Resistance to nematodes may be expressed by lack of reproduction, failure of the host to produce a feeding site and hypersensitive reactions. Resistance does not become apparent until after initial stages of infection. In varying types of infection the more intimate the interaction, the more often resistance is found. Ectoparasites require little host modification so resistance is rare, while endoparasites modify the host to a large extent and resistance is more common (Rhode, 1972). In alfalfa some resistance is found in most populations. There is little or no association between resistance and nematode penetration. The ability of the nematode to infect the plant may be altered by soil moisture, temperature and aeration (Hunt, *et al.*, 1972). Once inside alfalfa seedlings *D. dipsaci* causes a swelling of the cotyledonary node. This is a response to infection and does not appear to relate to reproductive capabilities (Elgin *et al.*, 1975). Griffin and Hunt (1972) found an inverse relationship between galling and age of the plants at inoculation. The older the plants at inoculation the fewer galls are produced.

Inheritance of nematode resistance is considered to be tetrasomic by Grundbacher and Stanford (1962) and by Hunt *et al.* (1972). Polyploidy produces a change in the phenotypic ratio of offspring from 3:1

in diploids to 35:1 which is called tetrasomic inheritance. This greatly reduces the chance of establishing recessive mutations but dominant mutations may be promoted (Stebbins, 1971). Tetrasomic inheritance occurs with increases in ploidy level as simple ratios become overshadowed by the increase in chromatin for each trait. Multiple genes may also be involved according to tests by Sherwood *et al.*, (1967) who showed a gradation of resistance comparing swelling and number of eggs produced in susceptible varieties. Resistant varieties are selected from a susceptible but adapted variety for a particular region. Outcrossing in alfalfa breeding is important in maintaining high yields so inbreeding to isolate resistance is often ineffective. Cytoplasmic male sterility in alfalfa has been achieved only recently (Lowe, *et al.*, 1974). Original highly resistant stock in the U.S. was from Turkestan (Bolton, 1962). By crossing Turkestan variety with other resistant varieties from the U.S. several varieties have been developed with resistance to a number of diseases and nematodes (Hunt, *et al.*, 1972). A number of resistant varieties have been developed from the Turkestan parent including Lahontan, Washoe, Caliverde 65, and Apachee (Hunt and Peaden, 1972; Hunt *et al.*, 1971).

Resistance to *D. dipsaci* in oats is associated with reproduction and is due to a dominant gene (Griffiths, *et al.*, 1957). Red clover resistance is probably due to several genes depending on the species (Bingefores, 1956). In tomato, resistance to root-knot nematode is felt to be due to a single dominant gene. Three different genes for resistance were discovered in different cultivars of tomato (Sidhu and Webster, 1973).

Studies of speciation and races in D. dipsaci have been made by several workers. Gibbens and Grandison (1968) were unable to detect definite differences in antisera produced from homogenates of several biological races of D. dipsaci but felt that in the future serology may be important in distinguishing the races. Hussey and Krusberg (1971) found distinct enzyme and protein patterns in electrophoresis gels of D. dipsaci and D. triformis, but soluble proteins of two populations of D. dipsaci were similar. Eriksson and Granberg (1969) found that disc electrophoresis would not distinguish between four races of D. dipsaci but showed distinctly different patterns for the genera Ditylenchus and Aphlenchoides.

Photometry

Photometry may be used for a wide variety of biological problems. It can provide adsorption curves on intact cells, aid in chemical identification of colored compounds, determine amounts of pigment present in areas varying in size from tissues to organelles, or provide quantitative data on intensity or rate of color production, amount of nucleic acid or histone present (Pollister, et al., 1969). Microphotometry is important for histochemists in judging the intensity of a reaction. Also it is important to the cytologist to determine if a cell or tissue is haploid, diploid or contains a polyploid genetic compliment. Microspectrophotometry can be used to cytochemically analyze cells as a basic unit rather than homogenates of tissues (Pollister, et al., 1969). Photometry has been used in a variety of nuclear studies. Halkka (1964) studied polyploidy, polyteny and agmaloploidy in Luzula and found that DNA increased only with the

degree of true polyploidy and remained constant with polyteny and agmatoploidy which involve divisions of single chromosomes rather than new sets. Rasch and Woodard (1959) found no significant alteration in DNA/histone ratios during differentiation or pathological cell duplication in crown gall tumor but did find that an increase in DNA content of tumor nuclei associated with duplication or aneuploidy was accompanied by similar increase in histone. Genotrophs of flax cultivar Stormant Cirrus were found to vary in dry mass, nuclear volume and nuclear DNA content after growth with various nutrients. Plants of the original variety were changed into large or small genotrophs by varying their mineral nutrition. The changes in size and DNA content remain stable for ten generations (Evans, 1968). Miksche (1971) found DNA values to vary directly with latitude in *Picea* sp. and Cecich *et al*. (1972) found that the relative amount of DNA per cell in cytohistological zones of spruce apices varied through the growing season and varied between zones during the same season. Rothfels *et al*. (1966) compared the DNA content with chromosome size in species of *Anemone*. Endo (1971) in a study of nematode infected soybean roots discovered an increase in size of infected nuclei to almost four times normal. By fluorescent photometry he found a DNA synthesis ratio of infected to normal plants ranging from 1.6 to 3.5 over a fifteen day period, with higher ratios more common in later treatments. Determination of polyploidy classes of mitotic nuclei can be related to interphase nuclei DNA measurements according to Patau and Das (1961). Homologous chromatids appear to have the same feulgen content and structural appearance.

The effects of fixation on cytophotometry were studied by Hillary (1939) who found no effect from fixatives if they were well washed out before hydrolysis began, and Greenwood and Berlyn (1968) who found that Craf III and gluteraldehyde depressed the absorption peak and interfered with DNA extraction. Carnoy's solution worked best but caused shrinkage. Formalin, even in the adhesive, increased resistance to hydrolysis due to denaturation of DNA. A great variety of techniques have been used in photometry. In order to make all data comparable, Dhillon *et al*. (1977) suggest all investigators use chicken red blood cells to calculate grams of DNA per nucleus. Techniques for photometry are described in several publications (Patau, 1952; Bloch and Godman, 1955; Pollister, *et al*., 1956; Berlyn and Miksche, 1976; and Berlyn and Cecich, 1976).

CHAPTER III

MATERIALS AND METHODS

General Methods

Seeds of resistant Washoe (Nevada Foundation Seed Stock Inc. DW966C, 1969) and susceptible Buffalo variety alfalfa were germinated by bubbling them for twenty-four to forty-eight hours in aerated water. The seedlings were then planted in fine sand in three centimeter diameter plastic cream containers which were vented at the base to provide drainage. The containers were placed in metal rectangular casings and surrounded by more fine sand to hold them in place. Unvented plastic containers were used to cap each small pot to provide a high humidity chamber for the seedlings (Figure 1). Seedlings were grown for two days and inoculated by dropping the nematode suspension between the cotyledons (Figure 2). Ten nematodes were placed on each plant.

Nematodes were extracted from alfalfa plants from infected fields near Coyle and Stillwater, Oklahoma. Extraction followed a modified Christie-Perry technique in which the plants were immersed in water for fifteen minutes and the supernatant water poured through a 222 micrometer strainer and a 44 micrometer mesh screen. Material remaining on the screen was rinsed onto a double thickness of facial tissue supported by a coarse screen over a container of water. By this technique, nematodes which crawled through the wet tissue and settled

to the bottom were separated from the debris which remained on top of the tissues. Individual D. dipsaci were identified with a dissecting microscope before transfer to the seedlings.

Seedlings were grown under fluorescent lights of 1000 ft-c intensity and twelve hour days at 20 C until harvest. Seedlings for attraction studies were grown in individual five cm plastic pots for two weeks before use.

Injury Studies

Mechanical injury by a glass rod was effected to one group of seedlings two days after planting. A 0.01 mm glass rod was inserted to approximately one-third of the stem diameter. Rods were prepared by drawing out a small diameter glass rod, producing a fine point. A point of approximately 0.01 mm, the approximate larval size of the nematode, was selected using an ocular micrometer (Figure 3).

Plants of both varieties were grown in a controlled environment chamber for ten days following treatment or inoculation. To insure that infestation had occurred in plants to be tested for nematode damage, only galled plants were used in those tests.

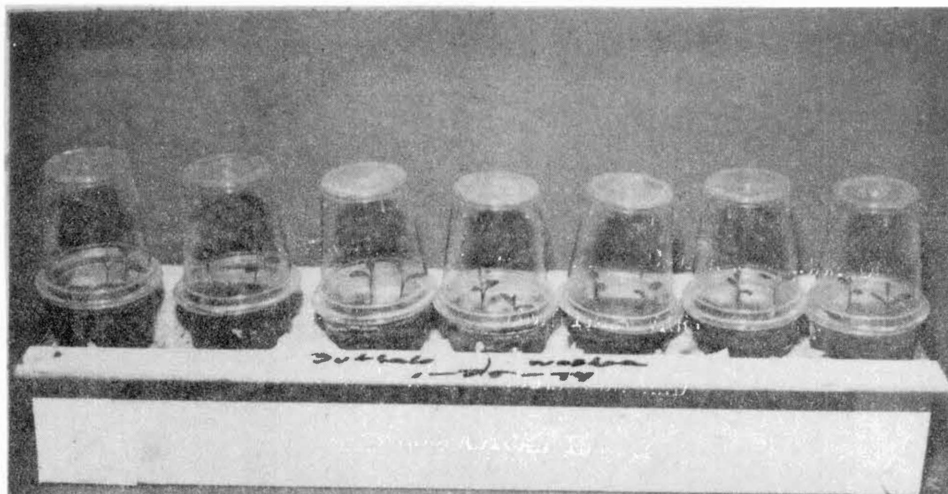
Histological Procedures

Plants were harvested ten days after inoculation. Plants were killed and fixed in FPA (Sass, 1958), a mixture of formaldehyde, propionic acid and alcohol and the tissues dehydrated with increasing concentrations of tertiary butyl alcohol (Johansen, 1940). Infiltration of the tissue followed dehydration in tertiary butyl alcohol. This was achieved by dissolving Paraplast in the pure tertiary butyl

Figure 1. Planting Scheme Used in the Study Showing Individual Moist Chambers

Figure 2. Planting Scheme Showing Size of Seedlings at the Time of Inoculation and Injury.

Figure 3. Planting Scheme Showing Glass Rod Used in the Mechanical Injury Studies.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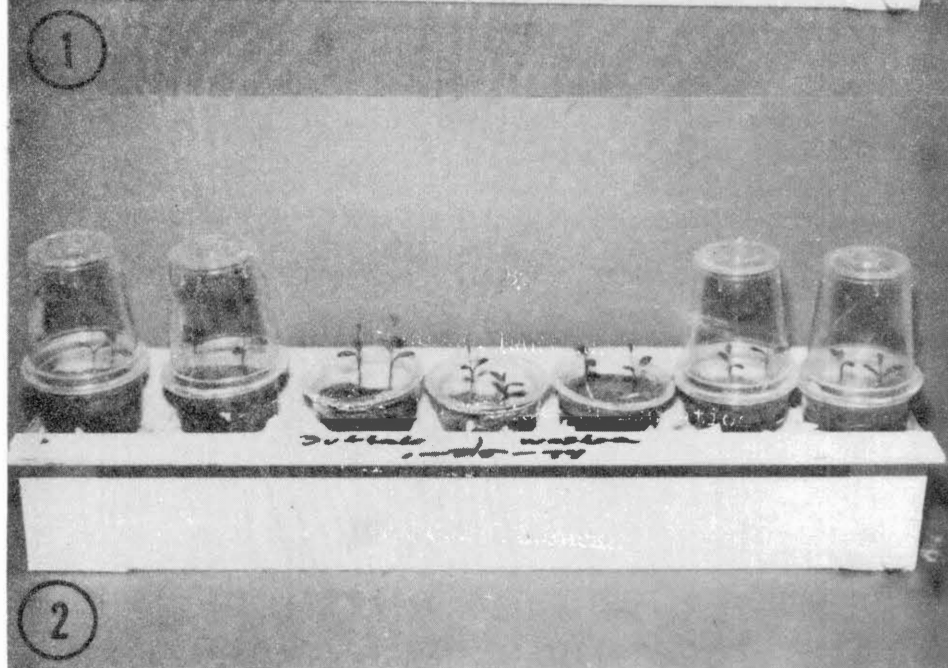
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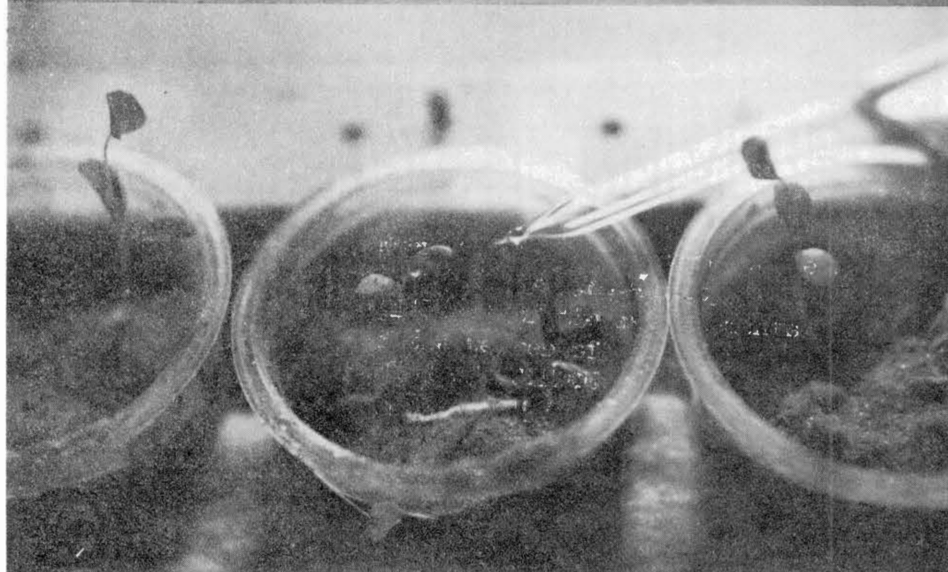


alcohol. Total infiltration was accomplished by increasing the Paraplast concentration while decreasing the alcohol concentration by evaporation and decantation (Johansen, 1940). Infiltrated tissue was embedded in melted Paraplast, poured into plastic molds, oriented, and allowed to harden around the tissues. Paraffin blocks were removed from the molds, trimmed and mounted for sectioning. Sections were cut on a rotary microtome to a thickness of ten micrometers. Serial sections were affixed to slides with Haupt's adhesive and allowed to dry for twenty-four hours (Johnsen, 1940). The slides were then stained using Johansen's quadruple stain and mounted in Histoclad. Identical procedures were used with the mechanically injured plants.

Histochemistry and Enzyme Localization

Tests for several broad compound classes were conducted on both varieties of alfalfa in control and experimental trials. Tests were made ten days after treatment. Reactions for saturated and unsaturated lipids, pectins, cellulose, and lignin were used.

Hypocotyl segments were quick frozen and sectioned at -18 C to a thickness of sixteen micrometers using a cryostat (model CTD International Harris Cryostat). Clean slides were coated with Meyer's albumen and sections were transfered from the cryostat blade to the coated surface. Sections were tested by placing a drop of stain or reagent on the sections affixed to the slides, rinsing, and mounting in Farrant's medium (Chayen, et al., 1969). Results were recorded photographically.

Lipids were examined by staining with Sudan Black B which is a fat soluble colorant. Within tissues it stains both lipids and

phosholipids a blue-black to grey color. Colorant partitions between the lipids within the tissue and the alcohol solvent, but it is more soluble in the lipids of the tissues than in the alcohol. Histochemically, fatty materials have the properties of lipids or unsaturated fatty acids. This makes it possible to test for them in two ways, by physical or partition methods and by chemical tests. The physical methods are generally considered more important and one definition of lipids is based on their ability to concentrate Sudan Black B. This test does not indicate steroids unless heating to melt the steroids is accomplished (Chayan, et al., 1969).

Ruthenium Red was used as indicator of pectic substances. Lignin was determined using phloroglucin and hydrochloric acid and cellulose was tested with iodine and sulfuric acid (Jensen, 1962).

One enzyme was tested, Dopa-oxidase (phenolase), due to the fact that it is often involved in wounding or cutting in plants. Phenolase catalyzes the oxidation of monophenols and diphenols. The enzyme contains four atoms of copper in each molecule which are its active centers and are inhibited by substances which complex with the copper (Chayen, et al., 1969). In controls the reaction was inhibited by adding potassium cyanide to the reaction medium.

Ultrastructural Studies

Plants were harvested eight, twelve, sixteen and eighteen days after inoculation. Hypocotyl sections of infected alfalfa seedlings and controls were harvested and fixed in Sabatini cacodylate buffered gluteraldehyde (Sabbatini, et al., 1963) for two hours at 6 C, rinsed with buffer for four changes over a one hour period and post-fixed for

one hour in one percent osmium tetroxide. Sections were again rinsed three times and dehydrated for fifteen minutes in each of five ethanol dilutions at 6 C. After a third change of absolute alcohol, sections were infiltrated with Spurr epoxy resin of the regular formulation and polymerized at 70 C for eight hours (Spurr, 1969). Blocks of resin were trimmed to expose the tissue and one micrometer sections were cut with a glass knife for orientation purposes. A Sorvall MT-2 ultramicrotome was used for both thick and thin sections. Thick sections were stained with a solution of 0.01% toluidine blue, heated to dryness on a hot plate, rinsed with distilled water and dried on a hot plate before viewing. Thin sections were cut from the same blocks. Light gold, silver and silver grey sections were mounted on 400 mesh copper grids, stained with uranyl acetate and lead citrate and viewed with a Phillips 200 electron microscope. Significant observations were recorded photographically for further study and documentation.

Microspectrophotometry

Alfalfa seedlings were grown and inoculated as before. Eight and twelve days after inoculation segments of hypocotyls near the cotyledonary node were killed and fixed in modified Bouin's fixative (Berlyn and Miksche, 1976) for 24 hours, dehydrated in tertiary butyl alcohol, infiltrated and embedded in Paraplast as described earlier. Ten micrometer thick longitudinal sections were cut with a rotary microtome and mounted in non-formalin gelatin adhesive (Berlyn and Miksche, 1976). DNA was stained by the Feulgen technique using a 20 minute hydrolysis in 5 N hydrochloric acid at 20 C and two hour staining time. Three ten-minute potassium metabisulfite bleaches were

used. Hydrolysis curves were generated by staining test slides. Maximum absorption was at 550 nm with a 20 minute hydrolysis. Spectral curves were generated to find the wavelength with the best absorption level. Cover slips were mounted in Eukitt (Calibrated Instruments, Inc. 713 Saw Mill River Road, Ardsley, N.Y. 10502). The plug method of visible wavelength microspectrophotometry was used with a Farrand monochrometer with slits at 5/5 and set at 550 nm. A Zeiss bright field microscope and analyzer slits at 0.13 mm entrance and 15 mm exit were also used. Illumination was with a xenon arc lamp of 150 watts and a Farrand photometer (Farrand Optical Co., Inc., N.Y.) galvinometer recorded the readings. One hundred nuclei were measured for each treatment except for one in which 38 were measured. Ten slides were measured with ten nuclei from each slide being recorded in most cases. The measuring plug was held constant with a diameter of 6 micrometers and section thickness was ten micrometers. Nuclear radii were measured with a calibrated ocular micrometer. Readings of control plants were taken in the cortex while those of infested plants were taken only from granular cells surrounding nematode-induced cavities in the cortex.

Attraction Studies

Attraction studies of *Ditylenchus dipsaci* to alfalfa plants were attempted. Plants were grown to an age of 14 days in five cm diameter plastic pots. Pots of plants to be tested were placed on opposite ends of 30 cm metal casings filled with coarse sand and eluted with 70 ml of water from each side until the sand was saturated. The water was allowed to equilibrate in the sand for six hours and the nematodes were added at a point midway between the pots. The pots and metal casings

were then covered with plastic wrap to retain moisture for 12 hours at 21 C. After 12 hours the pots were removed, sand washed from the roots, and any nematodes extracted. Sections of sand 2.5 cm long were extracted and nematodes counted. A second method of study involved placing seedlings in saturated sand in a petri dish. Nematodes were added two cm from the seedlings. After 24 hours nematodes were extracted from the sand within 1.5 cm of the seedlings and from the remainder of the dish and counts were made.

CHAPTER IV

RESULTS

Nematode infestation of both the susceptible Buffalo and the resistant Washoe variety alfalfa seedlings was followed by gall formation within seven to ten days. All inoculated plants which did not form galls were investigated and no nematodes were found upon examination. Galls were normally produced in the hypocotyl area of the stem at or slightly above the cotyledonary node. Leaves which were produced tended to be distorted and reduced in size and the stem apex was stunted in growth.

Histology

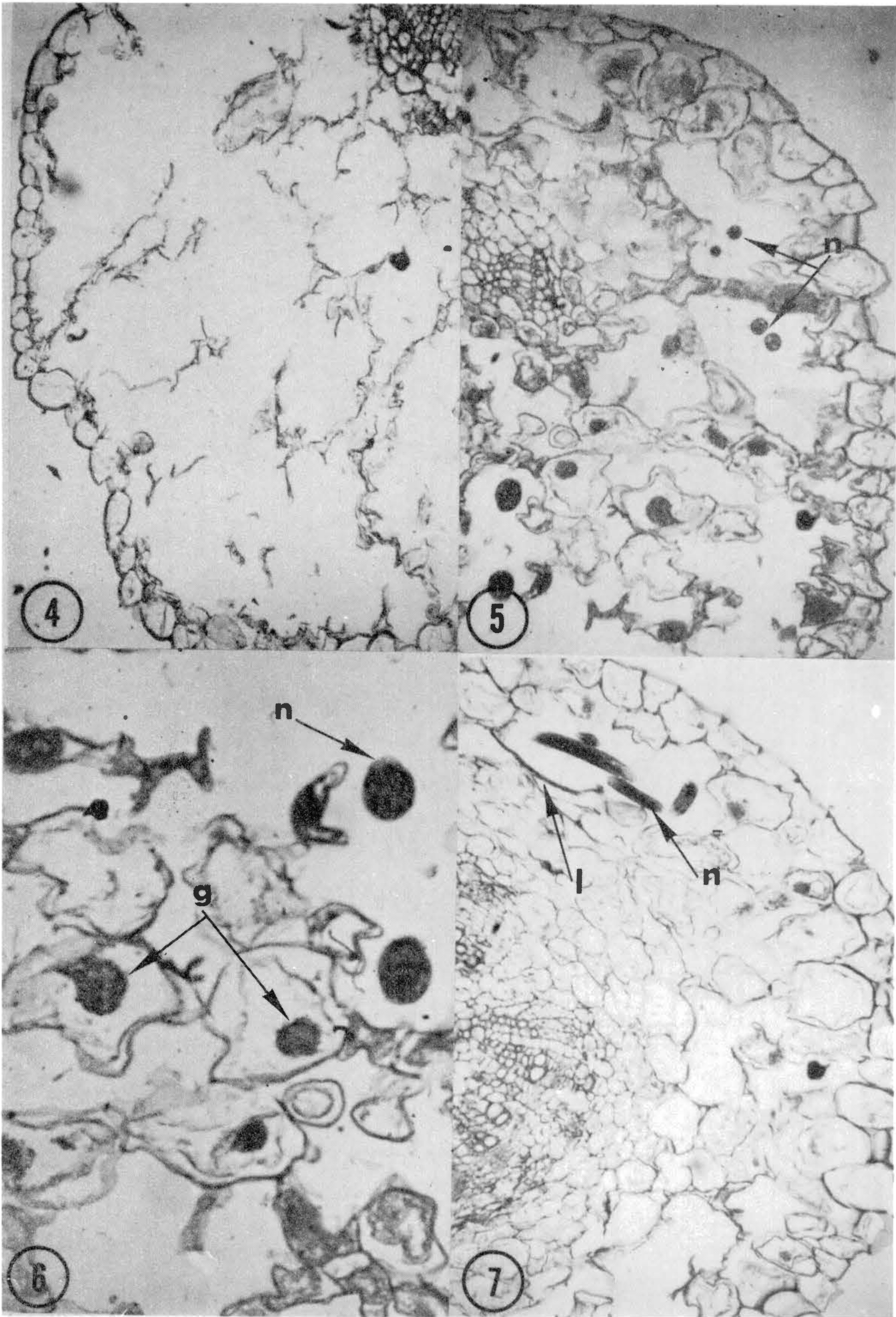
Examination of galls from plants infested with the stem nematode *Ditylenchus dipsaci* revealed many changes in the anatomy of the stems. Sections of galls from Buffalo variety contained portions of nematodes and eggs (Figures 4 and 5). Sections of galls from Buffalo also showed large areas of cellular destruction around the nematodes and most of the inside area of the gall remained as a large cavity in the cortex. Cells on the periphery of the cavities were often darkly stained and granular in appearance (Figure 6). These appear to be similar to the nutritive cells described by Goodey (1939). Some of the cavities contained heavily lignified walls (Figure 7). Some increase in the size of surrounding cells was also evident. Nematodes were

Figure 4. Transverse Section of Buffalo Variety Alfalfa Gall Showing Tissue Destruction in the Cortex. Magnification: X100.

Figure 5. Transverse Section of Buffalo Variety Alfalfa Showing Nematodes (n) Within a Gall. Magnification: X100.

Figure 6. Transverse Section of Buffalo Variety Alfalfa Showing Nematodes (n) and Cells with Granular Cytoplasm (g). Magnification: X130.

Figure 7. Transverse Section of Buffalo Variety Alfalfa Showing Nematodes (n) and Lignification of Cavity Walls (l). Magnification: X100.


n
4
5
n
g
6
l
n
7

located in all parts of the cortex from just inside the epidermis to near the vascular cylinder (Figures 8 and 9).

The resistant Washoe variety produced galls similar to those of the Buffalo variety. Transverse sections showed some cellular destruction in the cortex and some cavity formation although it appeared to be less than that found in the Buffalo variety. Very heavy lignification surrounded the cavities. Few enlarged cells with granular cytoplasm were noted (Figure 10). Heavy lignification surrounded the entry point of the nematode (Figures 11 and 12).

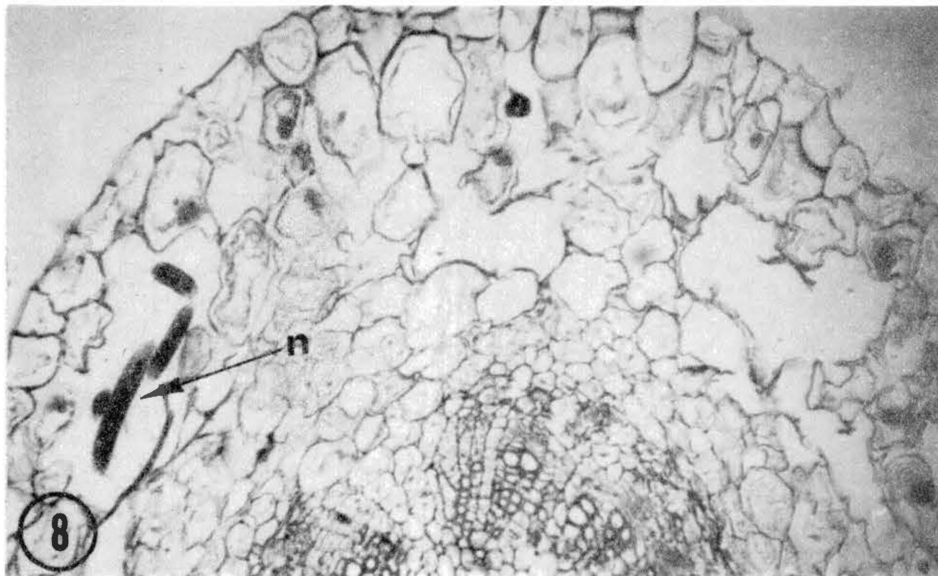
Galling did not occur on mechanically injured plants of either variety. Transverse sections of Buffalo variety alfalfa seedlings showed destruction of cells in the path of the glass rod but surrounding cells appeared normal. Lignification around the wound was evident. Washoe variety also showed destruction of cells around the wound. Heavy lignification surrounded the wound in Washoe. Lignification of Buffalo variety was light to moderate while Washoe variety was heavy (Figures 13 and 14).

Histochemistry and Enzyme Localization

Table I shows the relative responses of both varieties of alfalfa to the series of histochemical tests which were conducted. Sudan Black B used to stain lipids and phospholipids appeared similar in the control and the infested plants of both varieties. Pectin and cellulose tests showed equal intensity staining patterns in the infested and control plants of either variety, indicating equal distribution of these compounds. Lignin content in Buffalo variety increased around the gall and the entrance point of the nematodes in

Figure 8. Transverse Section of Buffalo Variety Alfalfa. Note the Position of the Cavities. Magnification: X100.

Figure 9. Transverse Section of Buffalo Variety Alfalfa with a Gall Extending from the Epidermis to the Vascular Cylinder and Almost Complete Tissue Destruction. Magnification: X100.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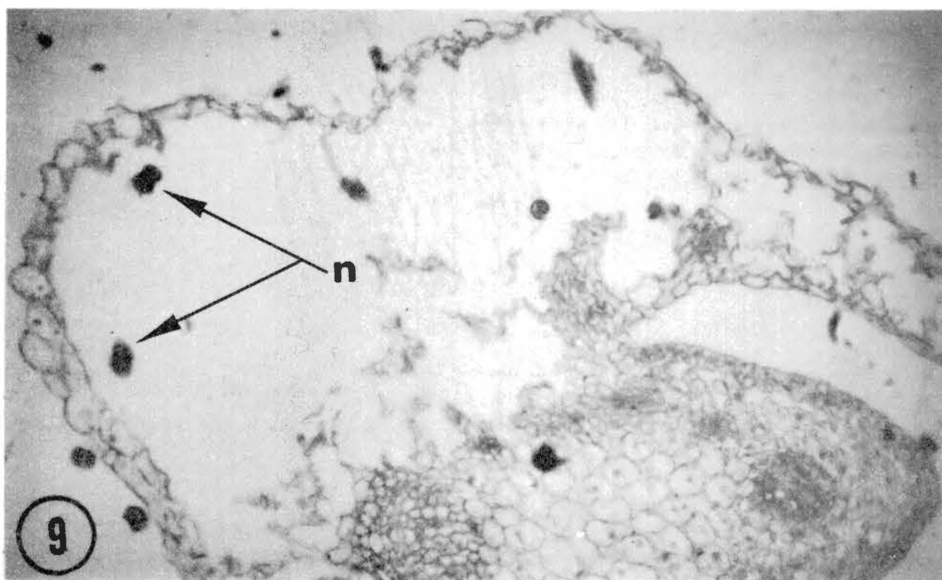
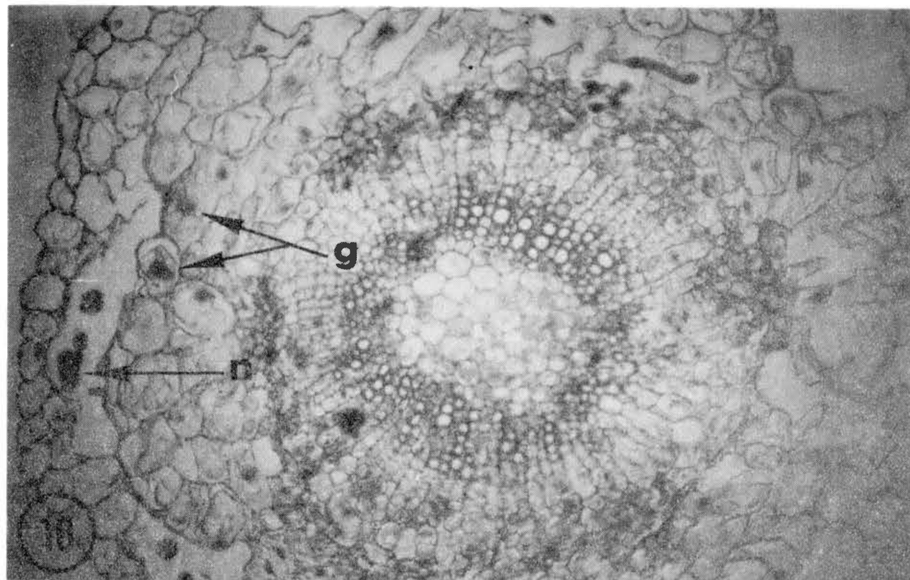
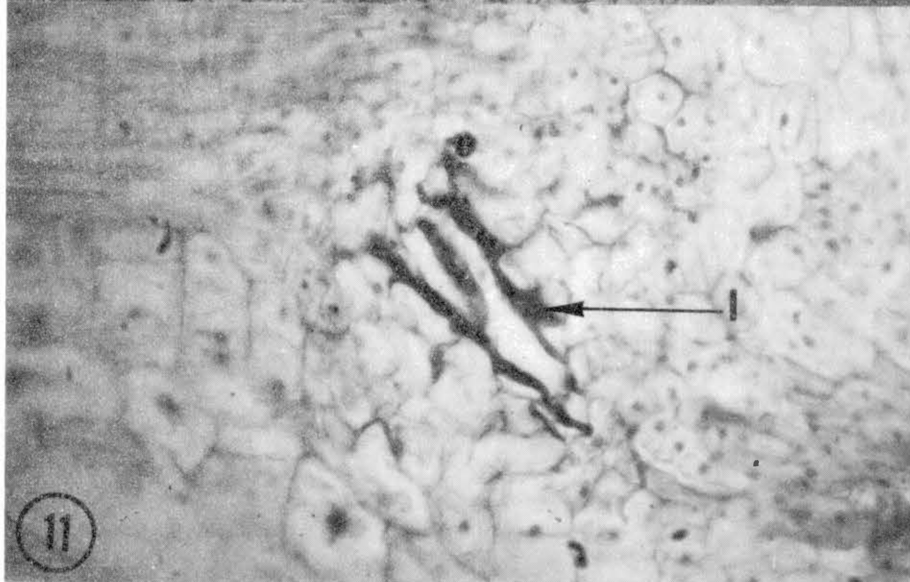

n
9

Figure 10. Transverse Section of Washoe Variety Alfalfa Showing Cell Destruction, Cavity Formation and a Small Number of Granular Cells. Magnification: X100.

Figure 11. Longitudinal Section of Washoe Variety Alfalfa Showing the Entry Point of the Nematode and Lignification (1) Surrounding the Wound. Magnification: X130.

Figure 12. Transverse Section of Buffalo Variety Alfalfa Showing Lignification (1) Surrounding the Entry Point. Magnification: X100.


g
n
10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11

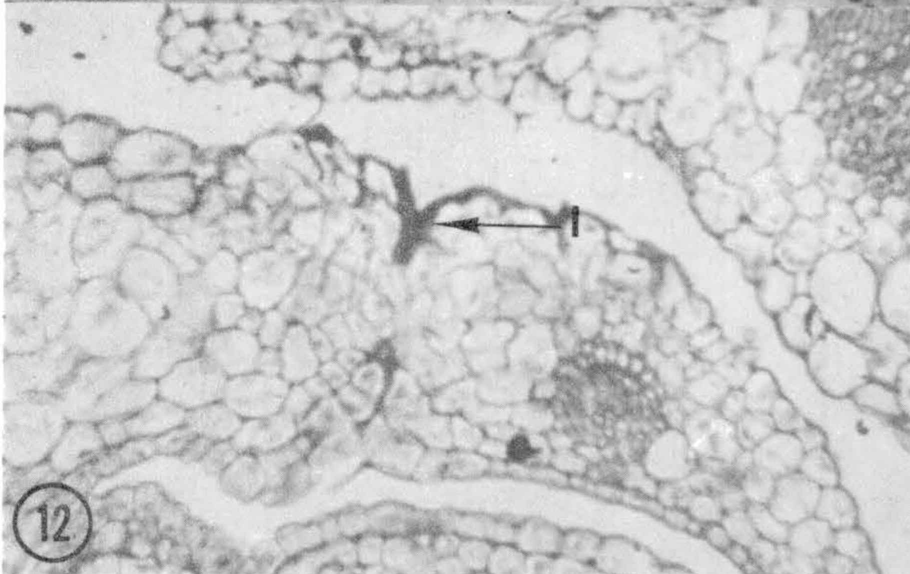
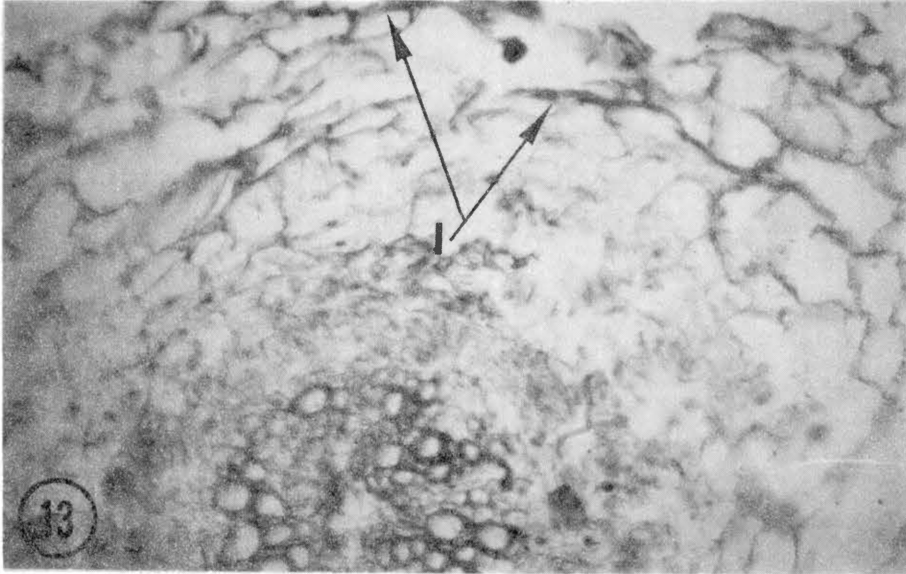

l
12

Figure 13. Transverse Section of Buffalo Variety Showing Wound and Lignification (1) Caused by Mechanical Injury. Magnification: X100.

Figure 14. Longitudinal Section of Washoe Variety Showing Wound and Heavy Lignification (1). Magnification: X100.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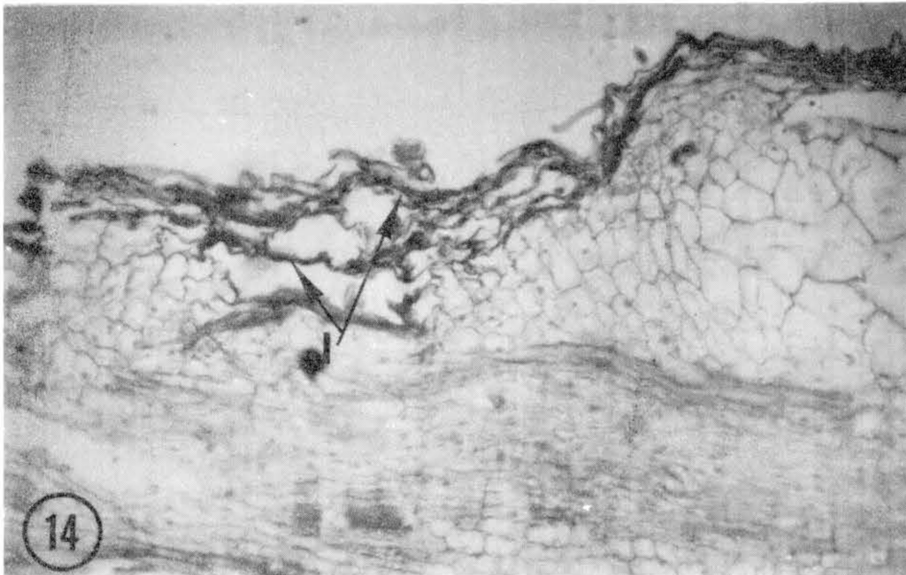
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14

infested plants as compared to no heavy lignification in control plants. Washoe variety showed a greater amount of lignification around galled portions and entry points than did Buffalo variety.

Histochemical responses of the mechanically injured plants are presented in Table II. Plant responses are similar to those of the nematode infested plants. Tests for pectins, cellulose and lipids showed the wounded plants to be similar to the controls in both the Buffalo and the Washoe varieties. Both varieties showed increased lignification in the injured plants as compared to the controls. Washoe variety exhibited a greater degree of lignification in response to injury than did the Buffalo variety (Figures 15 and 16).

Control and experimental plants of both varieties of alfalfa showed no change in Dopa-oxidase (phenolase) activity. Both the nematode infested and mechanically injured plants responded in the same manner as the controls. No increase in activity was found in galled or injured portions of a stem as compared to other portions of the same stem section.

Ultrastructure

Eight days after inoculation both varieties showed extensive damage as compared to control plants. In Buffalo, the susceptible variety, there were invaginations of the cell membranes, inflated endoplasmic reticulum forming vesicles, and an increase in the number of free ribosomes. Chloroplasts and mitochondria appeared normal at this stage (Figure 17). Washoe variety, which has been bred for resistance to the stem nematode, showed more extensive damage.

TABLE I

HISTOCHEMISTRY OF NEMATODE INFESTED PLANTS[1]

Test	Buffalo control	Buffalo experimental	Washoe control	Washoe experimental
Sudan Black B for lipids	+	+	+	+
Iodine, sulfuric acid for cellulose	+	+	+	+
Phloroglucin, HCl for lignin	+	++	+	+++
Ruthenium Red for pectin	+	+	+	+

[1]Multiple plus signs indicate increasing quantities of the compound.

TABLE II

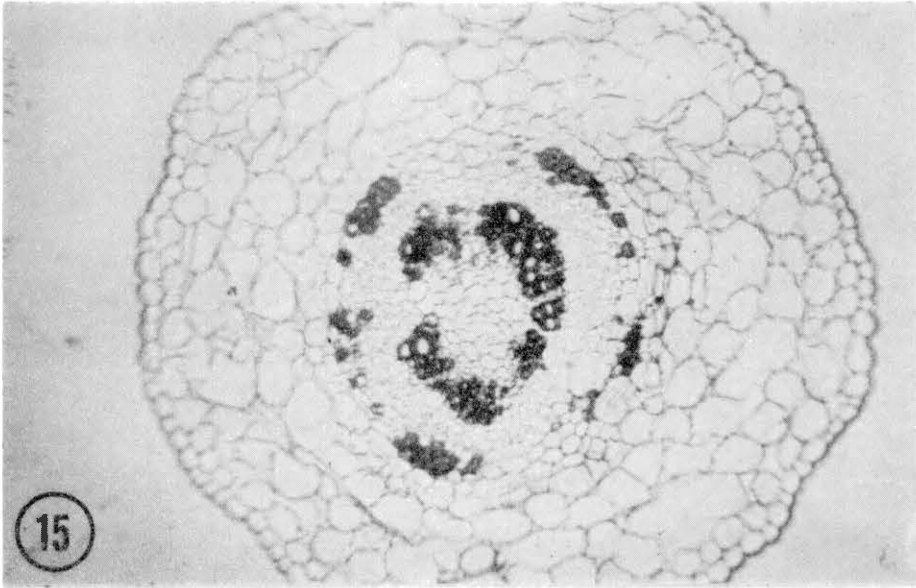
HISTOCHEMISTRY OF MECHANICALLY INJURED PLANTS[1]

Test	Buffalo control	Buffalo experimental	Washoe control	Washoe experimental
Sudan Black B for lipids	+	+	+	+
Iodine, sulfuric acid for cellulose	+	+	+	+
Phloroglucin, HCl for lignin	+	++	+	+++
Ruthenium Red for pectin	+	+	+	+

[1]Multiple plus signs indicate increasing quantities of the compound.

Figure 15. Transverse Section of Buffalo Variety Alfalfa Showing Normal Lignification. Magnification: X100.

Figure 16. Transverse Section of Buffalo Variety Alfalfa Showing Lignification (1) due to Mechanical Injury. Magnification: X100.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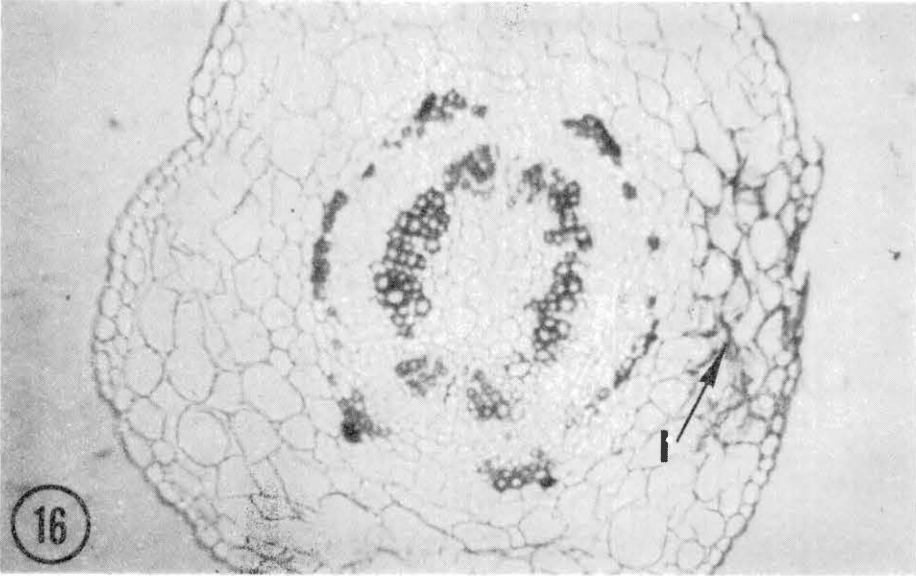
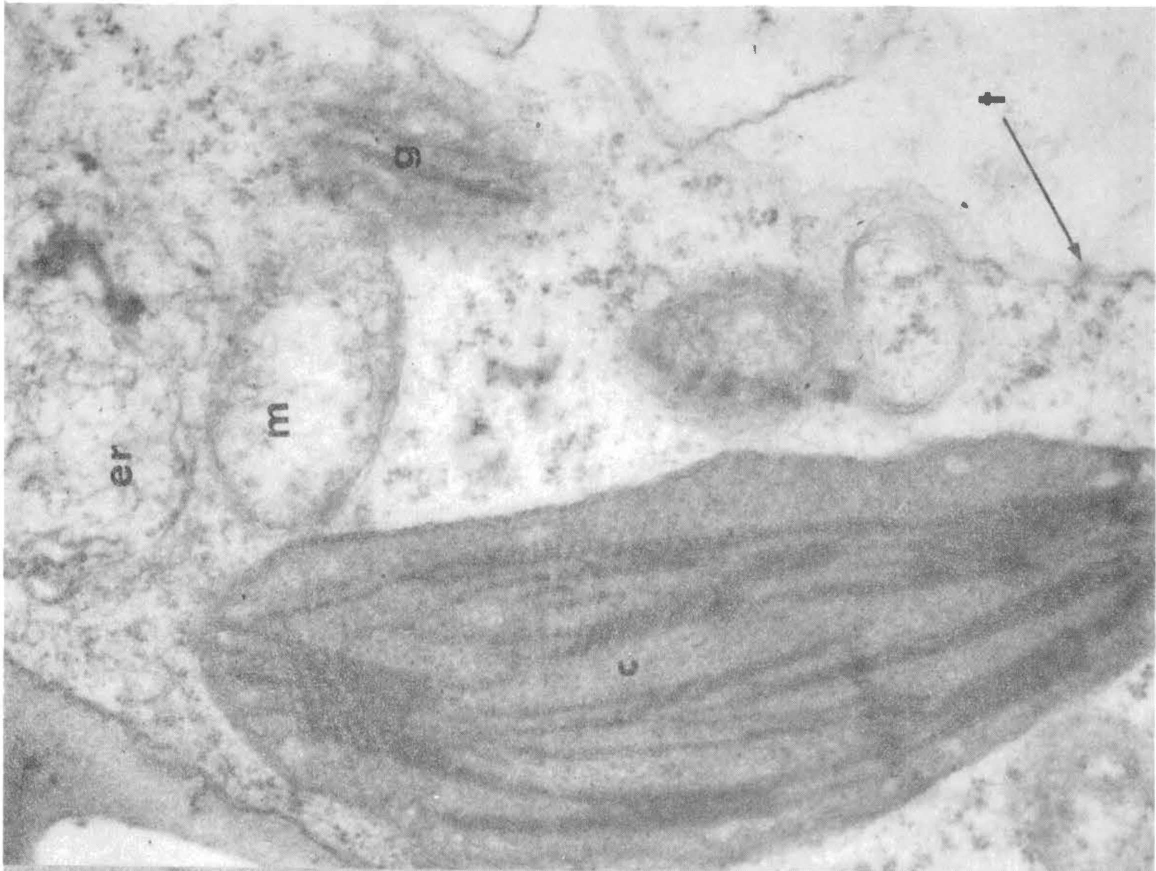
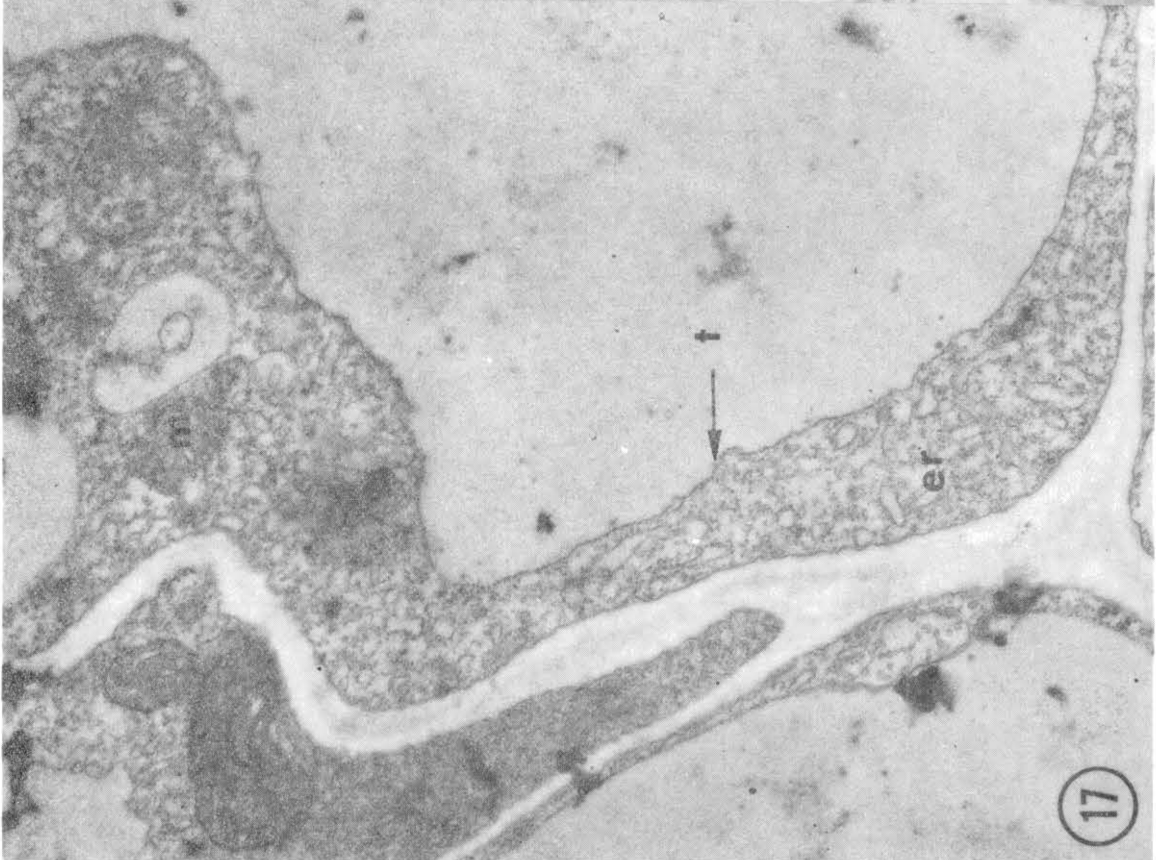

16
l

Figure 17. Buffalo Day 8: Left, Control X24,750. Right, Experimental X42,570. Note Endoplasmic Reticulum (er), Tonoplast (t), Mitochondria (m), Chloroplast (c) and Golgi (g).


er
m
g
c
t


m
t
er
17

Collapsed walls, folded and broken membranes and swollen endoplasmic reticulum vesicles were noted after eight days (Figure 18).

Twelve days after inoculation Buffalo variety cells contained broken membranes, swollen endoplasmic reticulum and an increase in the number of free ribosomes in the cytoplasm (Figure 19). Washoe after twelve days showed chloroplasts with separated lamellae, little endoplasmic reticulum, broken nuclear membranes and many free ribosomes (Figure 20).

Sixteen days after inoculation, Buffalo contained dark deposits on the wall near the nematode. Endoplasmic reticulum was nearly absent, chloroplast lamellae were separated and deformed in shape. The nuclear membrane was broken or absent and the tonoplast was only partially intact (Figure 21). Washoe at sixteen days displayed disorganized endoplasmic reticulum, large vacuoles, disrupted nuclear membranes, separated chloroplast lamellae and mitochondria lacking internal structure (Figure 22).

Buffalo on day eighteen showed little endoplasmic reticulum and that which was present was dilated. Large numbers of free ribosomes, disorganized mitochondria and separated chloroplast lamellae were also evident. No dense particles were noted on the walls or tonoplast (Figure 23). Washoe eighteen days after inoculation contained dilated endoplasmic reticulum, heavy deposits on the wall near the nematode and dark particles on the tonoplast. Stringy chloroplast lamellae, empty mitochondria and some cell wall outgrowths were also noted (Figure 24).

Figure 18. Washoe Day 8: Left, Control X12,150. Right, Experimental X33,660. Note Changes in Density of Cytoplasm and Nucleus (nu), Endoplasmic Reticulum (er) and Free Ribosomes (r).

nu
m
er
nu
18

Figure 19. Buffalo Day 12: Left, Control X34,650. Right, Experimental X32,670. Note Changes in Nucleus (n), Nuclear Membranes (nm) and Tonoplast (t).

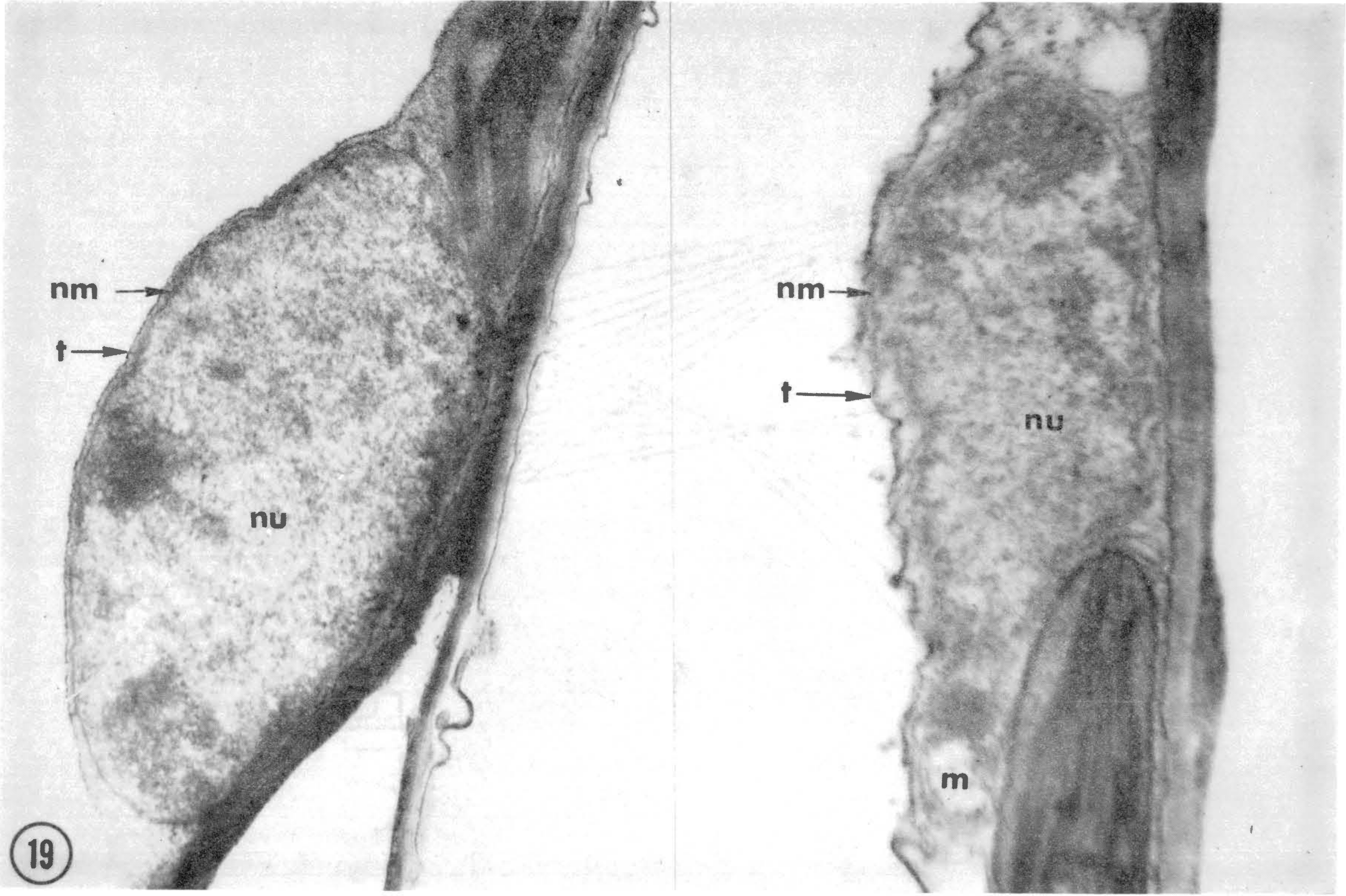
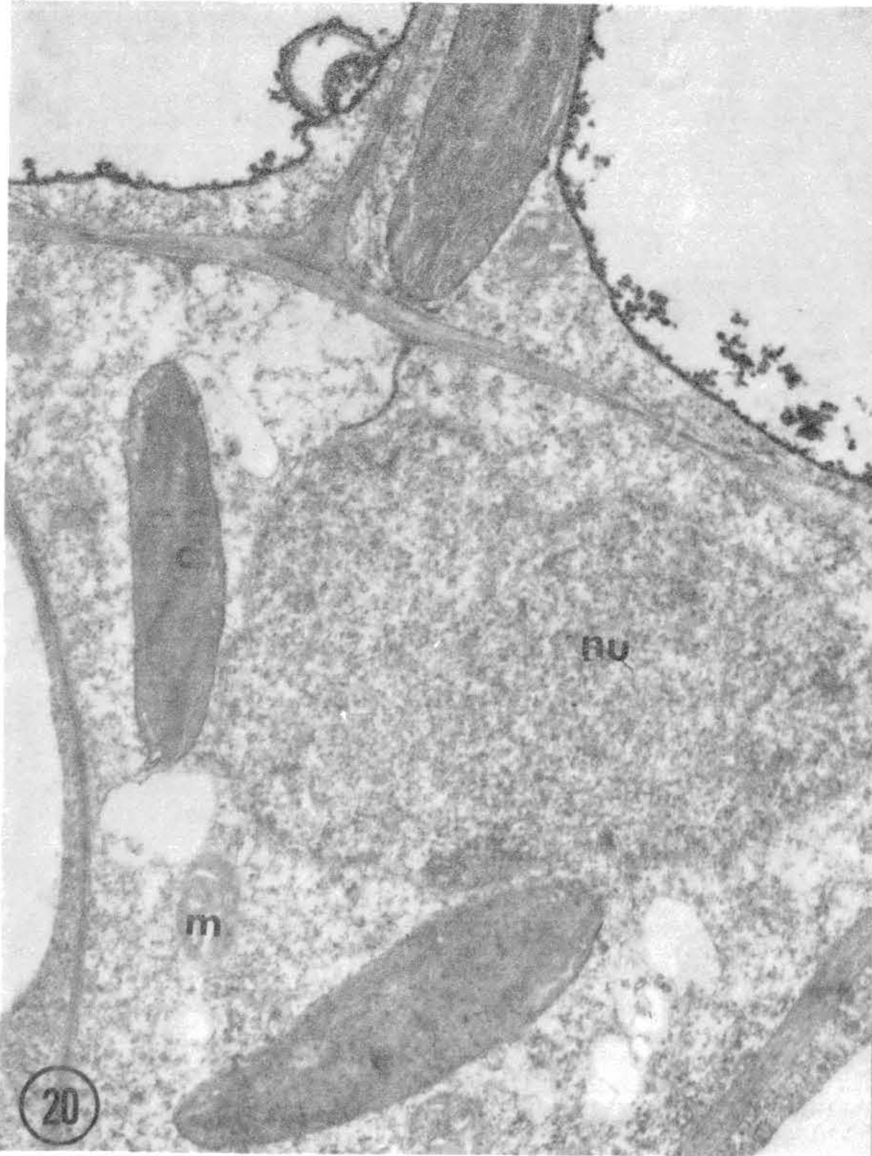

nm
t
nu
19
nm
t
nu
m

Figure 20. Washoe Day 12: Left, Control X21,150. Right, Experimental X29,690. Note Expanded Endoplasmic Reticulum (eer) and Chloroplasts (c).


c
nu
m
20

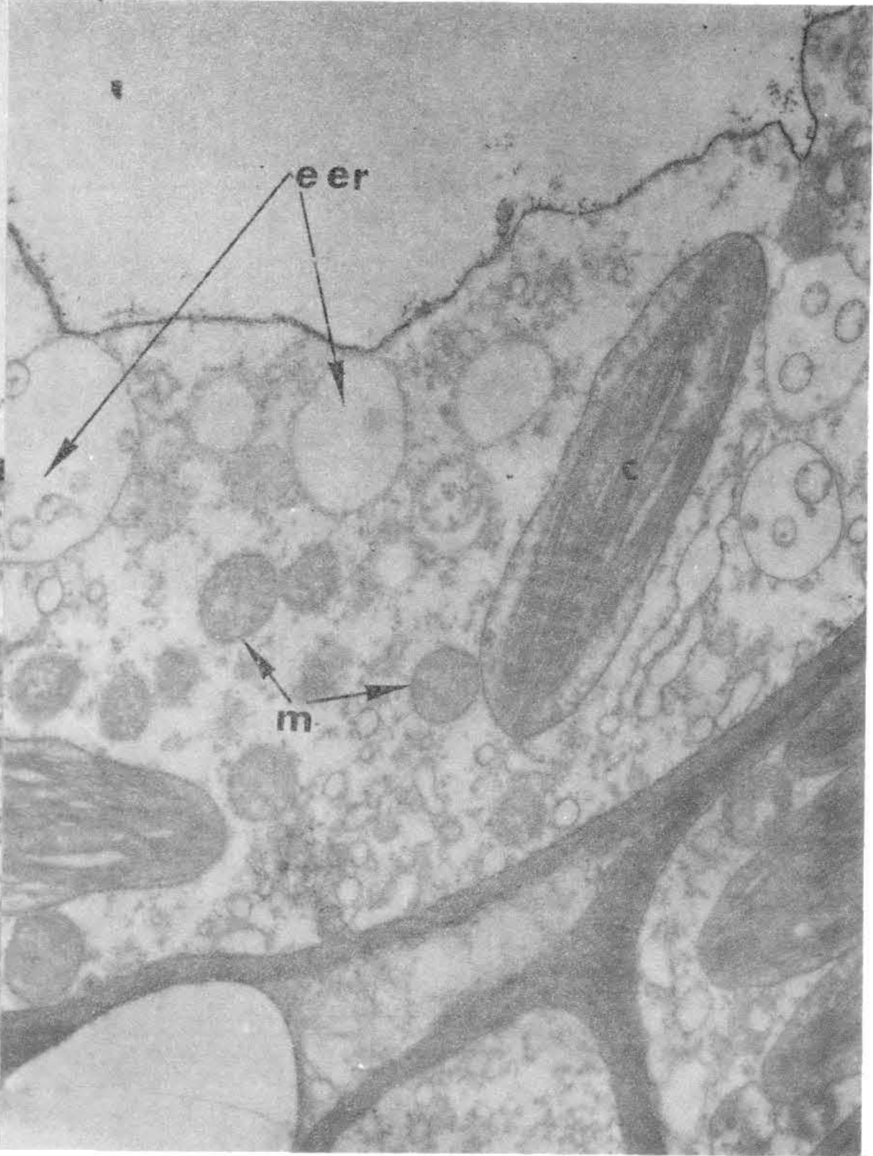

e er
c
m

Figure 21. Buffalo Day 16: Left, Control X37,620. Right, Experimental X29,690. Note Dark Deposits (d) on Wall Near Nematode Cavity (nc), Disrupted Chloroplast (c) and Nucleus (nu).

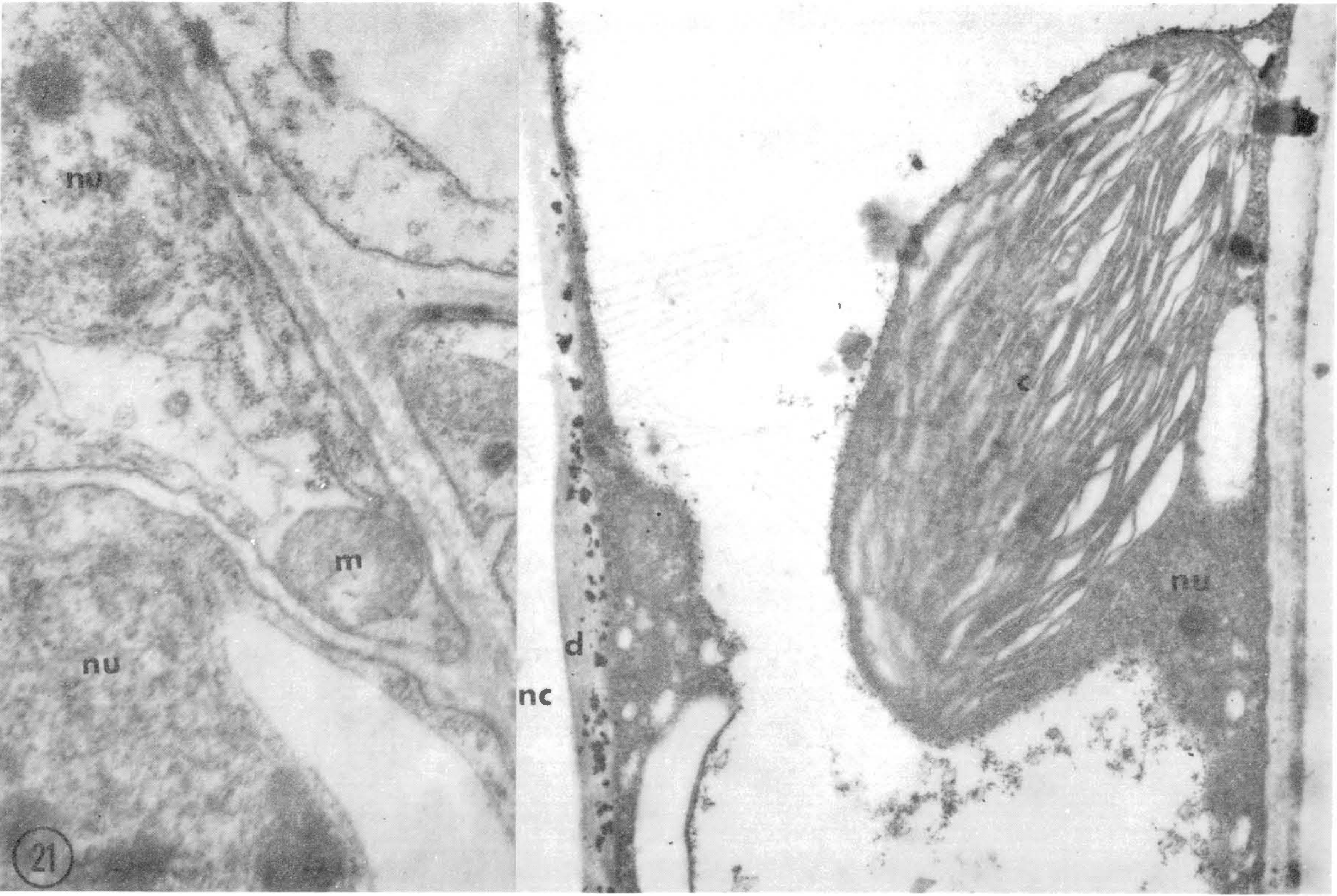

nu
m
nu
21
d
nc
c
nu

Figure 22. Washoe Day 16: Left, Control X39,600. Right, Experimental X16,100. Note Endoplasmic Reticulum (er), Tonoplast (t) and Expanded Endoplasmic Reticulum (eer).

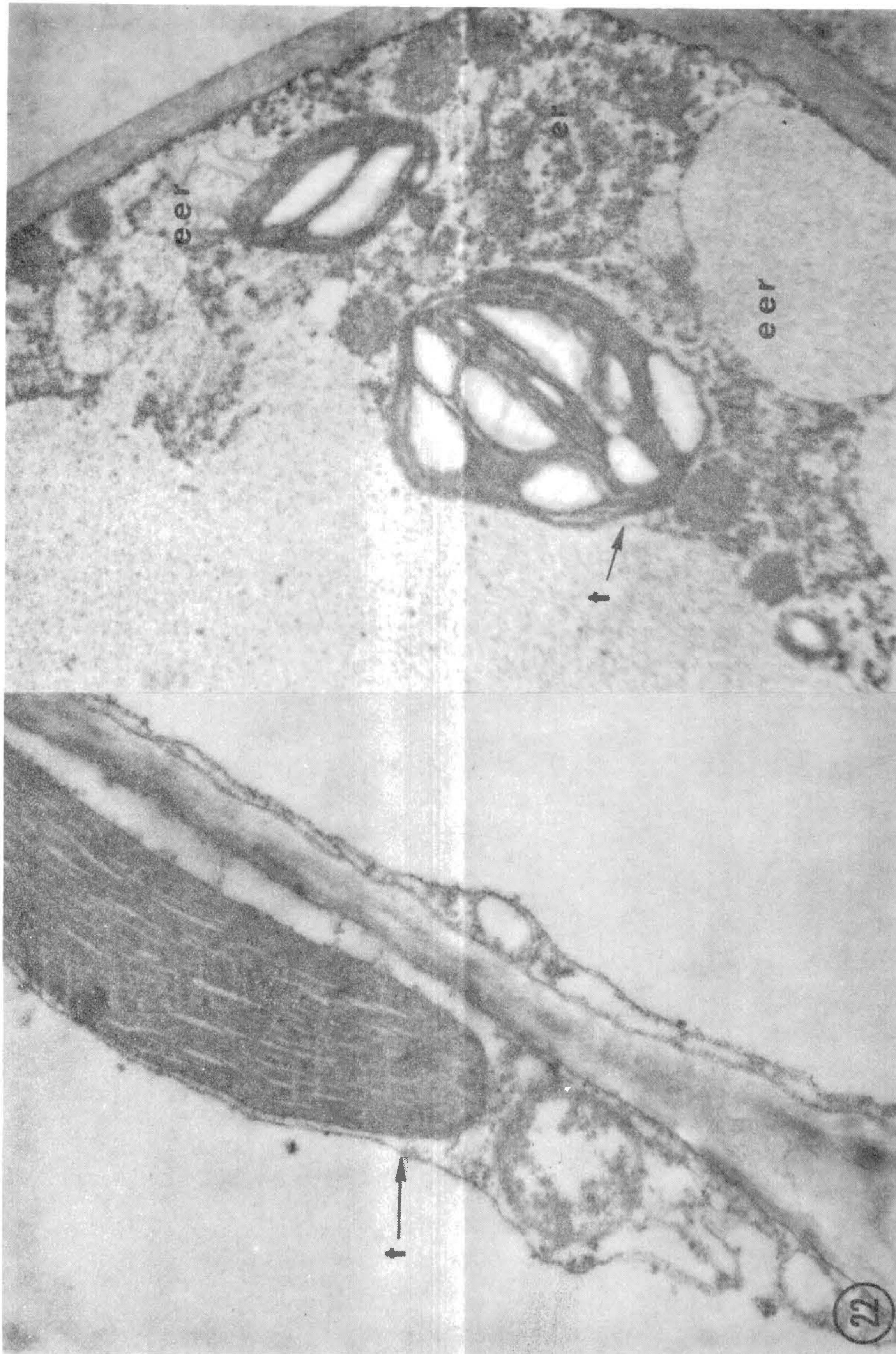

eer
eer
t
t
22

Figure 23. Buffalo Day 18: Left, Control X21,780. Right, Experimental X42,570. Note Mitochondrial Structure (m), Endoplasmic Reticulum (er) and Increase in Free Ribosomes (r).

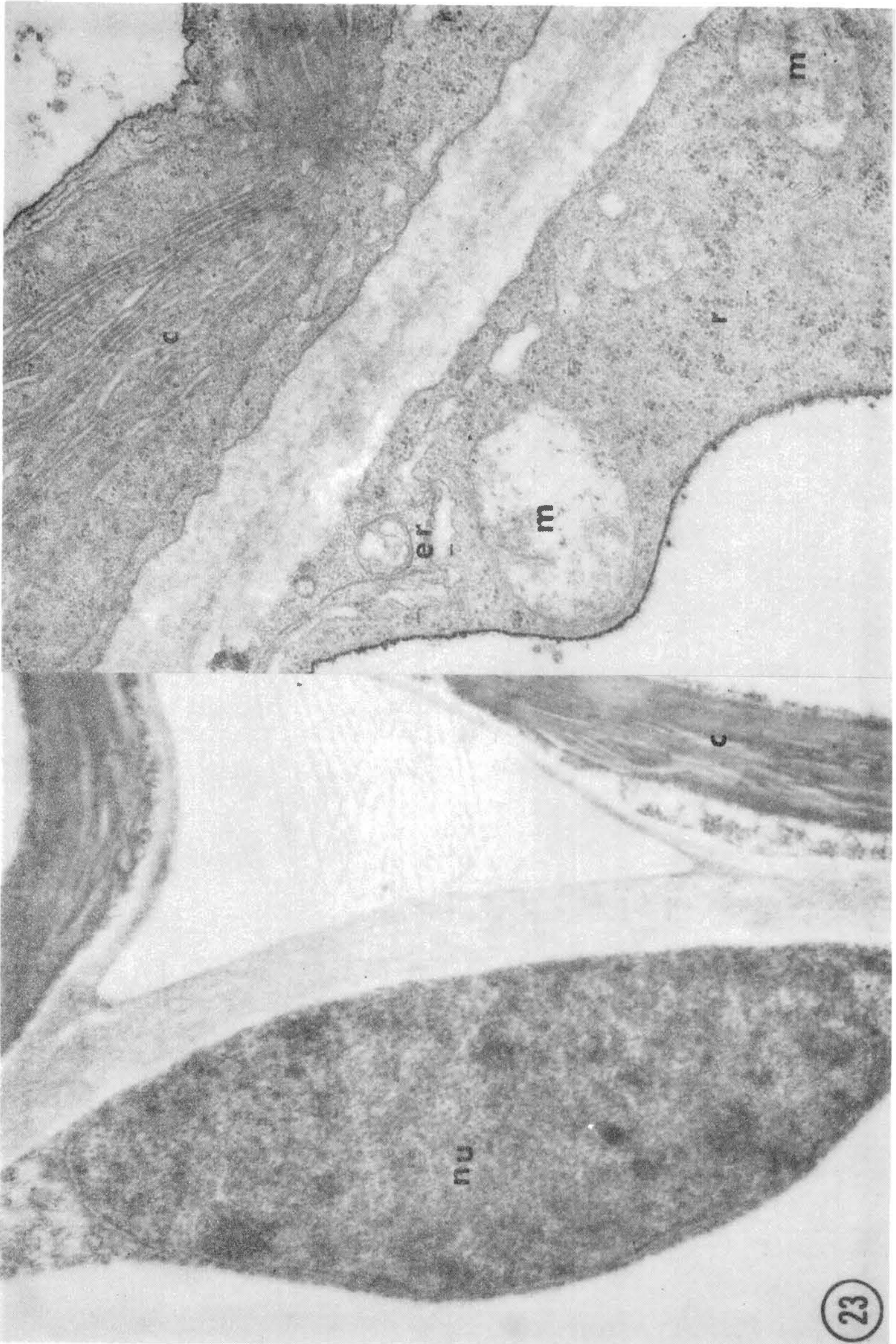
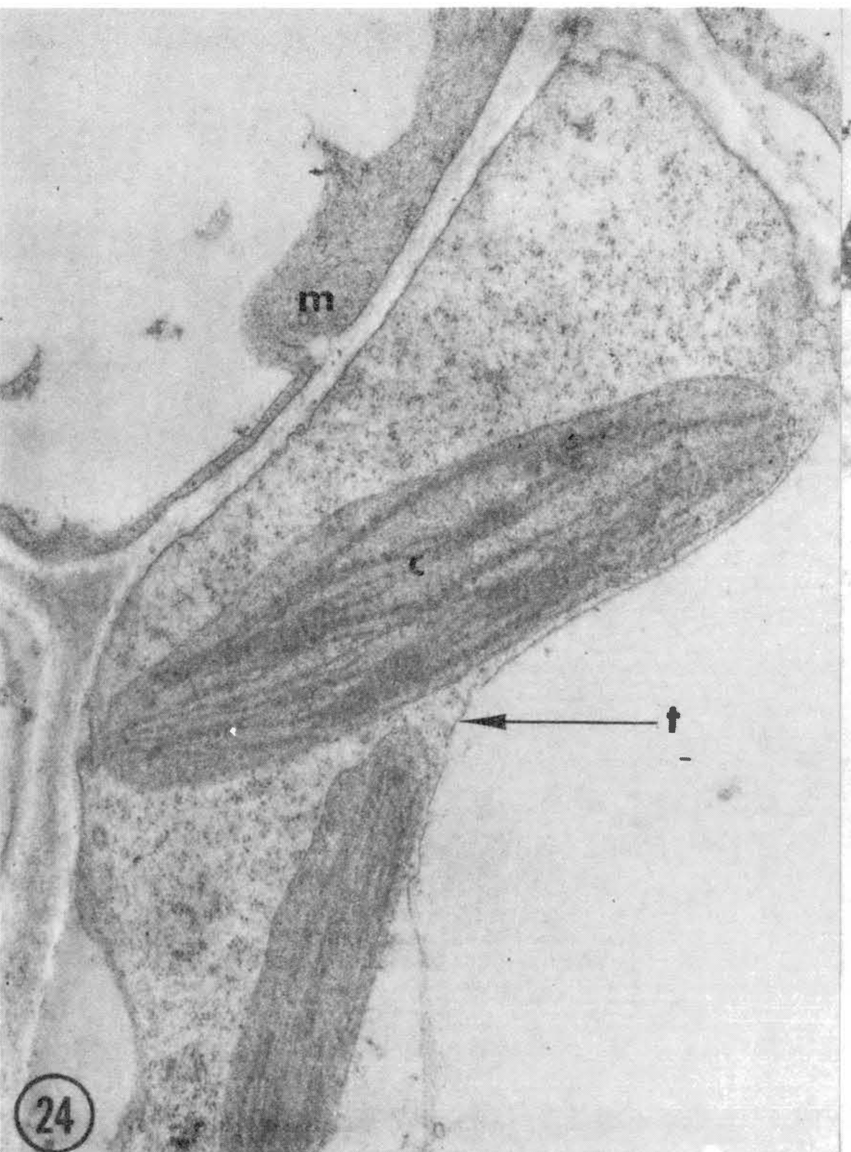
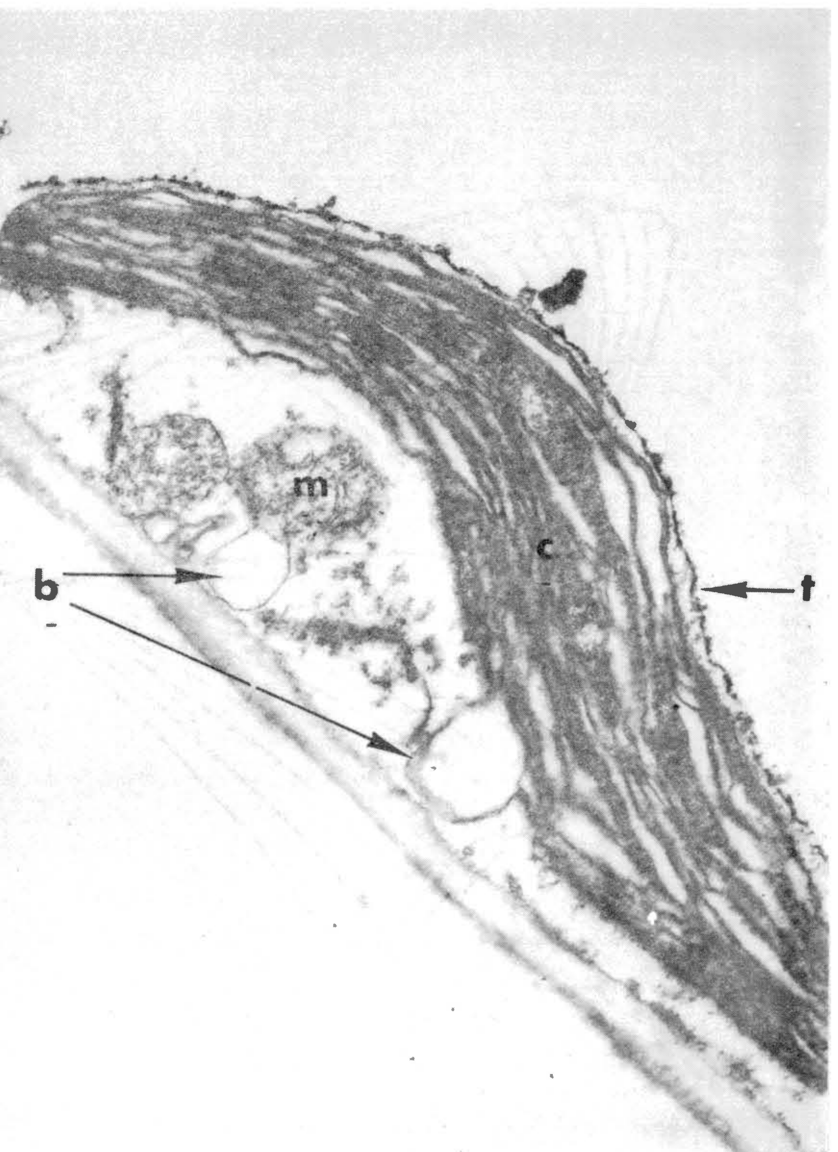

c
m
r
er
m
c
nu
23

Figure 24. Washoe Day 18: Left, Control X28,710. Right, Experimental X28,820. Note Broken Membranes, Separated Chloroplast Lamellae, Blebs (b) in Membranes of Mitochondria and Chloroplasts and Breaks in Tonoplast (t).


24
m
c
t


m
c
b
t

Microspectrophotometry

The relative amount of DNA per cell was determined by Feulgen cytophotometry in the two different varieties of alfalfa. Hydrolysis curves generated by staining test slides (Figure 25) indicated maximum absorption at 550 nm with a 20 minute hydrolysis. Plants studied eight days after inoculation showed similar increases in DNA content over controls with a 75% increase for Buffalo and a 61% increase over controls for Washoe variety. This compares a mean optical density of 0.32 units per nucleus in Buffalo controls to 0.56 in infected plants and 0.44 in Washoe controls with 0.71 in infected plants. In infected plants only granular cells surrounding the nematode cavities were measured.

Twelve days after inoculation greater differences occur between the two varieties. DNA content of Buffalo increased 153% over controls while Washoe increased 43% over controls. Optical density for nuclei of the two varieties varied from 0.32 for Buffalo control to 0.81 for infected and 0.44 for Washoe control and 0.63 for infected.

At both eight and twelve days Buffalo showed greater increases in DNA content per nucleus over the control than did the Washoe treatments (Table III).

Nematode Attraction

Six replicates of nematode attraction to Buffalo plants and an empty pot were run. No nematodes were found within five centimeters of the Buffalo plant and only two were found within ten. Nearly all of the nematodes remained within five centimeters of the inoculation point.

Figure 25. Hydrolysis Curves for Photometry Indicating Optimum Wavelength and Hydrolysis Time.

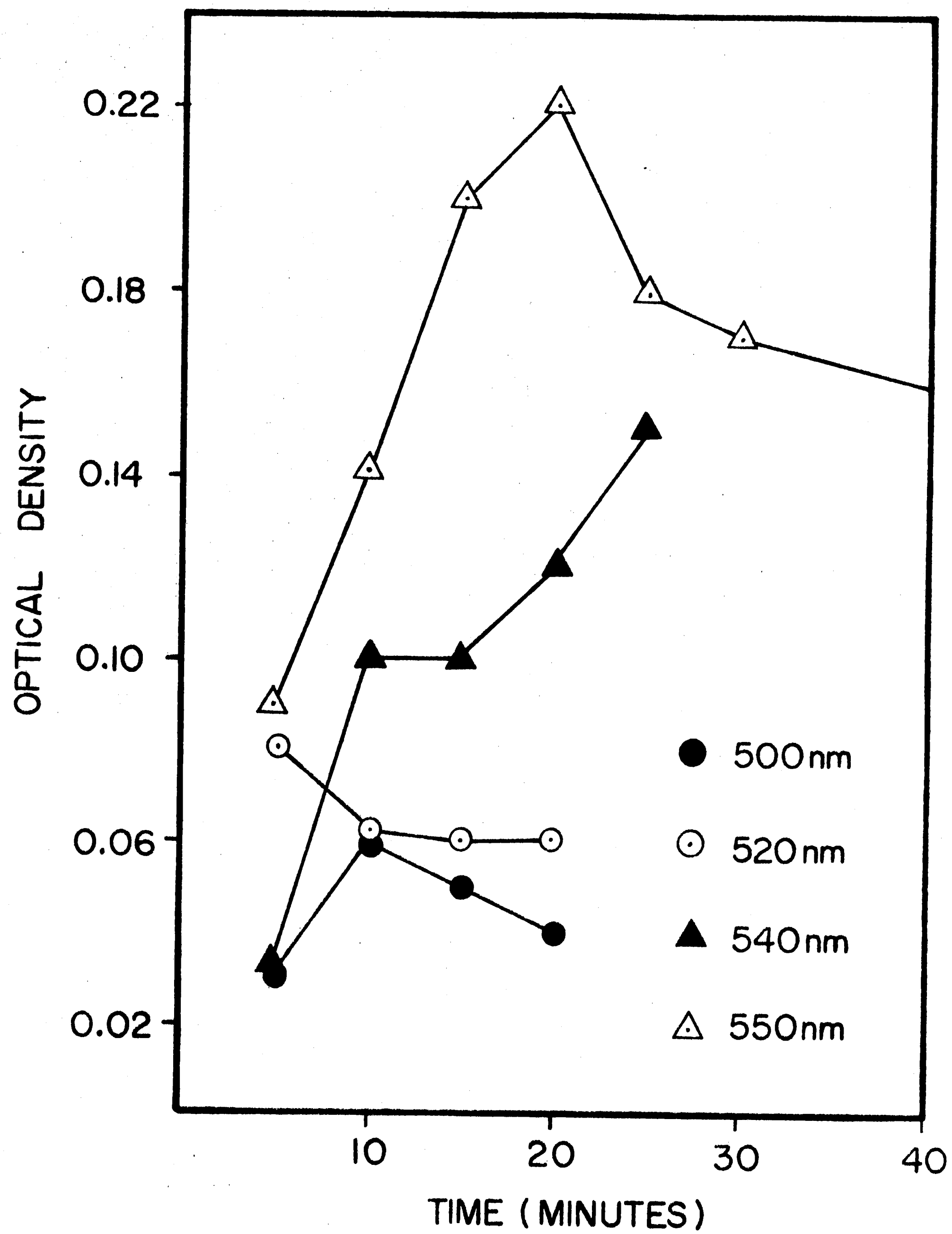

0.22
0.18
0.14
0.10
0.06
0.02
OPTICAL DENSITY
10
20
30
40
TIME (MINUTES)
500nm
520nm
540nm
550nm

TABLE III

OPTICAL DENSITIES OF ALFALFA NUCLEI

Treatment	Plug O.D.	Std. Dev.	Absorption units/nucleus	% Increase	# Tested
<u>Day 8</u>					
Buffalo					
Control	.13	.05	.32	--	111
Experimental	.23	.09	.56	75%	100
Washoe					
Control	.17	.05	.44	--	100
Experimental	.27	.07	.71	61%	100
<u>Day 12</u>					
Buffalo					
Control	.13	.04	.32	--	100
Experimental	.33	.11	.81	153%	88
Washoe					
Control	.17	.04	.44	--	100
Experimental	.24	.06	.63	43%	38

The petri dish studies showed an average of five nematodes within 1.5 cm of Buffalo and 3.3 nematodes for Washoe as compared with 25 inoculated and 11 for Buffalo and 11.6 for Washoe with one hundred inoculated (Table IV). A study of Buffalo versus Washoe in metal trays showed Buffalo to be more attractive than Washoe when nematodes were inoculated 5 cm from the pot. Of the nematodes recovered 81% remained more than 2 cm away from the pots. Equal numbers were within 2 cm of the two varieties (Table V).

TABLE IV

ATTRACTION STUDIES IN PETRI DISHES

Buffalo trial	Within 1.5 cm	Outside 1.5 cm	Washoe trial	Within 1.5 cm	Outside 1.5 cm
1[a]	3	10	1[a]	4	7
2	7	12	2	2	0
3	5	16	3	4	14
4[b]	18	44	4[b]	1	2
5	20	82	5	12	8
6	3	51	6	34	44
7	12	40	7	19	47
8	12	37	8	2	50
9	1	15	9	2	10
Totals	81	307	Totals	91	182
% of total recovered	21	79	% of total recovered	34	66

[a]Twenty-five nematodes inoculated two cm from each plant.

[b]One hundred nematodes inoculated two cm from each plant.

TABLE V

ATTRACTION STUDIES IN METAL CASINGS

Section of Casing	Trial A[1]	Trial B[1]	Trial C[1]	Trial D[2]	Trial E[2]	Trial F[2]	6 Trial Total
Buffalo Pot	3	0	7	1	2	4	17
2 cm to Pot	2	1	0	5	0	2	10
2 to 5 cm	1	0	2	2	2	0	7
Mid-section 5-5 cm	13	5	2	88	73	42	223
2 to 5 cm	1	2	0	0	1	5	9
2 cm to Pot	2	0	0	5	4	2	13
Washoe Pot	1	2	1	9	1	1	15

[1]Twenty-five nematodes inoculated per casing at 5 cm from pots.

[2]One hundred sixty nematodes inoculated per casing at 5 cm from pots.

CHAPTER V

DISCUSSION

Comparisons of the histological responses of susceptible Buffalo and resistant Washoe varieties of alfalfa revealed several important differences in their response to nematode attack. Washoe which was chosen for its resistant qualities produced smaller cavities within the galls which were formed. Washoe cavities were heavily lignified. It is possible that the lignification response functions in limiting the size of the cavity and confining the nematode to a particular portion of the stem. This may be part of the resistant response. Washoe also appeared to produce fewer cells with dark granular cytoplasm than did Buffalo. Goodey (1939) suggested that these cells are a food source for the nematode. This theory has not been confirmed but if true the lack of nutritive cells could also be a factor in limiting the growth of the nematode population within the stem by limiting its food supply. Results of this study indicate that nematode reproduction occurred in both varieties. Washoe is only moderately resistant to the Oklahoma biotypes of *D. dipsaci* used in this study.

The mechanical injury alone did not produce galling. This indicates that mechanical damage alone is not important in gall formation. Possibly nematode esophageal enzymes will be demonstrated as gall producing substances. A lignification response occurred following injury by either the glass rod or the nematode and may be of

value when testing for resistance. Nematodes which are walled off by heavy lignification may be unable to feed thus limiting the area of infestation and degree of reproduction.

Lack of differences in susceptible and resistant varieties in tests for lipids, pectins and cellulose seems to suggest that compounds other than these must be involved in the plant response to the nematodes' presence and esophageal enzymes. Lack of a change in pectins was in agreement with ideas of recent workers who have been unable to demonstrate pectinases as an important factor in gall production although they are found in nematode extracts (Krusberg, 1967).

Presence of Dopa-oxidase (phenolase) was studied due to its activity in wounding in other plants (Chayen, et al., 1969). The lack of activity in this instance indicates that it was not involved in the resistance or susceptibility of these two varieties under the conditions of this study.

The results of this study indicate that on the basis of comparative histology alone, Washoe does not exhibit a high level of resistance. Further enzyme assays of the nematode and the plants will be necessary for development of tests to screen for resistance on the tissue level. Resistance on the cellular level may also play an important role in delaying or stopping nematode development.

A progression of cellular disruption can be noted in nematode infected areas of alfalfa stems. The first noticeable effects are on cellular membranes and the loss of ribosomes from the endoplasmic reticulum. Later stages show disruption of the nucleus, chloroplasts and mitochondria. Differences in resistant and susceptible varieties

Washoe and Buffalo were not distinct. Washoe's resistance is expressed early with a large amount of cellular damage and continues through the ten day period. Buffalo shows the greatest damage at eighteen days with a slow progression from the early stages while Washoe starts with a large amount of cellular disruption and continues to a totally non-functioning state by sixteen to eighteen days. Early loss of cell function may be a clue to Washoe's resistance although it is difficult to pinpoint an ultrastructural marker for it at this time. Histochemical analysis at the cellular level may determine compounds responsible for the resistance displayed by Washoe. This fine structural study does not provide conclusive evidence as to why one of these varieties is susceptible and the other is moderately resistant.

Granular cells surrounding the nematode cavities appear to have increased chromatin content. Increases in chromatin levels over the control range from 43% in Washoe after twelve days to 153% in Buffalo at the same age. This increase in DNA may be an important clue to resistance in Buffalo as compared to Washoe. The increase would seem to indicate an increase in synthesis by the cell, producing products which may be used by the nematode for food or in reproduction. The resistant variety, although increasing its chromatin somewhat may not produce enough extra substances or the right substances to support the nematode and its reproduction. Perhaps those resistant plants which do not form galls do not have a great enough increase in chromatin and therefore do not support enough feeding by the nematode to cause galling.

There appears to be no attraction of _D. dipsaci_ by alfalfa seedlings at distances exceeding 5 cm. Some attraction becomes evident

at 5 cm and 2 cm from the pot. Petri dish studies seemed to indicate some attraction by both varieties with nearly equal percentages being attracted by each variety. These results are somewhat similar to other work which showed attraction at distances of .5 cm or less from the plant.

CHAPTER VI

SUMMARY

Histological, histochemical, ultrastructural, DNA and attraction studies of resistant and susceptible alfalfa varieties to nematode and mechanical injury indicated the following:

1. Galling occurred in both resistant and susceptible varieties due to *Ditylenchus dipsaci* infestation.

2. No galls were formed as a result of mechanical injury.

3. Cellular response to *D. dipsaci* resulted in large cavities and some lignification in susceptible Buffalo variety and smaller cavities and increased lignification in resistant Washoe variety.

4. Mechanical injury caused lignification in both varieties with heavier lignification in Washoe than Buffalo but little tissue destruction was evident next to the initially damaged cells in either variety.

5. There were no changes observed in the content of pectins, cellulose, lipids or Dopa-oxidase in nematode infested or mechanically injured plants of either variety.

6. One basis for resistance appears to be lignification of cell walls around the nematode but further studies are needed to develop methods of screening alfalfa varieties for resistance to the stem nematode, *Ditylenchus dipsaci*.

7. Ultrastructural changes occur faster in Washoe than in Buffalo. A probable result of this is loss of a feeding site for the nematode.

8. Photometric determinations show increased DNA content in the granular cells surrounding the nematode feeding site. Buffalo variety DNA increased to a greater extent than did Washoe, with the greatest difference at 12 days after inoculation.

9. Attraction studies showed no attraction of *Ditylenchus dipsaci* when inoculated 15 cm from the plant and some attraction at 5 cm. Greatest attraction was found when nematodes were inoculated 2 cm from the seedling with 21-34% being attracted.

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