THE HISTOLOGICAL AND HISTOCHEMICAL RESPONSES OF RESISTANT AND SUSCEPTIBLE VARIETIES OF ALFALFA TO NEMATODE AND MECHANICAL INJURY

By

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Lincoln, Nebraska

1971

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May 11, 1974

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ACKNOWLEDGEMENTS

I wish to express my appreciation to my major adviser Dr. Paul E. Richardson for his guidance and encouragement during the course of this study and the preparation of this manuscript. Acknowledgement is also due Dr. John E. Thomas, Head of the Department of Botany and Plant Pathology and committee members Drs. Charles C. Russell and Eddie Basler for their assistance and cooperation and critical review of this manuscript.

I am also grateful for the assistance of Mr. Theodore Alby, Mr. Thomas Cason and Mr. William Jackson in portions of this study.

I am most grateful for the continued encouragement, understanding and patience of my husband and my parents during the completion of this study.

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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 allowed 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 plant to a nematode pathogen is based on the amount of nematode reproduction which takes place in the plant and is not dependent on the plant survival rate. 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. Alteration of hormonal regulation may cause hypertrophy, lateral root or bud formation and hypersensitive reactions (Rhode, 1965).

Fast, reliable procedures for screening resistant and susceptible plants would greatly facilitate the development of resistant varieties. This study was designed to compare the reactions of two varieties of alfalfa, susceptible Buffalo variety and resistant Washoe variety, to various histochemical tests and histological observations. Tests were also conducted by mechanically injuring plants of both resistant and susceptible varieties and comparing their reactions by the same histochemical procedures and histological observations. The mechanical damage was an attempt to simulate mechanical damage caused by nematode movement in the absence of enzymatic secretions from the nematode. Plant response to injury could then be contrasted with the plant response to both mechanical and enzymatic damage caused by the nematodes.

CHAPTER II

REVIEW OF LITERATURE

The stem nematode Ditylenchus dipsaci has been known as a pathogen of alfalfa since the mid nineteenth century. Since 1881 it has been known that the race of D. dipsaci which attacks alfalfa is host specific (Thorne, 1961).

Many descriptions of the disease symptoms have been published (Barker and Sasser, 1959; Seinhorst, 1959; Thorne, 1961; Krusberg, 1962; Griffin, 1968; Dropkin, 1969; Hawn, 1969; Muse and Williams, 1969; Evans, 1971). Symptoms include enlarged stems, discoloration and fine transverse ridges or wrinkles on the stem (Thorne, 1961). Short internodes, blistering, petiole and leaf vein curvature may also be present (Hawn, 1969). White flagging, the occurance of an unpigmented branch, also occurs and is consistently associated with nematode infestation (Evans, 1971; Campbell and Griffin, 1973).

The nature of resistance must be understood in order to breed for resistant varieties. Rhode (1960) defined resistance as characteristics of the plant which impede the growth and reproduction of the parasite. Resistance has been tested by measuring the rate of nematode reproduction on seedlings (Sherwood, 1967). Resistance may be due to mechanical barriers to penetration or to resistance after entry into the plant. Three conditions may be important in the post-infection response (Rhode, 1965). These conditions include nutritional incompatibility, lack of host response,

and hypersensitivity. According to Barker and Sasser (1959) resistant plants become necrotic and the nematodes fail to reproduce. Griffin (1968) found a relationship between temperature and plant susceptibility. Resistance is reduced at 25 to 30° C for even the most resistant varieties. Both resistant and susceptible varieties were infected over a temperature range of 5 to 30° C. Barker and Sasser (1959) also noted that varieties resistant in one area of the country may not be resistant in other areas due to differences in populations of the pathogen.

In early stages of infestation the nematodes enter the young tissues and congregate near developing leaves and at the stem apex (Thorne, 1961). According to Krusberg (1960a) damage was evident six hours after innoculation. Nematodes were found between portions of the terminal bud, in the cotyledons slightly above the axils, and attacking the embryonic leaves and the shoot apex. Affected cells failed to stain normally. After twenty-four hours many cell and tissue changes had taken place. Cortical cells were irregular in shape and often separated from one another. The cytoplasm withdraws and walls collapse causing large cavities to form. Dropkin (1969) indicated that in resistant varieties of alfalfa, D. dipsaci causes cells near the nematode to enlarge and separate from one another. Effects were evident before actual contact between the cell and the nematode (Krusberg, 1961). Some cells surrounding the cavities contained dense cytoplasm. J.B. Goodey (1939) described these dense cells as nutritive cells on which the nematodes later feed. Nematodes initiate galls only in very young, active, growing and differentiating tissue and galls do not form in older tissues (Krusberg, 1960).

There have been few studies of enzyme activity in galls caused by D. dipsaci. Pectinases have been studied more than other types. 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. In 1963 Krusberg 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.

CHAPTER III

MATERIALS AND METHODS

General Methods

Seeds of resistant Washoe variety alfalfa (Nevada Foundation Seed Stock Inc. DW966C, 1969) and susceptible Buffalo variety alfalfa were imbibed for twenty-four to fourty-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 rectangular metal 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). The seedlings were then grown for two days in a controlled environment chamber (Model CEL 37-14 Sherer-Gillett) under fluorescent lights of 1000 ft-c intensity and fourteen hour days at 85° C and ten hour nights at 65° C.

Two days after planting, seedlings of both varieties were innoculated with Ditylenchus dipsaci or left as controls. Initially plants which were to be innoculated were treated immediately after planting with a suspension of nematodes in water. The nematode suspension was pipetted onto the seed bed immediately after planting. This method yielded low infestation levels and was discontinued. A more effective technique of innoculation was adopted. In this procedure a drop of water was placed between the cotyledons of each seedling two days after planting. Experimental plants

were innoculated by hand picking nematodes to the drop of water with a modified dental pulp canal file. A total of ten nematodes including males, females and larvae were transfered to each plant except the controls. Control seedlings were spaced alternately in the metal trays and a drop of water without nematodes was placed between the cotyledons (Figure 2).

Nematodes were obtained by extraction from plants from an infested field near 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 coarse strainer and a 325 mesh screen. Material remaining on the screen was rinsed onto a double thickness of facial tissue supported by a screen over a container of water. By this technique nematodes which crawled through the tissue and settled to the bottom were separated from the debris which remained on top of the tissues (Russell, 1972). Individual Ditylenchus dipsaci were identified with a dissecting microscope before transfer to the seedlings.

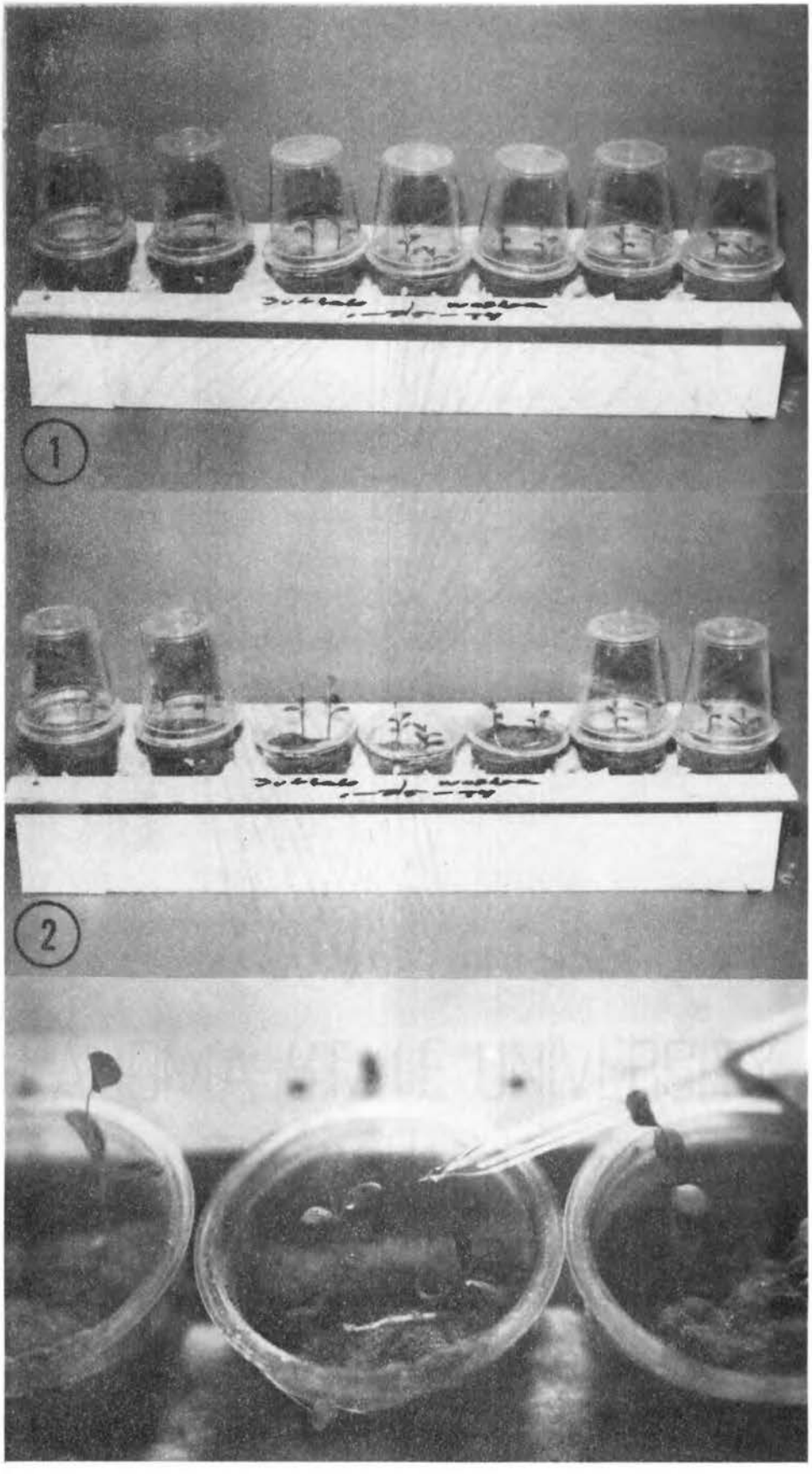
Mechanical injury by a glass rod was effected to the seedlings two days after planting. A 0.01 mm glass rod was inserted approximately one-third of the diameter of the stem while the experimenter viewed the procedure under a dissecting microscope. Rods were prepared by drawing out a small diameter glass 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 innoculation. To insure that infestation had occured in plants to be tested for nematode damage, only galled plants were used in those tests.

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

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

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

Plants were harvested ten days after innoculation. All histological work followed standard techniques. Plants were killed and fixed in FPA (Sass, 1958), a mixture of formaldehyde, propionic acid and alcohol. Then the tissues were dehydrated with increasing concentrations of tertiary butyl alcohol (Johansen, 1940). Infiltration of the tissue followed dehydration in the tertiary butyl alcohol by dissolving Paraplast in the pure tertiary butyl alcohol. Total infiltration was accomplished by increasing the Paraplast concentration while decreasing the alcohol by evaporation and decantation (Johansen, 1940). Infiltrated tissue was embedded in melted Paraplast poured into plastic boats, oriented, and allowed to harden around the tissues. Paraffin blocks were removed from the boats, 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 set for twenty-four hours (Johansen, 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 $-18^{\circ}C$ 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, 1969). Results were recorded photographically for future reference.

Lipids were examined by staining with Sudan Black B which is a fat soluble colorant. Within tissues it stains both lipids and phospholipids a blue-black to grey color. Colorant partitions between the lipids within the tissues and the alcohol solvent, but 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 (Chayen, 1969).

Ruthenium Red was used as an 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 at its active center and are inhibited by substances which complex with the copper (Chayen, 1969). In controls the reaction was inhibited by adding potassium cyanide to the reaction medium.

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 innoculated plants which did not form galls were found to be uninfested and no nematodes were found upon examination. Galls were normally produced in the hypocotyl area of the stem 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 of Nematode Infested Plants

Examination of galls from plants infested with the stem nematode Ditylenchus dipsaci revealed many changes in the gross anatomy of the stems. Sections of galls from Buffalo variety contained portions of nematodes and eggs (Figures 4 and 5). Sections of Buffalo galls 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 located in all parts of the cortex from just inside the epidermis to near the vascular cylinder (Figures 8 and 9).

Figure 4. Transverse Section of Buffalo Variety Alfalfa Gall Showing Tissue Destruction in the Cortex. Magnification: 100X.

Figure 5. Transverse Section of Buffalo Variety Alfalfa Showing Nematodes (n) Within a Gall. Magnification: 100X.

Figure 6. Transverse Section of Buffalo Variety Alfalfa Showing Nematodes (n) and Cells with Granular Cytoplasm (g). Magnification: 130X.

Figure 7. Transverse Section of Buffalo Variety Alfalfa Showing Nematodes (n) and Lignification of Cavity Walls (l). Magnification: 100X.

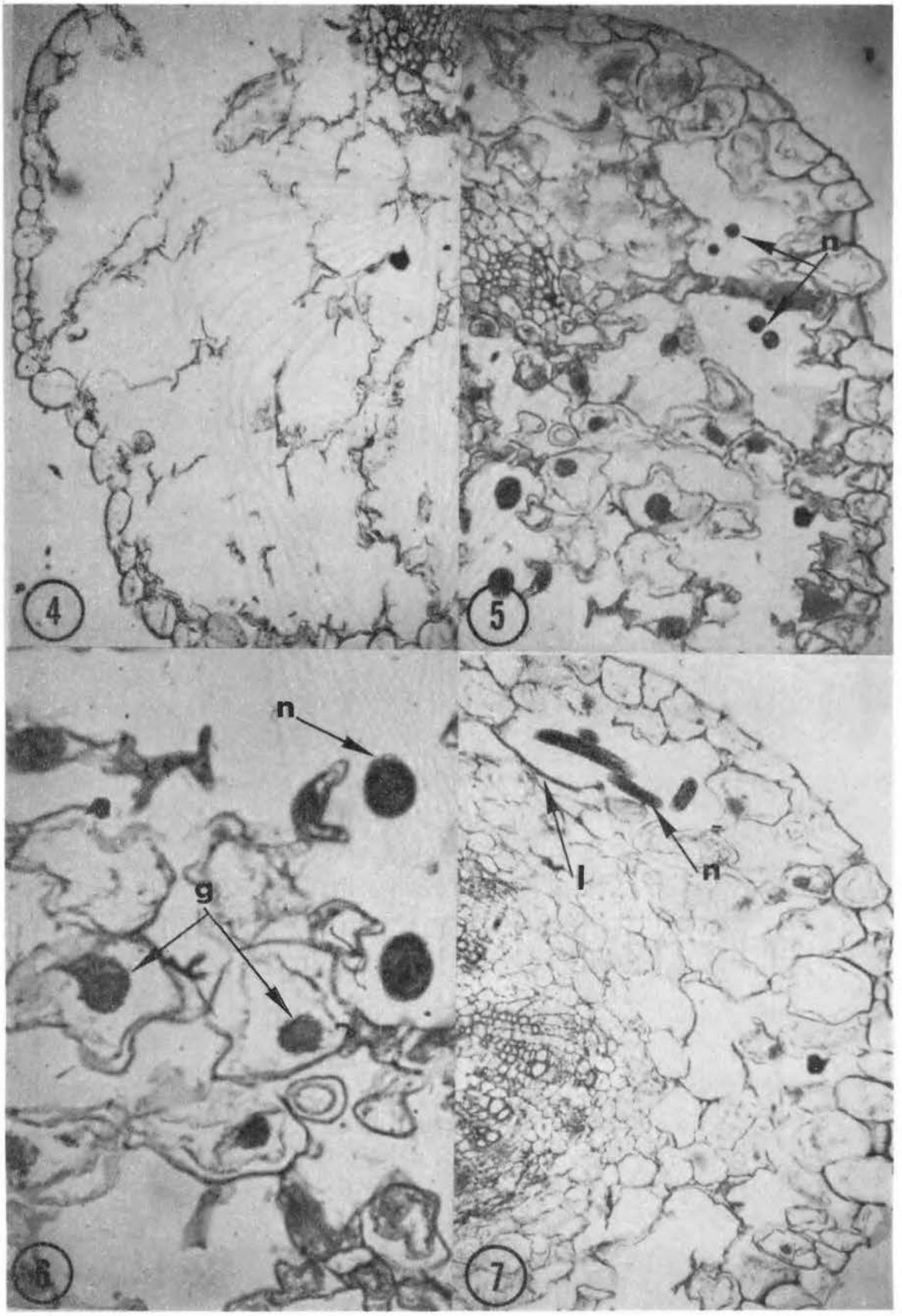
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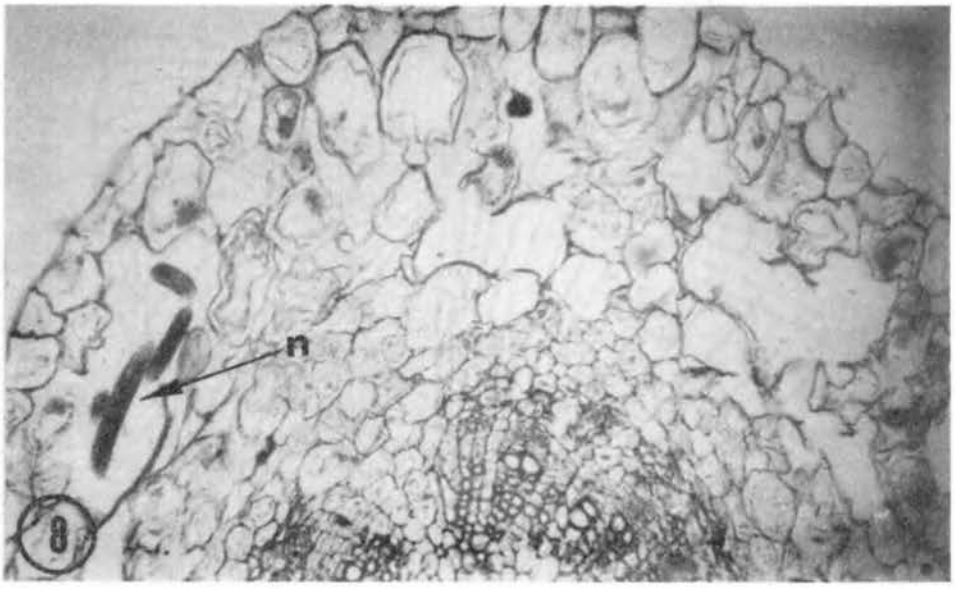
Figure 8. Transverse Section of Buffalo Variety Alfalfa. Note the Position of the Cavities. Magnification: 100X

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: 100X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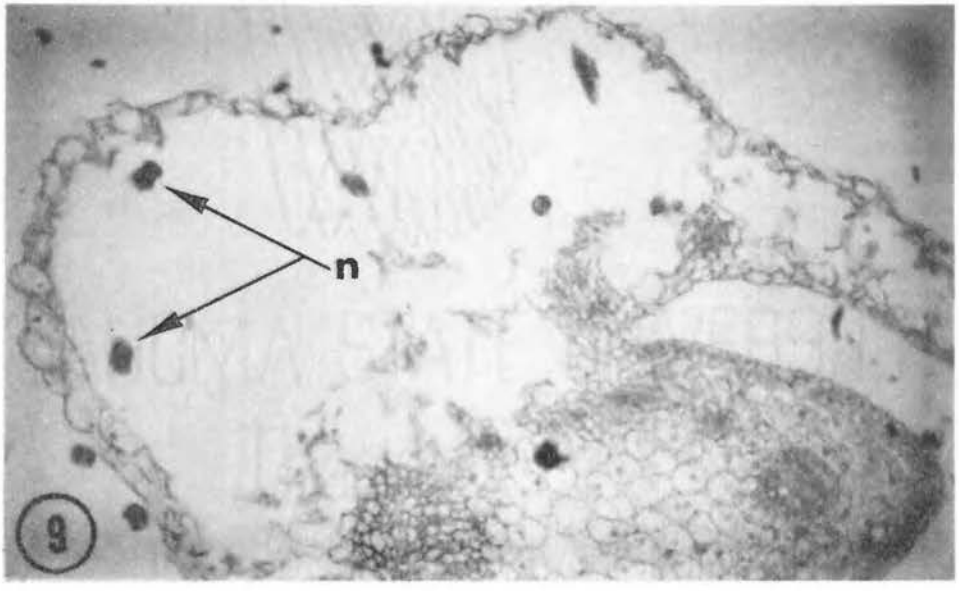
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The resistant Washoe variety produced galls similar to those of the Buffalo variety. Transverse sections showed some cellular destruction in the cortex and some cavity formation although it appeared to be less than that found in the Buffalo variety. 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).

Histology of Mechanically Injured Plants

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 of Nematode Infested Plants

Table I shows the relative response 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. Pectins 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 nematode in infested plants as compared to no heavy lignification in control plants. Washoe variety showed a greater amount of lignification

Figure 10. Transverse Section of Washoe Variety Alfalfa Showing Cell Destruction, Cavity Formation and a Small Number of Granular Cells. Magnification: 100X.

Figure 11. Longitudinal Section of Washoe Variety Alfalfa Showing the Entry Point of the Nematode and Lignification (l) Surrounding the Wound. Magnification: 130X.

Figure 12. Transverse Section of Buffalo Variety Alfalfa Showing Lignification (l) Surrounding the Entry Point. Magnification: 100X.

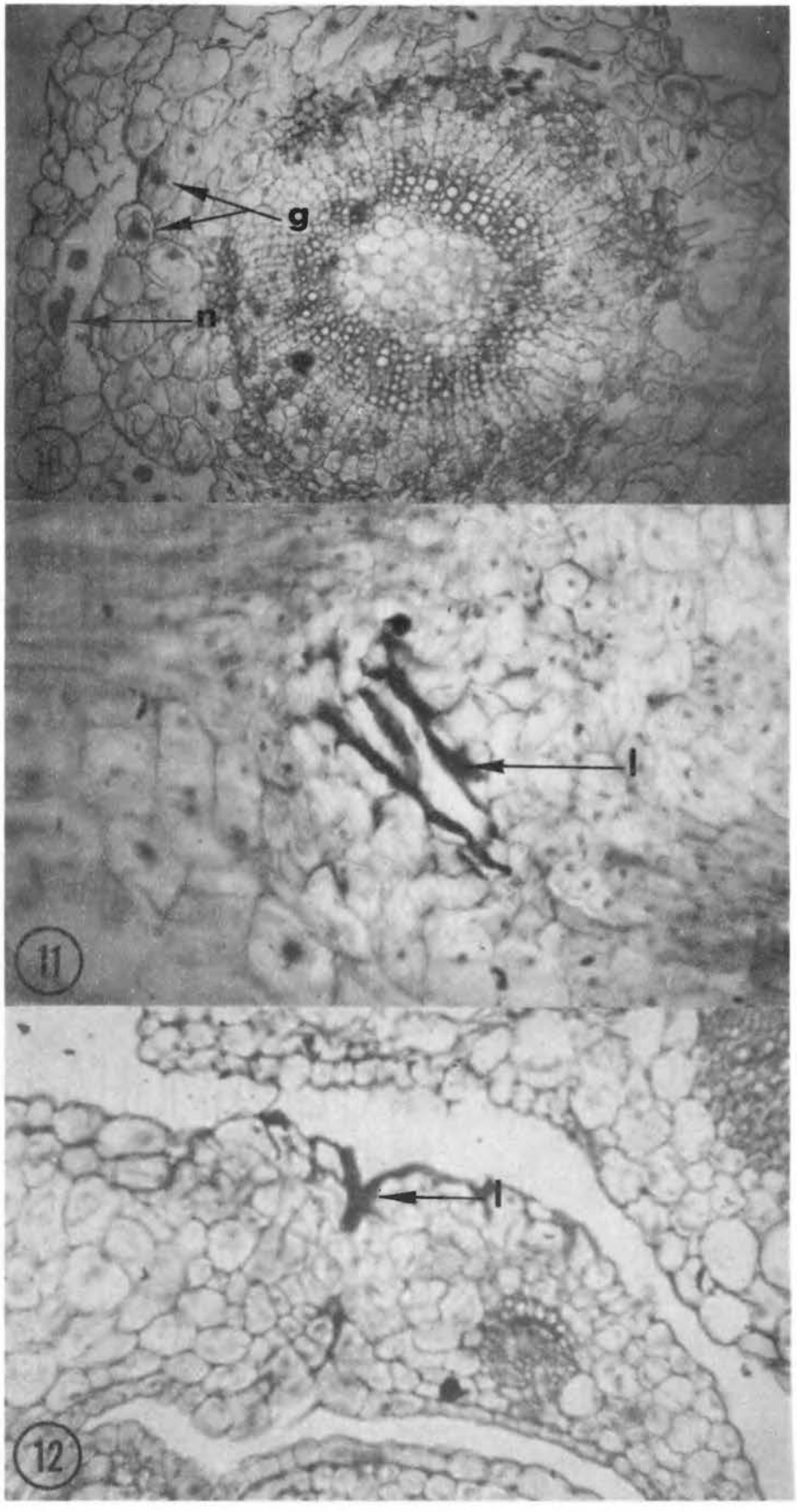
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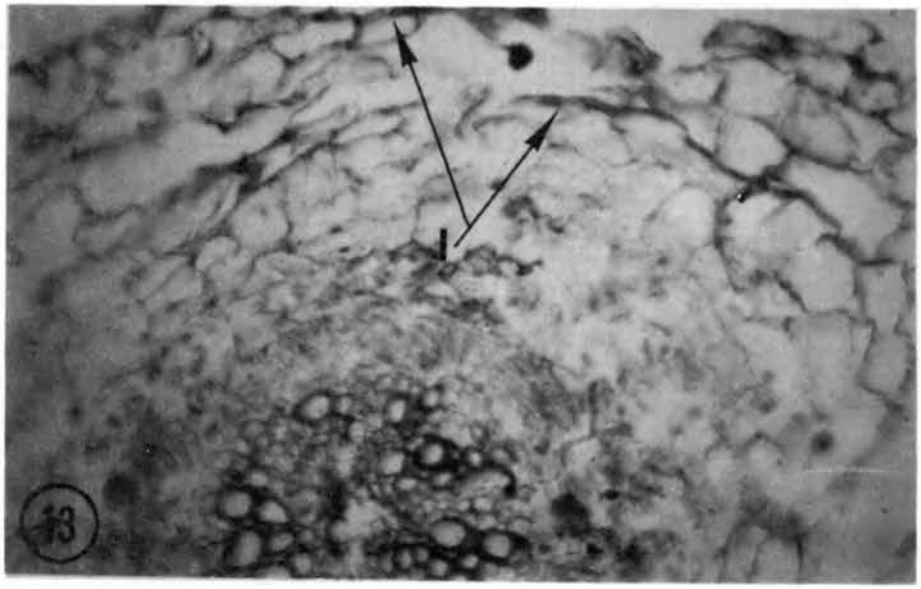
Figure 13. Transverse Section of Buffalo Variety Showing Wound and Lignification (1) Caused by Mechanical Injury. Magnification: 100X.

Figure 14. Longitudinal Section of Washoe Variety Showing Wound and Heavy Lignification(1). Magnification: 100X.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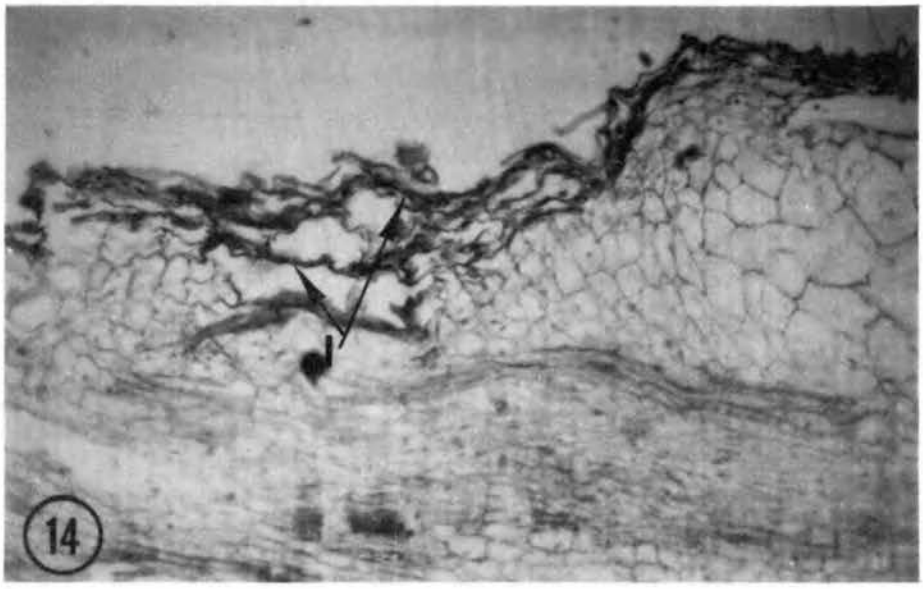
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around galled portions and entry points than did Buffalo variety.

Histochemistry of Mechanically Injured Plants

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).

Enzyme Localization

Control and experimental plants of both varieties of alfalfa showed no change in DOPA-oxidase (phenolase) activity. Both the nematode infested plants 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.

TABLE I

HISTOCHEMISTRY OF NEMATODE INFESTED PLANTS[1]

Test	Buffalo		Washoe	
	control	experimental	control	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

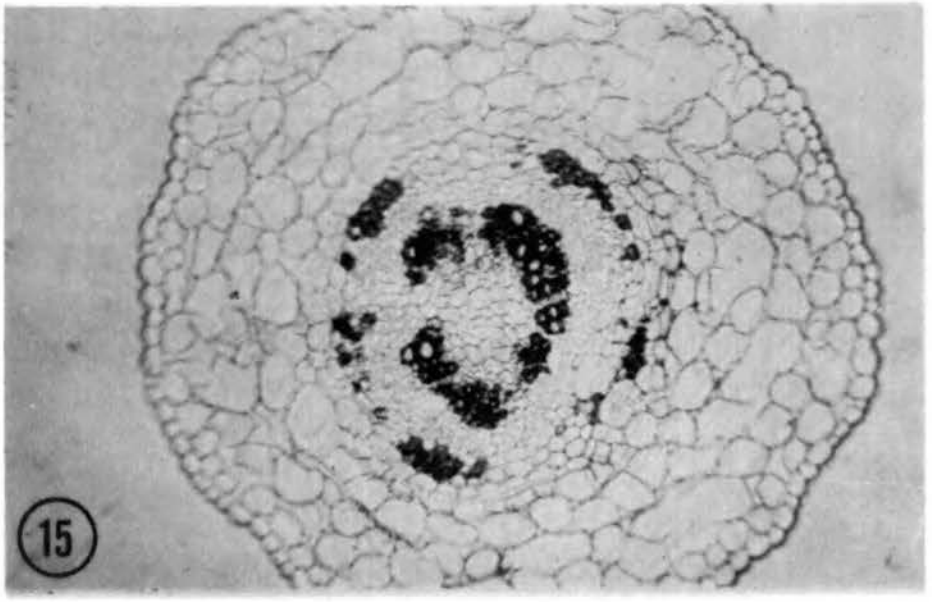
HISTOCHEMISTRY OF MECHANICALLY INJURED PLANTS[1]

Test	Buffalo		Washoe	
	control	experimental	control	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: 100X.

Figure 16. Transverse Section of Buffalo Variety Alfalfa Showing Lignification (l) due to Mechanical Injury. Magnification: 100X.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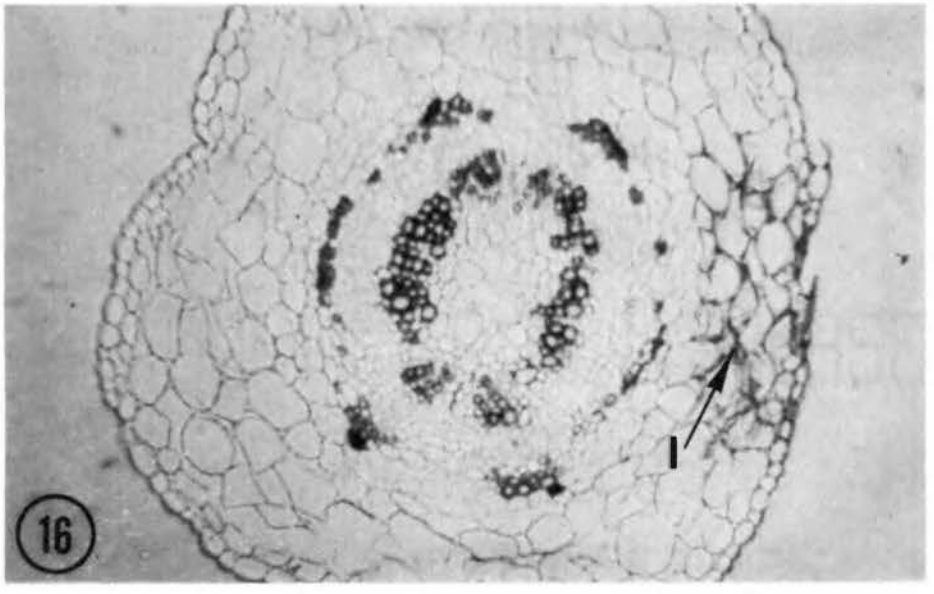
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CHAPTER V

DISCUSSION

Comparisons of the histological response 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 likely 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 occured in both varieties. This would tend to place Washoe in the category of partially resistant rather than completely resistant plants.

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 occured 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 reproduction.

Lack of positive 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, 1969). The lack of activity in this instance indicates that it is not involved in the resistance or susceptibility of these two varieties.

The results of this study seem to indicate that on the basis of comparitive histology alone, Washoe does not exhibit a high level of resistance. Further anatomical work as well as 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.

CHAPTER VI

SUMMARY

The results of histochemical and histological studies of the response of resistant and susceptible alfalfa varieties to nematode and mechanical injury indicated the following:

1. Galling occured 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 caused 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 for resistance in alfalfa varieties to the stem nematode, Ditylenchus dipsaci.

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