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Phenolic compounds associated with host reaction in tomato in injury caused by root-knot and lesion nematodes.

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PHENOLIC COMPOUNDS ASSOCIATED WITH HOST REACTION IN TOMATO TO INJURY CAUSED BY ROOT-KNOT AND LESION NEMATODES

by

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CHAPTER I

INTRODUCTION

Recently, much attention has been focused on the occurrence and metabolism of phenolic substances in plants, especially in response to injury or invasion by pathogens such as fungi, bacteria, viruses, or nematodes. Changes in the metabolism of phenolic compounds are associated with most plant diseases. Oxidized compounds produced in plants after invasion by pathogens often show considerable biological activity, which is the basis for a current hypothesis concerning resistance to plant diseases (Lyr, 1965; Farkas and Kiraly, 1962). A higher concentration of phenolic compounds in resistant varieties as compared to susceptible varieties has been reported (see review by Farkas and Kiraly, 1962). On the other hand, some investigations have indicated a lack of correlation between phenol content and resistance (Cruickshank and Swain, 1956; Hulme and Edney, 1960).

The discoloration and necrosis which usually occur in chrysanthemum leaf tissues in response to nematode invasion have been attributed to the formation and subsequent accumulation of relatively large concentrations of phenolic compounds (Wallace, 1961).

Root-knot nematodes stimulate formation of giant

cells in the vascular tissue of susceptible plants while in resistant plants a necrotic reaction takes place instead. Nematode larvae surrounded by these necrotic areas fail to develop normally and soon die (Dropkin and Nelson, 1960). Tissue resistance has been noted in several plants attacked by lesion nematodes in which the cortex is invaded, but the endodermis serves as a resistant barrier (Pitcher et al., 1960).

The present studies were undertaken for several purposes. The first objective was to compare histologically the effects of two nematode species on resistant versus susceptible tomato varieties. The nematodes chosen were root-knot nematodes, Meliodogyne incognita acrita (Kofoid and White, 1919) Chitwood, 1949, and lesion nematodes, Pratylenchus penetrans (Cobb, 1917) Filipjev and Stekhoven, 1941. The tomatoes chosen were Nemared (resistant to root-knot nematodes), Hawaii 7153 (moderately resistant), and B-5 (susceptible). At the beginning of these experiments, nothing was known about the pathogenicity of lesion nematodes to these varieties.

The second objective was to determine any changes in phenolic compounds which might occur in these varieties after inoculation and to evaluate the implications in the host-parasite relationship of any such changes.

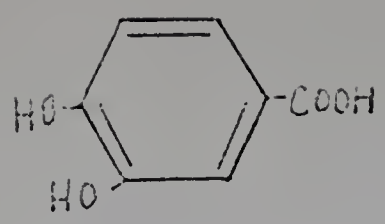
CHAPTER II

LITERATURE REVIEW

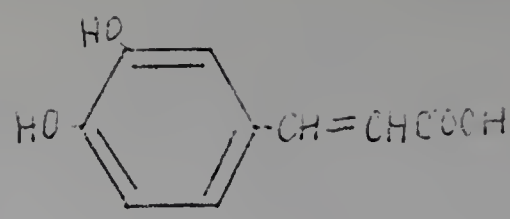
The accumulation of aromatic compounds in host tissues invaded by parasites has been postulated for many years as a factor in chemical resistance of plant tissues. A variety of plant diseases induced by fungi, bacteria, and viruses has been investigated, but the experimental results often have been contradictory. There have been very few studies specifically on the chemical reaction of hosts to injury by lesion and root-knot nematodes.

Phenolic substances occur throughout the plant kingdom, largely in the form of glycosides or esters. They may be classified into the following three groups based on the number of aromatic rings in the molecule (Figs. 1 and 2).

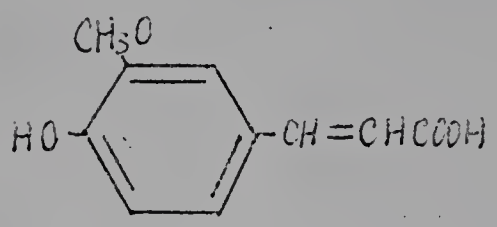
First: phenols and phenolic acids with one aromatic ring, such as catechol and protocatechuic acid. Phenols with 9 carbon atoms, the phenylpropanes, may be separated into the cinnamic acids and coumarins. Examples of common cinnamic acids are p-coumaric, caffeic acids, and the methylated derivatives, ferulic acid and sinapic acid. Caffeic acid occurs chiefly in combination with quinic acid as the depside chlorogenic acid. The coumarins are regarded as lactones of cinnamic acids. Phenolic aldehydes carry both free aldehyde and phenolic groups. Phenolic ketones have the



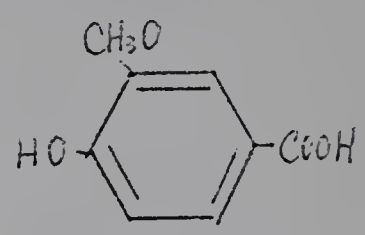
Protocatechuic Acid
(Simple Phenol)



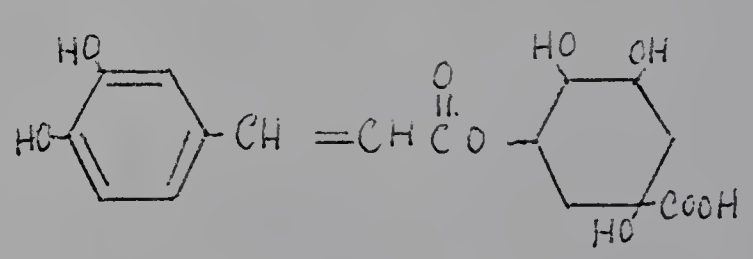
Caffeic Acid
(Derivative of Cinnamic Acid)



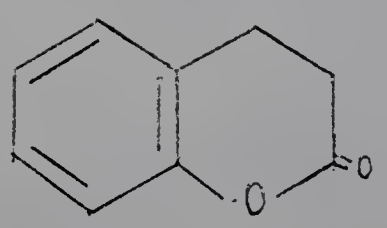
Ferulic Acid
(Methylated derivative
of Cinnamic Acid)



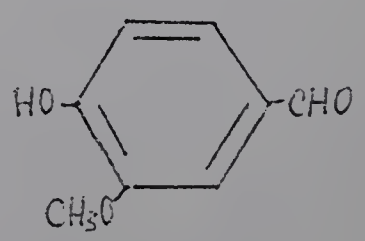
Vanillic Acid
(Phenolic Carboxylic
Acid)



Chlorogenic Acid
(Ester of Caffeic Acid)

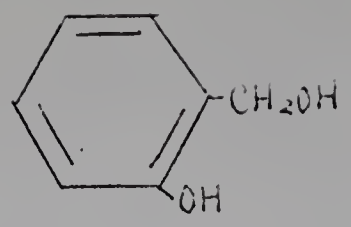


Coumarin
(Lactone of Cinnamic Acid)

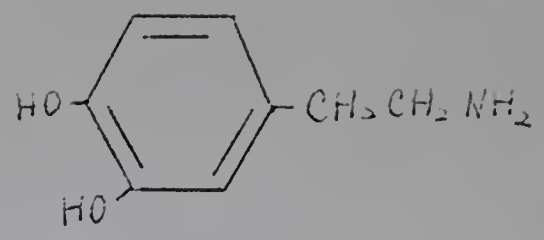


Vanillin
(Phenolic aldehyde)

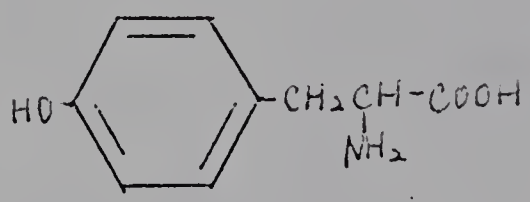
Fig.1.--Structures of typical phenolic compounds



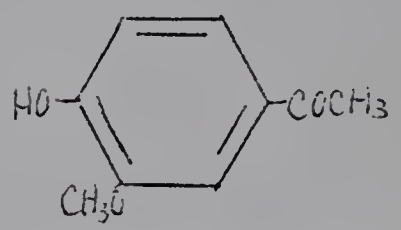
Saligenin
(Phenolic Alcohol)



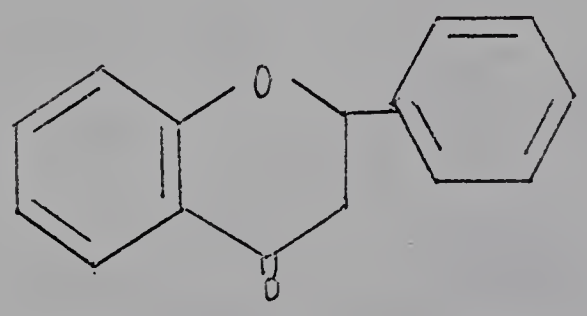
Tyramine
(Phenolic amine)



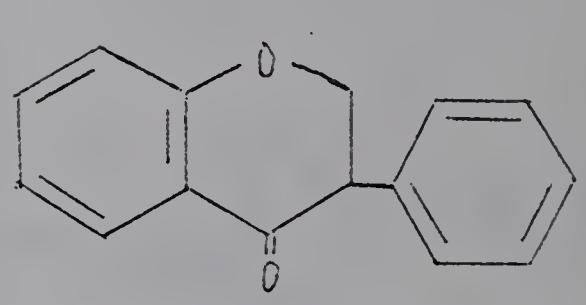
Tyrosine
(Phenolic amino acid)



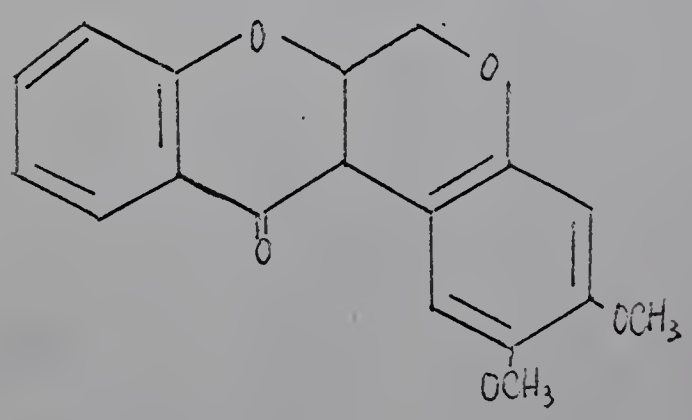
Acetovanillone
(Phenolic Ketone)



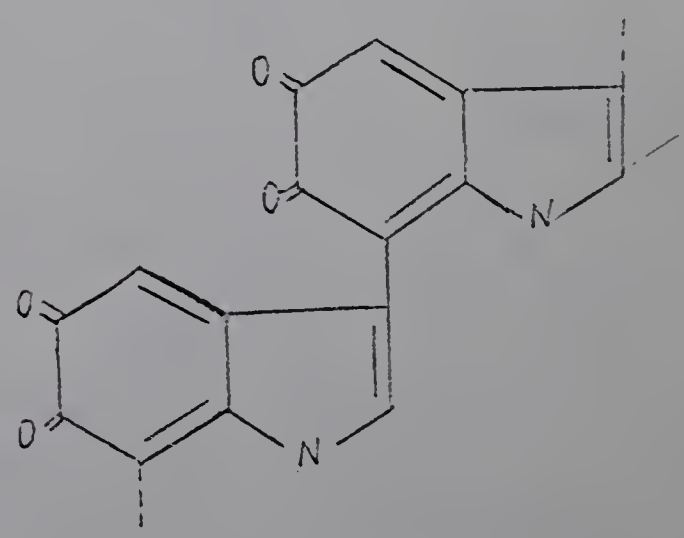
Flavonoid Derivative



Isoflavonoid Derivative



Rotenone
(Rotenoid)



Melanin

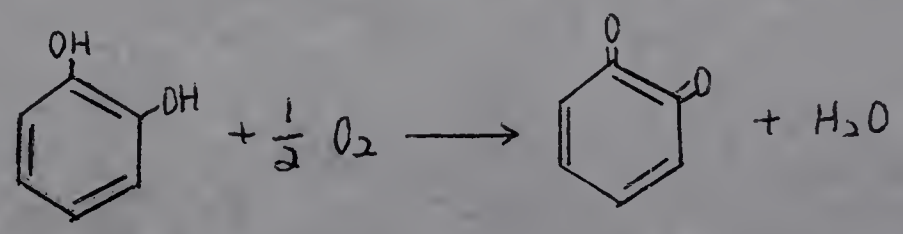
Fig. 2.--Structures of typical phenolic compounds

carbonyl group attached directly to the aromatic ring. Phenolic amines, such as tyramines, carry both a phenolic group and an amino group. Tyrosine and phenylalanine are phenolic amino acids.

Second: phenols with two aromatic rings. Flavonoids, isoflavonoids, and rotenoids are included in this group.

Third: polymeric phenolic compounds. The tannins are a heterogeneous group of polyhydroxy phenolic compounds with molecular weights in the range 600-2000. Lignin is a high molecular weight polymer made up of several different types of phenylpropane units. Melanins are polymer pigments based on indole-5, 6-quinone.

Phenol oxidases are widely distributed in plants. The quinones are formed in the oxidase reaction of o-dihydric phenols to corresponding o-dihydric-quinones which are further polymerized to colored products.



Burges (1962) mentioned three pathways by which phenols may be derived: (1) via prephenic and shikimic acid, (2) via mevalonic acid, and (3) via an acetate condensation. The most widespread route both in microorganisms and higher plants is the shikimic acid pathway. Farkas and Kiraly (1962) hypothesized that the stimulation of biosynthesis of phenolic compounds is due to a pathogen-enhanced operation

of the shikimic acid pathway.

Fungus glycosidases appear to function in the accumulation of phenolic compounds in many diseased tissues. Enzymes may release bound phenolics by hydrolyzing the glycosidic linkage. For example, Davis, Waggoner, and Dimond (1953) demonstrated β-glucosidase activity in the sap of Fusarium-infected tomatoes which was absent in healthy plants. Oku (1959) stressed the ability of Cochliobolus miyabeanus Ito & Kuribay to produce β-glucosidase and to liberate phenols from glucosides in self-inhibiting concentrations.

The earliest investigation of the phenol content in a healthy plant as a factor in preformed resistance was done by Walker (1923). The resistance of onion varieties to the onion smudge fungus, Colletotrichum circinans (Berk.) Vogl. is correlated with red and yellow pigments of the bulb scales. The flavones and anthocyanins in colored scales occur together with the simple phenols, protocatechuic acid and catechol (Angell et al., 1930). These phenols are water soluble and diffuse from the dead cell layers of the scales into the infection drop where they are highly toxic to spores of the fungus (Walker and Link, 1935).

Since the first paper by Walker, many studies have shown that various phenolics localized in non-living protective layers of plant organs play a fungistatic role in preventing infection. Martin and Batt (1958) found that the waxes extracted from leaves of mildew-resistant apple

varieties are inhibitory to the powdery mildew organism because they contained ether soluble substances.

The hypothesis of postinfectional changes in the amount of phenolic substances was first proposed by Cook et al. (1911). He felt that "protective" enzymes might release phenolic substances at the site of parasite attack and these would accumulate in fungitoxic concentrations. Rubin, however, felt that polyphenols accumulated in infected tissue through parasitically enhanced phenol biosynthesis (Farkas and Kiraly, 1962). In the case of resistance of potato to Phytophthora infestans (Mont.) d By., they found a markedly higher polyphenol concentration in the resistant combinations than in the susceptible ones. The nature of the compounds which accumulate is partly clarified. Scopolin, scopoletin, caffeic acid, and chlorogenic acid were synthesized in high amounts after infection.

Nienstaedt's (1953) studies on the tannin content in three chestnut species indicated that differences in the chemical composition of the tannins concerned might explain the differences in resistance. The actual fungitoxic compounds were presumed to be the phenolic breakdown products of tannins liberated after infection. Bazzigher (1955, 1957), however, found tannin splitting enzymes in infected tissues but not in healthy tissues. Strangely enough, the tannin extracted from Castanea sativa Mill. or C. mollissima Blume proved to be 8 times more toxic than the breakdown products by Endothia-enzymes. As the tannins in the heavily

infected tissue portions were almost fully degraded, Bazzigher (1955, 1957) concluded that the enzymes released by the fungus may detoxify the tannins rather than convert them into toxic molecules.

Chlorogenic acid in potato may play an important role in resistance to infection by many organisms. Johnson and Schaal (1952, 1957) found that potato-scab resistance is associated with chlorogenic acid in the periderm. Le Tourneau *et al*. (1958), Lee *et al*. (1957), and Patil, Powelson, and Young (1964) showed that potato varieties resistant to Verticillium wilt contain unusually high amounts of chlorogenic acid in the roots. Higher amounts of chlorogenic acid are found in Phytophthora-resistant potato leaves than in those of susceptible varieties (Valle, 1957). Sokolova, Savelieva, and Solovieva observed that cultures of *Phytophthora infestans* seem to grow well on chlorogenic acid-containing media (Farkas and Kiraly, 1962). Concentrations of 1-2 mg/ml were reported to stimulate growth. It is possible, however, that upon infection, chlorogenic acid is split into its components and thus caffeic acid, a compound more toxic to fungus, is released.

In addition to potato, there are some other plants in which resistance is related to higher amounts of chlorogenic acid. Echandi and Fernandez (1962) found that the canker-resistant coffee hybrids were higher in chlorogenic acid content than the susceptible *C. arabica* L. Resistant young limbs of *C. arabica* contained more chlorogenic acid

than did susceptible old trunks. Harrey et al. (1965) found that increases in chlorogenic acid and chlorogenic acid oxidase occurred during crown gall tumor formation in tomato plants.

Gill (1965) studied the fluorescent metabolites in virus- and rust-infected bean leaves. Seventeen fluorescent materials were found in normal leaves. Tobacco leaves contained additional fluorescent materials when infected with tobacco necrosis virus (TNV) and fifteen more when infected with tobacco mosaic virus (TMV). Thirteen of the new materials appeared to be the same in both TMV and TNV infections, although they were found in greater amount in the more extensively necrotic TNV lesions. One of the most prominent metabolites associated with the hypersensitive virus infection was occasionally present in trace amounts following mild leaf abrasion.

Only eight materials in addition to those in healthy leaves were detected in extracts from non-necrotic rusted tissue. No abnormal fluorescent materials were found in inoculated leaves bearing symptomless infections of a bean strain of TMV. Gill concluded that a rough correlation was apparent between the degree of necrosis and formation of new fluorescent compounds (1965).

Farkas and Solymosy (1965) studied the host metabolism and symptom production in virus-infected plants. They concluded that the symptom-linked enzyme changes might be partially responsible for the alterations of metabolic

patterns in the virus-infected plant and might lead to such biochemical symptoms of virus diseases as the accumulation of phenolics (stimulation of the pentose phosphate shunt) and the accumulation of organic acids (stimulation of dark fixation of CO_2).

The activity of polyphenoloxidase and peroxidase is increased in infected tissues (Farkas and Kiraly, 1962). Mechanical and physical damage can induce polyphenol oxidase activity even in the surrounding uninjured tissues. Excessive heat treatment results in browning of tobacco leaves associated with a decrease in chlorogenic and caffeic acids (Shiroya and Hattori, 1955).

In special cases, the activity of phenol oxidizing enzymes in healthy plants gives some indication of the degree of resistance due to a higher potential for a fast necrotic reaction associated with phenol oxidation. Quinones, the primary oxidation products of phenol exidation, are often highly fungitoxic compounds (Oku, 1960; Schaal and Johnson, 1955). Szent-Gyorgyi and Vietarisz (1931) assumed that the bactericidal and fungicidal effect of oxidation products of phenols depends on nonspecific tanning of host proteins which makes them unavailable for utilization by microorganisms.

Schaal and Johnson (1955) reported that autoxidation products of chlorogenic and caffeic acid are more inhibitory to the growth in vitro of Streptomyces scabies (Thaxt.) Waks. & Henrici than are the parent phenols. Lindeberg (1949) and

Stahmann (Lyr, 1965) found that some fungi are inhibited in their growth by oxidized gallic acid, catechol, and other polyphenols, whereas the unoxidized compound had no inhibitory effect. Oku (1960) obtained similar results with catechol and chlorogenic acid. Noveroske, Kuć, and Williams (1964) stated that oxidation products of phloridzin and phloretin are inhibitory to spore germination of Venturia inaequalis (Cke.) Wint. apud Thuem.

Patil, Powelson, and Young (1964) proposed that roots of young potato plants are resistant to cortical invasion by Verticillium alboatrum Reinke & Berth. because the pathogen is inhibited at the site of root penetration by oxidation products of chlorogenic acid. Patil et al. (1964) postulated that when wounds occur on susceptible varieties, the amount of chlorogenic acid available is small in relation to the high levels of polyphenol oxidase and is quickly oxidized and then polymerized at the site of the wound, leaving less quinone available for fungitoxic activity than would be available in a resistant variety.

Wallace (1961) studied the chemical nature of browning of chrysanthemum infested by the foliar nematode, Aphelenchoides ritzemabosi (Schwartz) Steiner. He indicated that browning in leaves was caused by an enzymatic oxidation of polyphenols to quinones and subsequent polymerization to brown pigments, presumably melanins. He found that chlorogenic acid and isochlorogenic acid are probably the chief substrates for browning in the chrysanthemum. He detected

no differences between uninfected resistant and susceptible chrysanthemum varieties in their phenolic contents and polyphenol oxidase concentrations. Resistance was attributed to a factor other than phenols.

Pitcher, Patrick, and Mountain (1960) compared the pathogenicity of Pratylenchus penetrans (Cobb, 1917) on the roots of apple and peach. Apple feeder roots reacted to invasion by rapid discoloration of outermost (epidermis and hypodermis) and the innermost (inner cortex and endodermis) cortical tissues, but showed little or no reaction in the intervening cortical parenchyma. In contrast, all cortical tissues in the roots of peach readily became discolored upon invasion by the nematode. Histochemical tests indicated that sensitivity to Pratylenchus penetrans is correlated with the presence and concentration of phenolic substances in the various tissues of the two hosts. The cortical parenchyma of apple roots, which is tolerant to nematode colonization, is relatively free from phenolic substances. The dermal and endodermal layers, which show a rapid necrotic reaction to P. penetrans, contain high concentrations of these substances (Mountain and Patrick, 1959; Pitcher et al., 1960). In contrast, peach root sections showed high concentrations of phenolic substances in all tissues. Similar reactions have been shown with strawberry and celery (Townshend, 1963).

Mountain and Patrick (1959) found that when lesion nematodes feed on peach roots the phenolic glycoside

amygdalin is hydrolyzed and hydrocyanic acid is released, much to the detriment of both host and parasite. Resistant peach rootstocks are characterized by high concentrations of amygdalin. The enzyme demonstrated in this reaction, a β-glucosidase, may be important in other host-parasite interactions since many phenolic compounds in plants occur naturally as less-toxic glycosides.

Root-knot nematodes (Meloidogyne spp.) differ from lesion nematodes (Pratylenchus spp.) in that larvae move intracellularly through the cortex to the vascular cylinder where they become sedentary during development. Vascular cells around the head of the nematode coalesce to form syncytia or giant cells.

Dropkin and Nelson (1960) classified host reaction to the invasion of root-knot nematodes into the following four categories:

Type 1. Cells around the head of the nematode died quickly.

Type 2. Cells undergo moderate cell fusion and display an unusually great number of cell inclusions of peculiar kinds.

Type 3. Cells are very large with many nuclei and with a diffuse, highly vacuolated cytoplasm.

Type 4. Giant cells with dense cytoplasm and thickened walls are formed.

Christie (1949) showed that production of giant cells is necessary for development of female root-knot nematodes.

While the necrosis and browning commonly found in root-knot-resistant hosts has never been shown to be linked with phenols, it would be logical to expect that reactions would follow the same pattern as most other types of plant injury.

CHAPTER III

MATERIALS AND METHODS

Preparation of tomato seedlings.

The three varieties were genetically similar except that Nemared is resistant to Meloidogyne incognita acrita (Kofoid and White, 1919) Chitwood, 1949, Hawaii 7153 is partially resistant and B-5 is susceptible. Seeds of these three varieties of tomato were given by Professor R. E. Young of the Waltham Field Station. The resistance in both of the nematode-resistant lines originally came from the same wild tomato. The breeding of Nemared has been conducted in Oklahoma, whereas Hawaii 7153 came from Hawaii.

Seeds of the three varieties of tomato were surface-sterilized for 15 minutes in sodium hypochlorite solution (prepared by mixing Clorox bleach with an equal volume of water), washed in sterile distilled water, and germinated on nutrient agar in petri dishes. After about five days, seedlings were transplanted in groups of ten into petri dishes containing nutrient agar (Krusberg, 1961). Seedlings were inoculated two days later.

Preparation of nematodes for inoculum.

Specimens of M. incognita acrita were cultured aseptically in tomato roots grown in nutrient agar in petri dishes. Larvae to be used for inoculum were obtained by

picking egg masses from the surfaces of galls and hatching them in sterile water.

Pratylenchus penetrans (Cobb, 1917) Filipjev and Stekhoven, 1941, were cultured aseptically in alfalfa callus tissue grown in nutrient agar containing 2,4-dichlorophenoxy-acetic acid (Krusberg, 1961).

Nematodes for inoculum were collected by placing the callus tissues on cotton plugs in 15 ml, conical, contrifuge tubes. Each tube had been filled with water to the level of the cotton and autoclaved. The callus tissues, cotton, and most of the water were discarded after 24 hours, when the nematodes had migrated through the cotton and settled. The resulting concentrated suspension of nematodes was poured over the roots of seedlings in petri dishes.

The inoculated seedlings were maintained in a plant growth room at 20° C. Uninoculated seedlings served as controls.

Mechanical injury. Roots of week-old tomato seedlings were punctured in several areas with a sterile micro-needle and then allowed to grow in nutrient agar in petri dishes where development of symptoms was observed.

Histochemical tests. Portions of infected roots containing lesions or galls were placed on a clean glass slide; a few drops of diazotized sulfanilic acid (DSA), a general histochemical reagent for phenolic compounds, were added. Any color changes in lesions or galls were observed. Punctured roots were tested in the same manner.

Cross or longitudinal sections of living roots, 30 μ thick, were cut from lesion areas and galls in a cryotome at -20° C. Portions of infected areas were mounted in liquid O.C.T., a commercial embedding medium. Tissue sections were transferred to a glass slide and stained with DSA.

Histological studies. Paraffin sections of root lesions, galls, and punctured roots were prepared following standard procedures (Jensen, 1962).

Root samples were fixed in CRAF solution for 24 hours, washed in running tap water for two hours, and dehydrated through a tertiary butyl alcohol series (Jensen, 1962). Dehydrated roots were embedded in Fisher's tissuemat and 10 μ cross- and logitudinal sections were cut with a rotary microtome. Sections were fixed to slides with Haupt's adhesive, stained with safranin and fast green, and mounted with Fisher's permount.

Preparation of crude extracts. Crude extracts were prepared from lesion areas, swollen areas, punctured roots, and healthy roots. Root tissues were cut and weighed separately, dropped into boiling methanol for 1 minute, cooled, and homogenized in a glass tissue grinder for 3-5 minutes. The homogenates were centrifuged at 31000 x G. for 5 minutes to remove cell fragments. Each extract was concentrated to 1 ml. extract per 1 g. tissue in a rotary evaporator under reduced pressure at 40° C. The concentrated extracts were stored at -15° C.

Paper chromatography. Concentrated methanolic

extracts were analyzed chromatographically on Whatman No. 1 paper, using methods described by Block et al. (1958).

Chromatograms were developed by descending chromatography using the following solvents:

(1) n-butanol-acetic acid-water (BAW), 4:1:2 v/v/v, organic phase, or

(2) n-butanol-acetic acid-water (BAW), 4:1:5 v/v/v, organic phase, a suitable solvent for many phenols.

(3) 2 per cent acetic acid, used for separation of cinnamic acid derivatives;

(4) hydrochloric acid-acetic acid-water (HAW), 3:30:10 v/v/v, used to separate phenolic compounds.

Chromatograms were observed under ultraviolet (UV) light (320-400 mμ), before and after exposure to NH_4OH fumes, for detection of aromatic compounds.

The following color reagents were used for detection and identification of compounds:

(1) diazotized sulfanilic acid (DSA) (Block et al., 1958), a general reagent for most groups of phenols;

(2) ferric chloride reagent (Smith, 1960), a reagent for phenols and tannins;

(3) ammoniacal silver nitrate (Block et al., 1958), for detecting o-dihydroxy compounds, although not specific for this group;

(4) Arnow reagent (Arnow, 1937), for detecting di- or tri-hydroxy phenolic compounds;

(5) anthrone reagent (Block et al., 1958), used for detecting ketohexoses and ketopentoses; and

(6) 0.3% ninhydrin in 95% ethanol (Block et al., 1958), reagent for detecting amino acids.

Spectrophotometric methods. Unidentified phenolic compounds were detected by color reagents. These same spots detected in unsprayed paper chromatograms using UV light, were cut out and eluted in methanol. The absorption spectra of eluates were measured in a Bausch and Lomb Spectromic 505 recording spectrophotometer.

Quantitative assay of chlorogenic acid in three varieties of tomato. Methanol extracts of equal weights (0.0477±0.0001 g.) of root tissues of the same age from each of the three tomato varieties were analyzed for chlorogenic acid by the method of Ruckenbrod (Block et al., 1958). The optical density of each extract was measured at 324-326 mμ and compared with a standard curve prepared with known chlorogenic acid.

Assay of polyphenol oxidase activity. Equal weights (0.0620±0.0001 g.) of healthy roots of each variety and infected roots six days after inoculation with P. penetrans were homogenized with 1 ml. phosphate buffer (0.02 M, pH 7), and centrifuged to remove debris.

The assay for polyphenol oxidase activity is based on the formation of a dark-colored polymeric compound from catechin. One hundred and fifty λ of extract were added to a cuvette containing three ml. of a 0.25% catechin solution. The formation of brown pigment was measured spectrophotometrically in a Bausch and Lomb Spectromic 505 recording

spectrophotometer at 400 mμ during the interval from 30 to 210 seconds after addition of the extract (Winstead et al., 1954).

Toxicity of chlorogenic acid to the root-knot and lesion nematodes. Individuals of each species were placed in the vials containing different concentrations of chlorogenic acid (0 ppm, 100 ppm, 200 ppm, 500 ppm, 1000 ppm, 2000 ppm) for 24 hours. Viable and killed larvae were counted under the microscope. Only those moving were counted as viable.

CHAPTER IV

RESULTS

Symptoms.

(1) Injury caused by root-knot nematodes. Three to four days after inoculation with M. incognita acrita larvae, galls appeared on the roots of the susceptible tomato variety, B-5. At first there was only a slight swelling on the root, but enlargement of epidermal cells could be observed microscopically (Fig. 3A). Sometimes the gall surface was broken by the rapid development of female nematodes and giant cells. Female nematodes matured after two to three weeks within the roots and during this time galls became spherical. Egg laying began from twenty to thirty days after penetration of the root by larvae, and gelatinous egg masses then formed on the surface of the gall.

No galls formed on the resistant tomato variety, Nemared. Dark brown flecks appeared on the roots where larvae had penetrated. Many larvae failed to penetrate and died outside the roots, a point which may be of considerable significance.

The moderately resistant tomato variety, Hawaii 7153, showed a variety of symptoms. Either necrotic lesions or galls were found. Those galls which formed were no different from the galls formed on the B-5 tomato plants, except

that few females produced egg masses.

(2) Injury caused by lesion nematodes. All three varieties of tomato seedlings had the same reaction to injury caused by lesion nematodes. Twenty-four hours after inoculation, large numbers of nematodes in all stages of development could be found in limited areas, causing a yellow lesion (Fig. 3B). The lesions were small at first, but they gradually enlarged as the nematodes moved, fed, and reproduced in the cortical tissues of root.

(3) Mechanically induced injury. Root punctures inflicted by sterile microneedles became discolored after twenty-four hours. The visible symptoms were similar to the symptoms caused by nematodes except that lesions were limited to the site of injury and did not later spread.

Histochemical test with DSA.

(1) Injury caused by root-knot nematodes. Whole galls from the B-5 variety tomato root were crushed slightly and stained with DSA reagent for detection of phenols. Little or no reaction occurred in the gall tissues in any stage of development (Fig. 4A). The addition of DSA caused the necrotic flecks formed on the Nemared variety tomato root to become intensely brown.

Cross and longitudinal frozen sections of living B-5 galls showed that larvae fed on giant cells with no browning of cells. Tissues did not react to the addition of DSA (Fig. 4B). Sections of lesions on Nemared variety revealed brownish contents in the endodermal cells which reacted to

DSA (Fig. 4C). Necrotic lesions or galls formed on Hawaii 7153 variety had the same reactions to DSA as those described above.

(2) Injury caused by lesion nematodes. The three infected tomato varieties had the same reaction to staining with DSA. Injured tissues became yellow to brown in color. Cross sections of infected tissue stained with DSA showed an overall brownish color with the endodermis staining much deeper than any other tissues (Fig. 5A and B). This reaction indicated the presence of a relatively large concentration of phenolic substances in the endodermal layer.

(3) Mechanically induced injury. The reactions of mechanically injured roots were similar to those of roots injured by lesion nematodes. A yellowish color, intensified by DSA, surrounded the site of puncture.

Histological studies.

(1) Injury caused by root-knot nematodes. Cross sections of the galls which formed on the B-5 variety infected with M. incognita acrita showed the giant cells with thick walls and dense cytoplasm, distortion of the vascular elements, and proliferation of the parenchyma which are characteristic of root-knot galls (Fig. 6A). Giant cells resembled those of type IV as described by Dropkin and Nelson (1960).

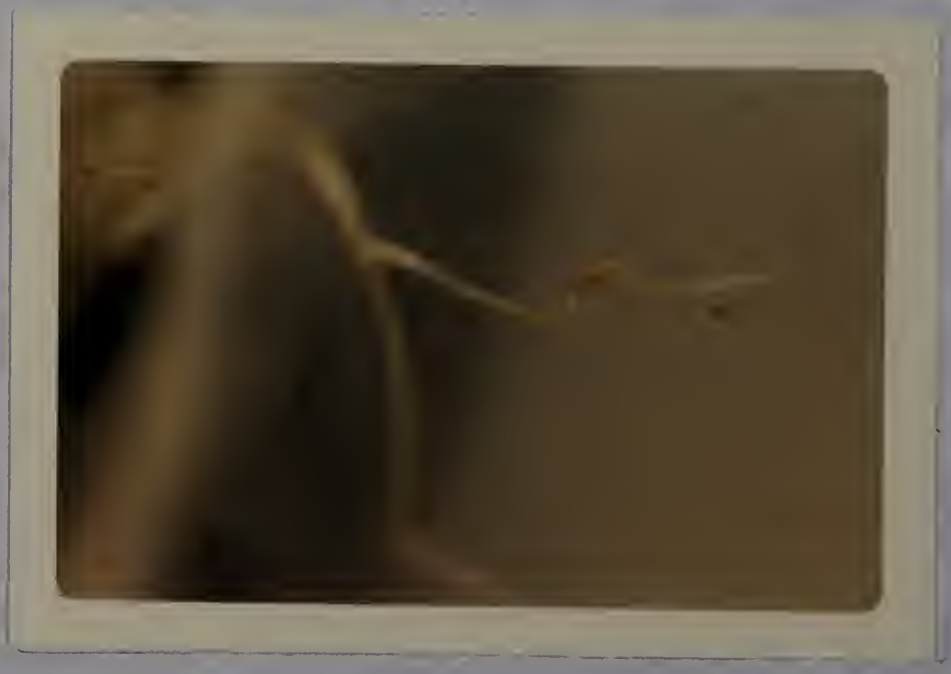
In the Nemared plants, the nematodes were found in the midst of necrotic cells which were deeply stained with safranin. Larvae did not develop and giant cells were not

Fig. 3.--Symptoms on tomato seedlings grown axenically in nutrient agar.

A. Gall caused by root-knot nematode (M. incognita acrita) on B-5 variety growing on nutrient agar four days after inoculation.

B. Lesion caused by lesion nematode (P. penetrans) on Nemared variety growing on nutrient agar three days after inoculation. Lesions are typical for all varieties (Hawaii 7153 and B-5). Much smaller lesions than this are caused by root-knot nematodes on Nemared variety.

A



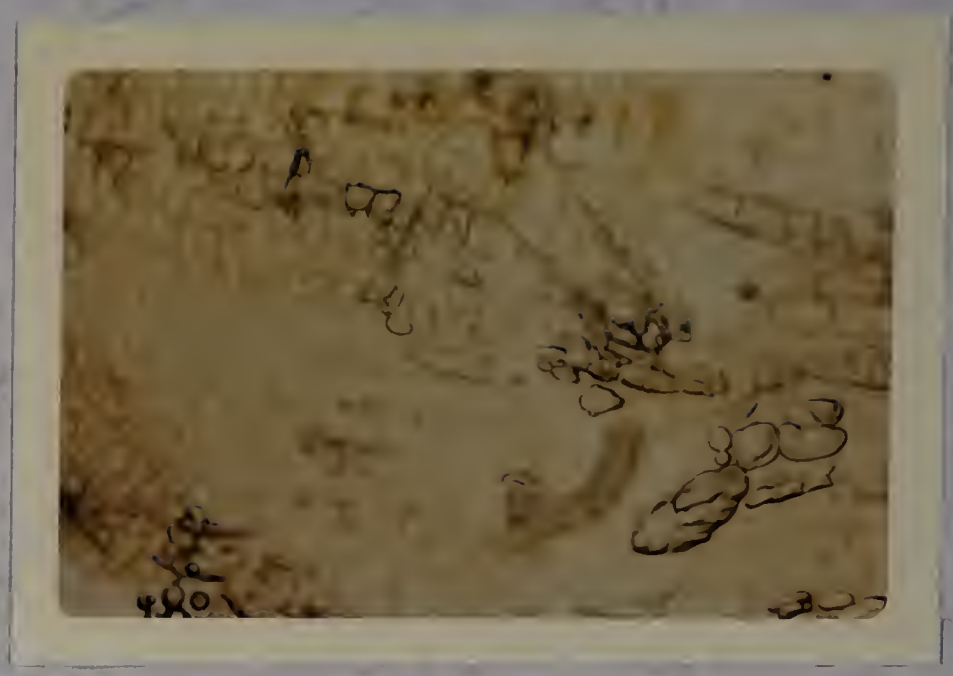
B

Fig. 4.--Histochemical test for phenolic compounds in tomatoes inoculated with M. incognita acrita, using DSA reagent.

A. Portion of crushed gall on B-5 variety. Note the larvae inside the gall and absence of staining reaction.

B. Longitudinal frozen section of root-knot gall on B-5 variety, showing head of larvae and giant cells.

C. Frozen cross section of lesion on Nemared variety, stained with DSA showing intense browning reaction of endodermis.



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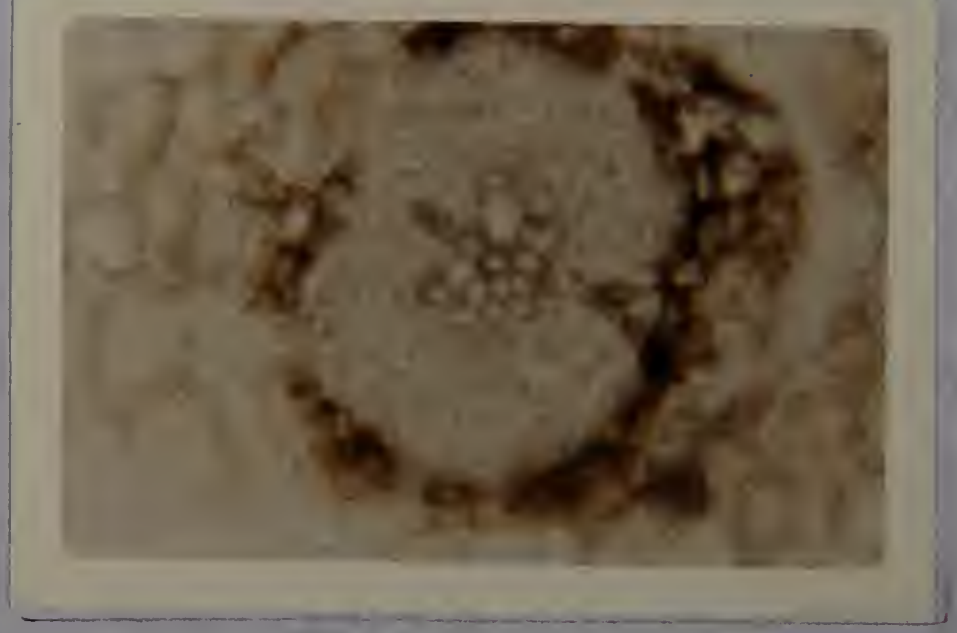
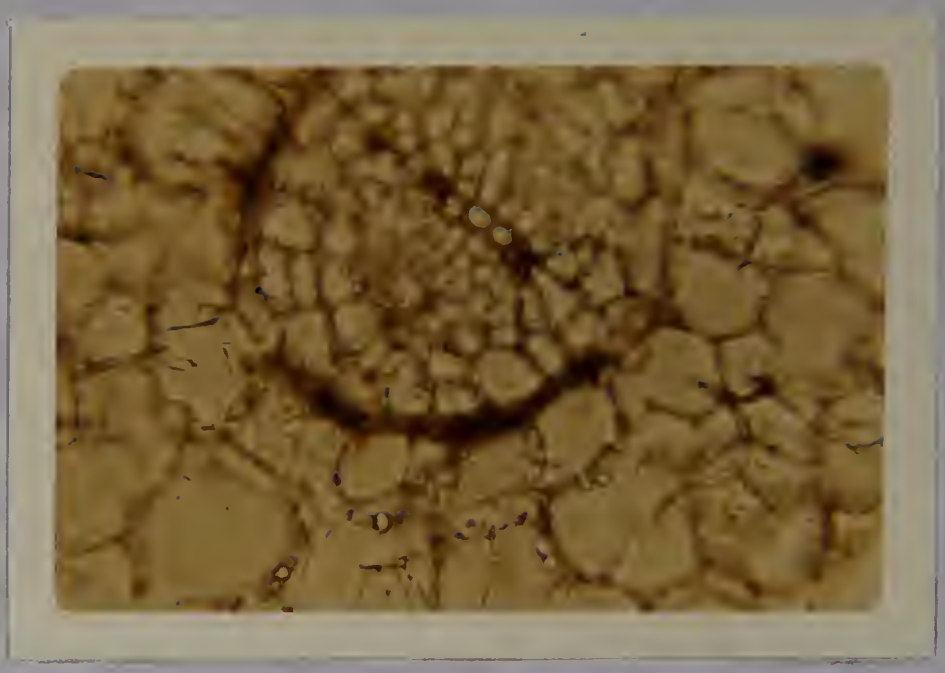


C

Fig. 5.--Histochemical test for phenolic compounds in tomatoes inoculated with Pratylenchus penetrans, using DSA reagent.

A. Frozen cross section of Nemared root lesion. The brownish color on the endodermis was intensified with DSA.

B. Longitudinal frozen section through B-5 lesion. Note lesion nematode and brownish cells in the endodermal region.



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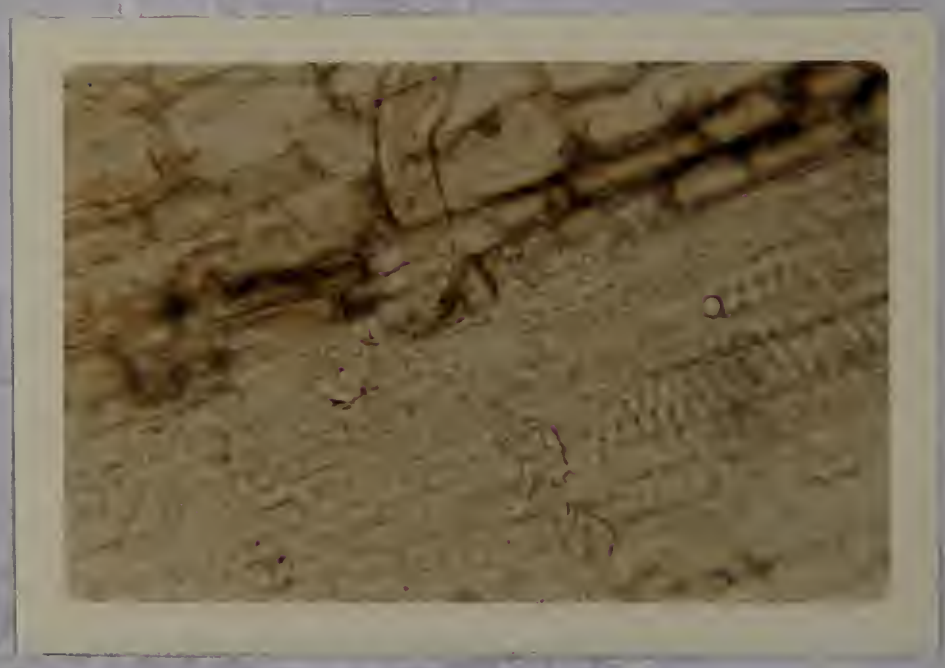
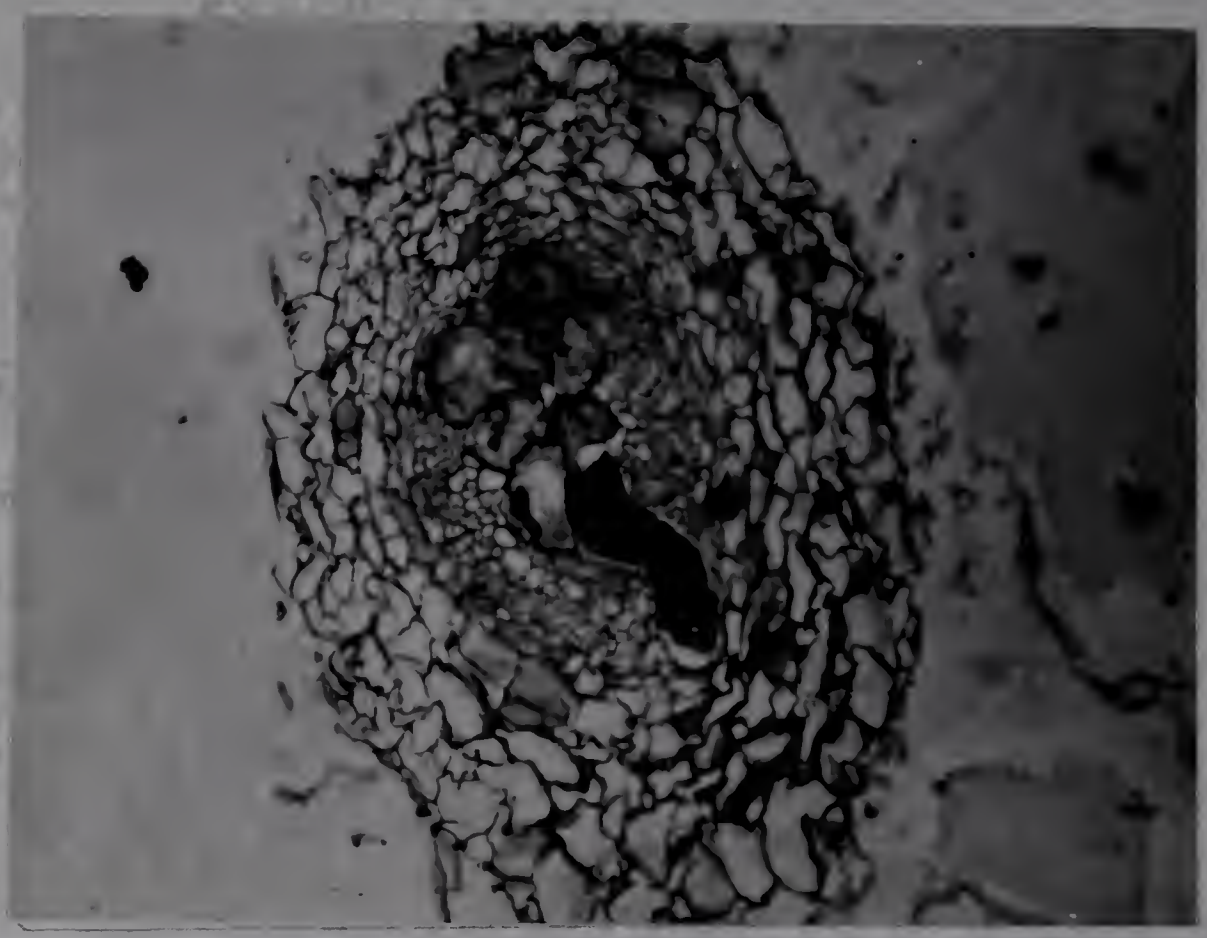


B

Fig. 6.--Paraffin sections of tomato roots infected with root-knot nematode (M. incognita acrita).

A. Cross section of gall on B-5 variety. Note developing larva and giant cells.

B. Cross section of lesion on Nemared variety. Note necrotic cells surrounding larvae which did not develop.



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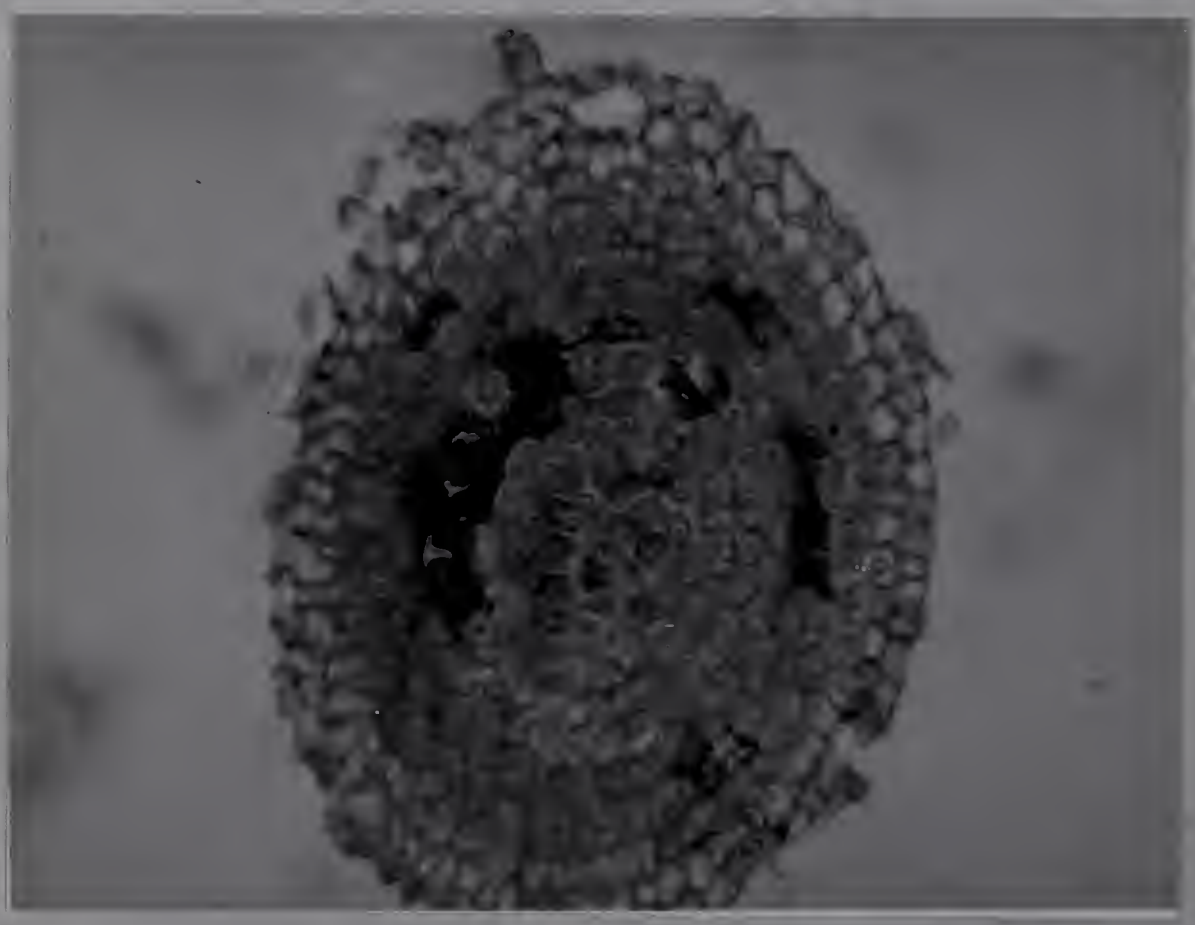


B

formed (Fig. 6B). This reaction was similar to type I of Dropkin and Nelson (1960).

(2) Injury caused by lesion nematodes. Cross and longitudinal paraffin sections of lesions caused by P. penetrans showed similar injury in all three varieties. All stages of nematodes, including eggs, could be found in the cortex. Cells adjacent to nematodes were deeply stained with safranin (Fig. 7A). In invaded areas, several cells in the cortex were often badly damaged by several nematodes, resulting in a cavity (Fig. 7B). After five to six weeks (the later stages of infection), nematodes were found in the vascular tissues of B-5 (Fig. 8A), but none was ever found in the vascular tissues of Nemared (Fig. 8B).

Identification of phenolic compounds by paper chromatography and spectrophotometry.

Paper chromatography of methanol extracts from healthy tomatoes revealed seven spots which fluoresced under UV light (Fig. 9A). The same spots were obtained from all three varieties. An additional spot was observed in extracts from all three varieties when inoculated with lesion nematodes.

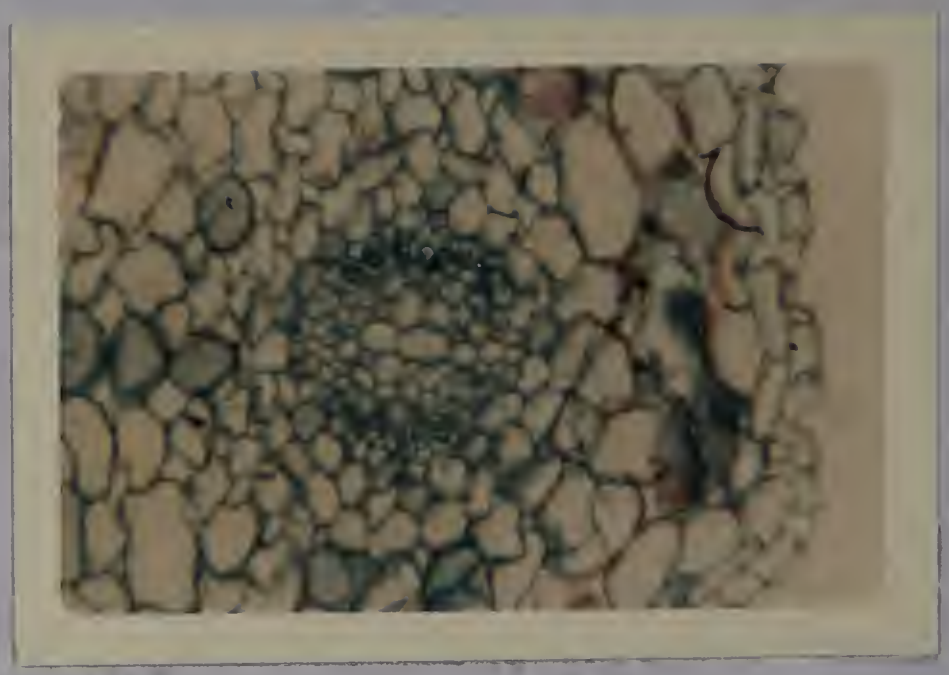
The Rf values of the fluorescent compounds found in healthy and infected tissue extracts are reported in Table 1.

By spraying chromatograms with several reagents for detection of phenolic compounds, it was determined that one spot, present in both healthy and infected extracts at Rf 0.58, was the main phenolic compound in methanol extract of tomato roots. A weakly reacting spot (Rf 0.46 in B.A.W.)

Fig. 7.--Paraffin sections of tomato roots infected with lesion nematodes three to four days after inoculation.

A. Cross section of lesion on Nemared variety. Stained with safranin and fast green. Note staining of cell walls in area of feeding.

B. Cross section of lesion on Nemared variety. Note cavity in cortex containing several nematodes.



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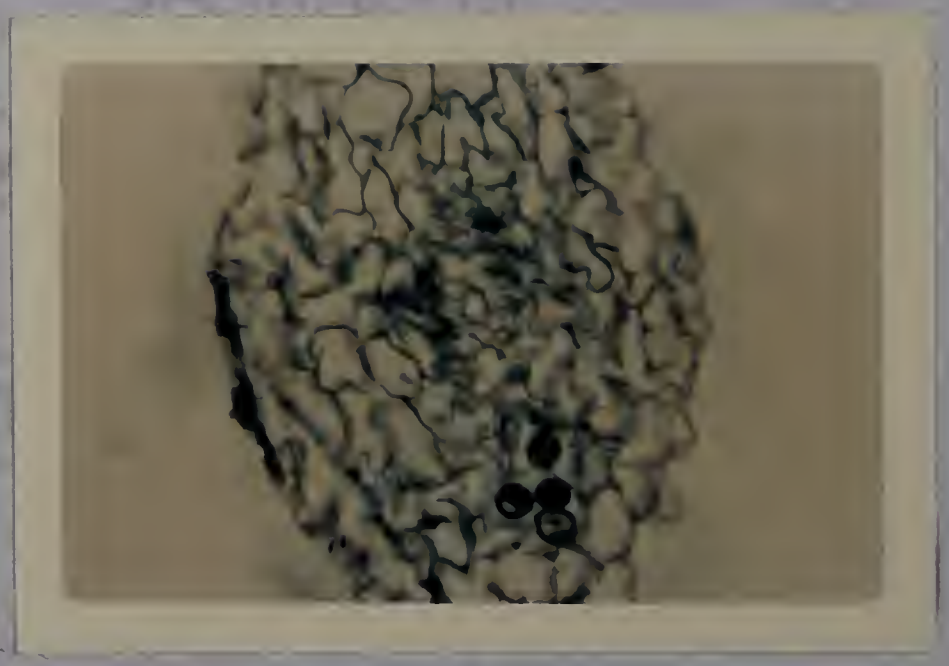
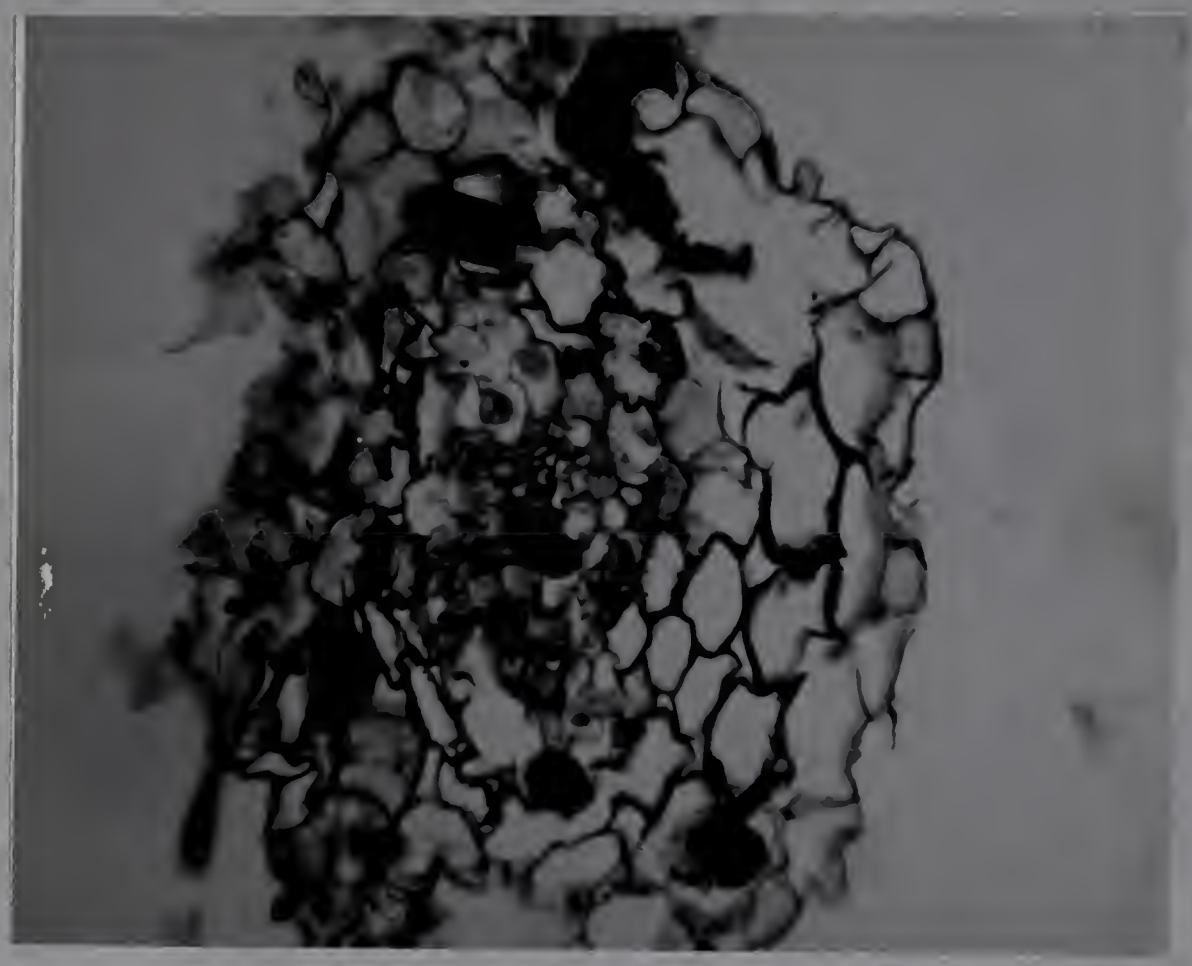


B

Fig. 8.--Paraffin sections of tomato roots infected with lesion nematodes five weeks after inoculation.

A. Cross section of lesion on B-5 variety. Note nematodes within vascular cylinder.

B. Cross section of lesion on Nemared variety. None of nematode was ever found in the vascular tissue even after extensive cortical breakdown.



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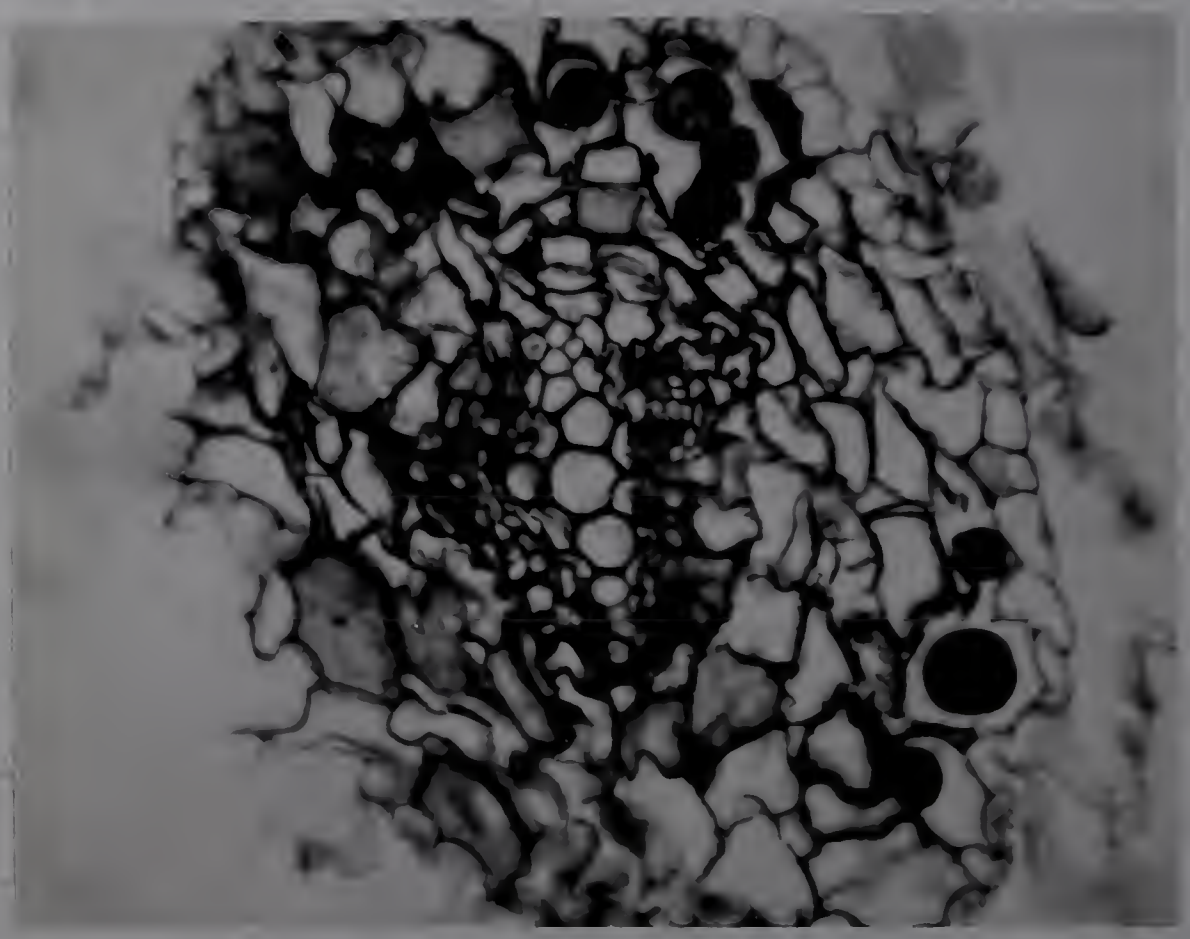

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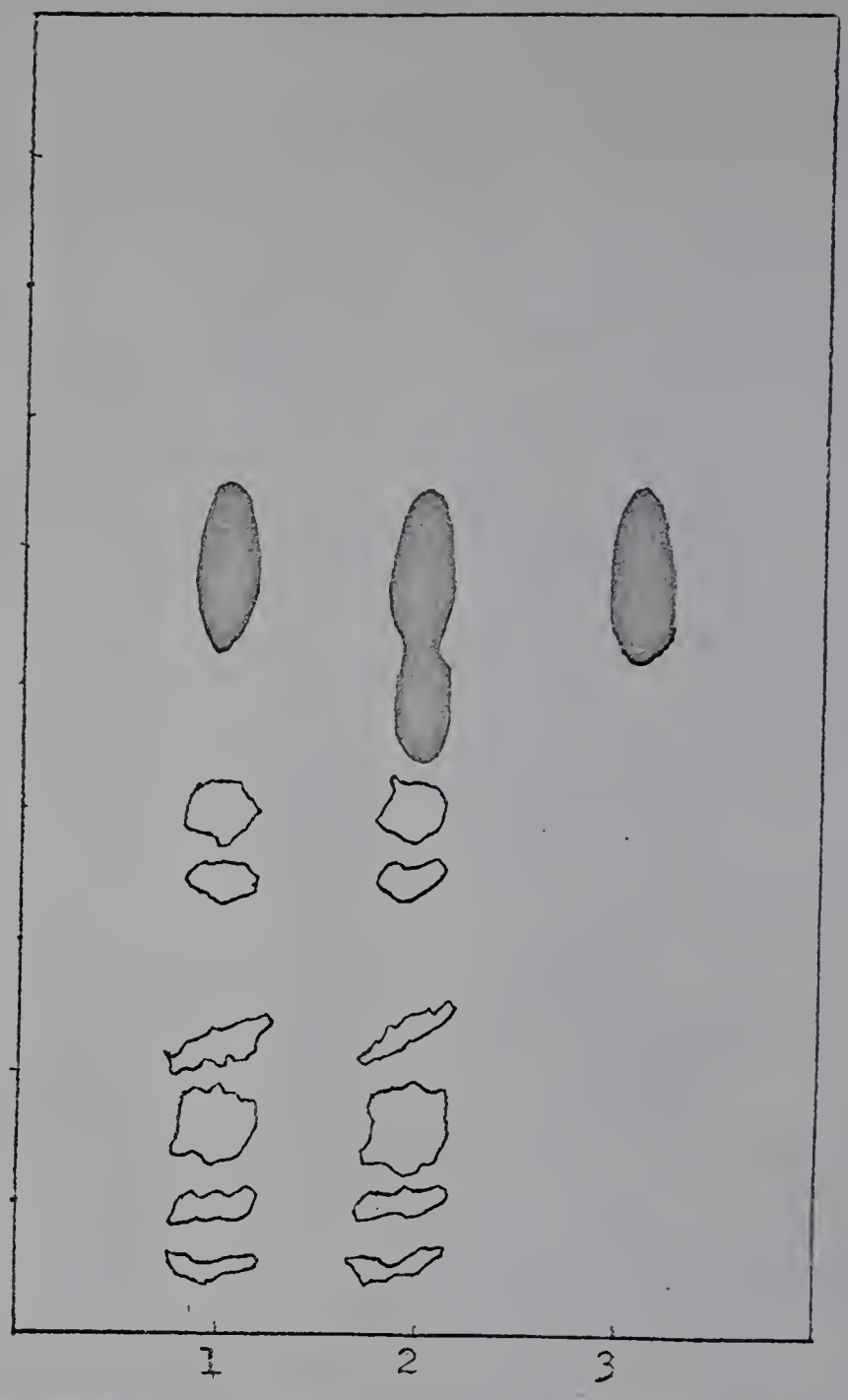

Fig. 9.--One dimensional paper chromatogram showing fluorescent spots under UV light.

Unshaded spots indicate a faint fluorescence. Butanol:Acetic acid:Water=4:1:2 was used as solvent.

1. Uninfected tomato root.

2. Tomato roots infected with Pratylenchus penetrans (Cobb).

3. Authentic chlorogenic acid.

TABLE 1.--Rf values of fluorescent compounds extracted by paper chromatography from three varieties of healthy tomato roots (Nemared, Hawaii 7153, B-5) and roots infected with Pratylenchus penetrans (Cobb)

Spots	Solvents			
	BAW (4:1:2)	BAW (4:1:5)	2% acetic acid	HAW (3:30:10)
1	.58	.63	.82	.69
1'	.46	.53	.75	--
2	.42	.45	.66	.79
3	.35	.31	--	--
4	.22	.24	--	--
5	.16	.17	--	--
6	.09	.14	--	--
7	.04	.10	--	--
8*	.58	.63	.82	.68

Spots 1 to 7 were present in both healthy and infected tissue extracts of all varieties. Spot 1' was present only in infected extracts. Spot 8* was authentic chlorogenic acid.

-- No spots obtained.

present only in extract of infected tissue was also identified as a phenolic compound (Table 2).

By comparison with authentic phenolic compounds, chromatographically (Fig. 9A) and spectrophotometrically (Fig. 9B), the spot at Rf 0.58 was identified as chlorogenic acid. The additional spot (Rf 0.46) in infected extracts was unidentified. This light pale blue spot was connected to chlorogenic acid. The maximum absorption peak of chlorogenic acid is at 326 mμ and the lowest peak is at 265 mμ. Chlorogenic acid had been reported previously as the major phenol in the tomato plant by Harvey et al. (1965).

Seven spots, obtained by paper chromatography of gall and fleck extracts of B-5 and Nemared varieties which had been inoculated with root-knot nematodes, were not different from the spots obtained from methanolic extracts of healthy varieties. This was perhaps because the lesions on Nemared varieties were so small and limited to only the site of invasion. If phenols did accumulate, not enough materials could be extracted to be shown by paper chromatography.

Quantitative assay of chlorogenic acid.

Differences were found between the chlorogenic acid concentrations in uninfected roots of the three varieties of tomato. Nemared roots contained 0.58 mg. of chlorogenic acid per gram of root tissue; Hawaii 7153, 0.44 mg.; B-5, 0.38 mg. (Fig. 11).

Differences in amount of chlorogenic acid were also demonstrated between the extracts of healthy and infected roots within each variety. Roots infected by nematodes

TABLE 2.--Color reactions of fluorescent compounds on paper chromatograms (BAW=4:1:2)

Reagents	Spots 1	1'	2	3	4	5	6	7	8*
Ninhydrin	-	-	+	+	+	++	+	+	-
Anthrone	-	-	-	-	-	+	-	+	-
$FeCl_3$	+	+	-	-	-	-	-	-	+
Arnow	+	+	-	-	-	-	-	-	+
$AgNO_3$	+	+	+	+	+	++++	+++	++	+
DSA	+	+	-	-	-	-	-	-	+

Spots 1 to 7 were present in extracts of both healthy roots and roots infected with Pratylenchus penetrans (Cobb). Spot 1' was present only in infected extracts. Spot 8* was authentic chlorogenic acid.

\+ positive reaction.
++ +++ ++++ stronger positive reactions.
\- negative reaction.

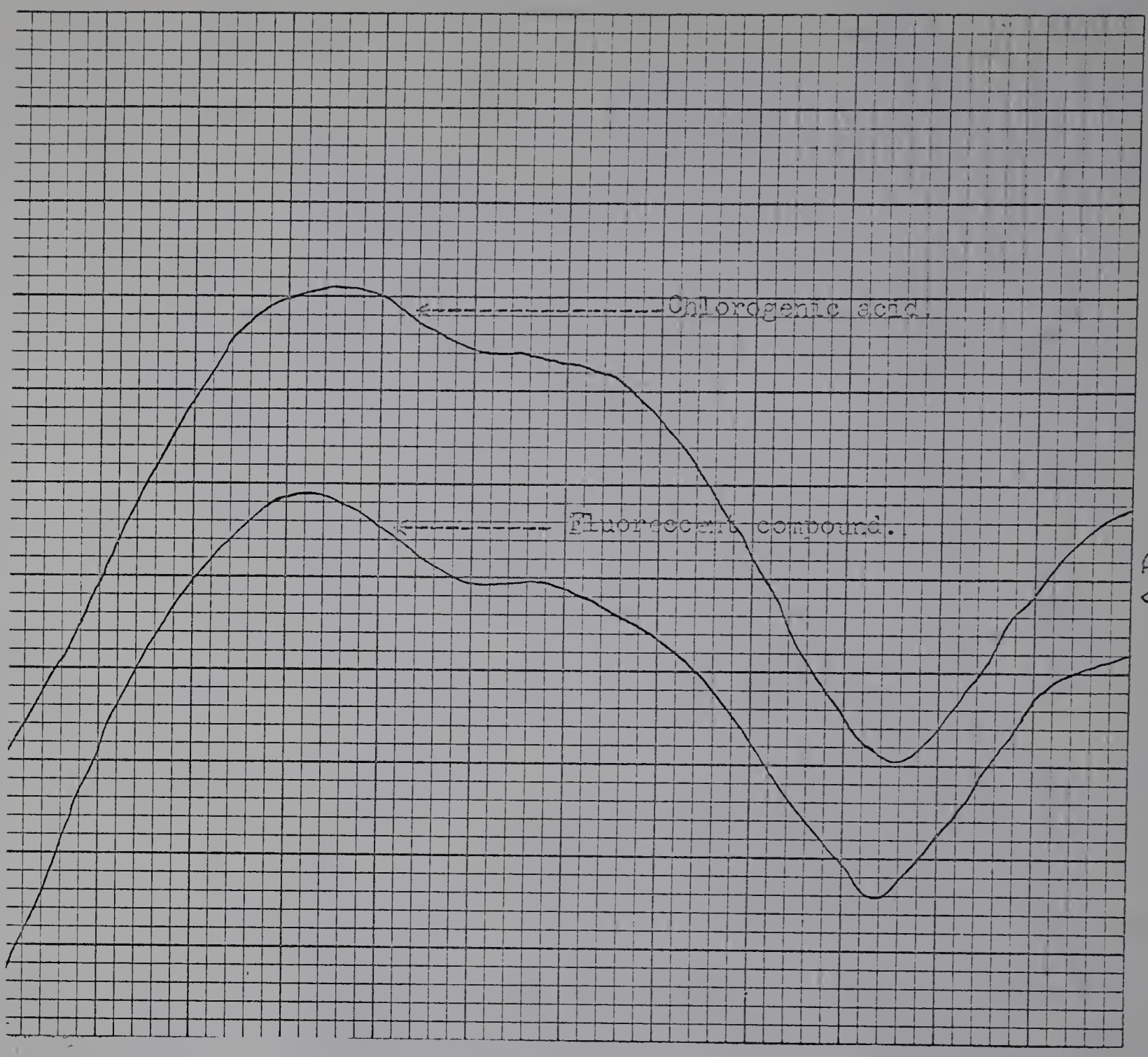

Fig. 10.--Absorption curves of authentic chlorogenic acid and fluorescent compound present in both healthy and infected extracts of Rf 0.58, eluted from paper chromatograms.

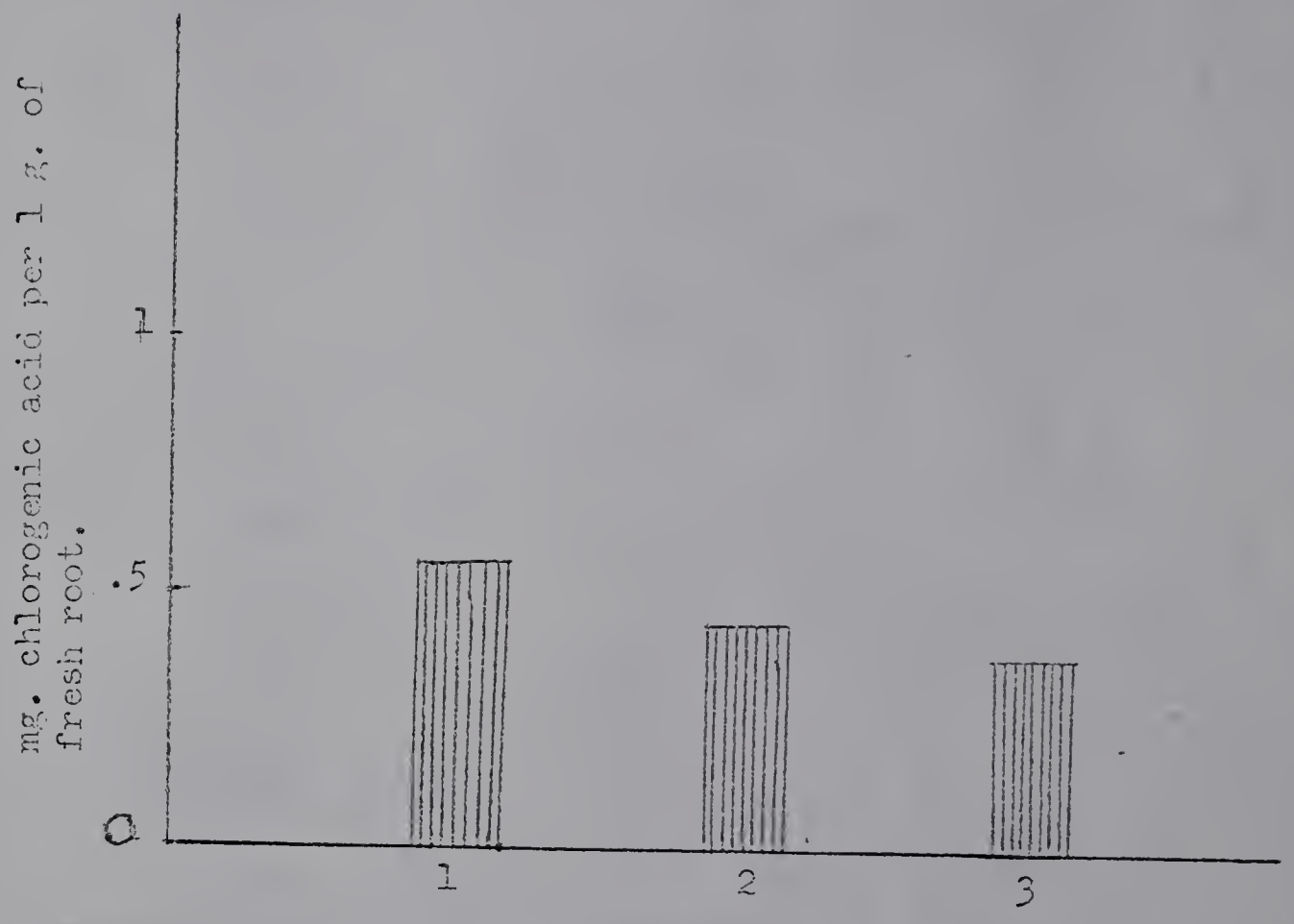

Fig. 11.--Concentrations of chlorogenic acid in methanol extracts of equal weight of three healthy tomato varieties' roots were measured at 324-326 mμ in a Bausch and Lomb Spectromic 505 recording spectrophotometer.

1. Nemared (resistant variety).
2. Hawaii 7153 (moderately resistant variety).
3. B-5 (susceptible variety).

contained more chlorogenic acid than did healthy roots of the same variety in proportion to the amount of infection (Fig. 12).

Assay of polyphenol oxidase activity.

Polyphenol oxidase activity in roots infected with Pratylenchus penetrans was higher than in healthy roots of the same variety (Fig. 13). In diseased roots, the polyphenol oxidase activity rose at higher levels of infection. In heavily infected tissue, however, the enzymatic activity was reduced as the cortex collapsed.

No varietal differences in polyphenol oxidase activity were found (Fig. 14).

Effect of chlorogenic acid on root-knot and lesion nematodes.

Attempts were made to demonstrate the toxicity of different concentrations of chlorogenic acid to the two species of nematodes in vitro. Under the conditions of the experiment, only 5 nematodes died of 158 tested (Table 3).

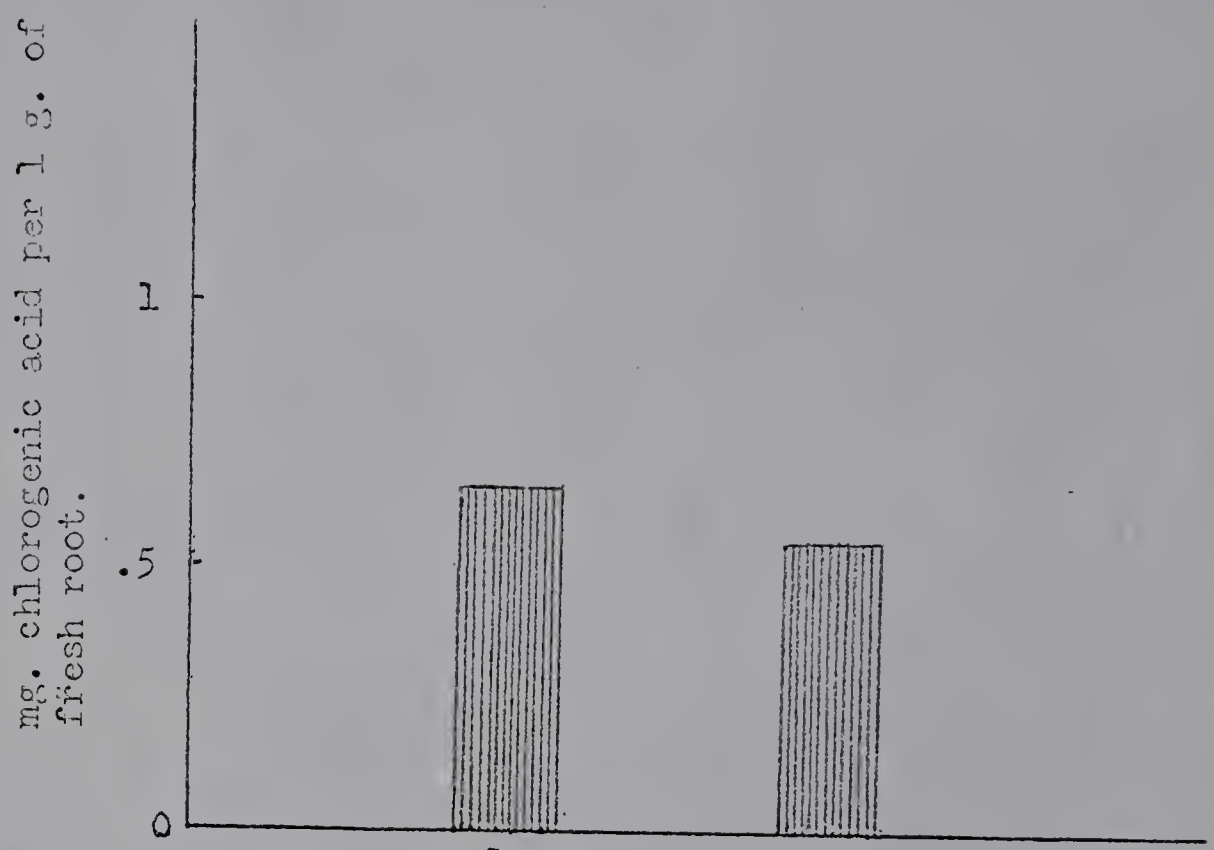

Fig. 12.--Concentration of chlorogenic acid in methanol extracts of equal weight of healthy Nemared variety and P. penetrans infected roots seven days after inoculation were measured at 324-326 mμ in a Bausch and Lomb Spectromic 505 recording spectrophotometer.

1. Nemared variety infected with P. penetrans.

2. Healthy Nemared roots.

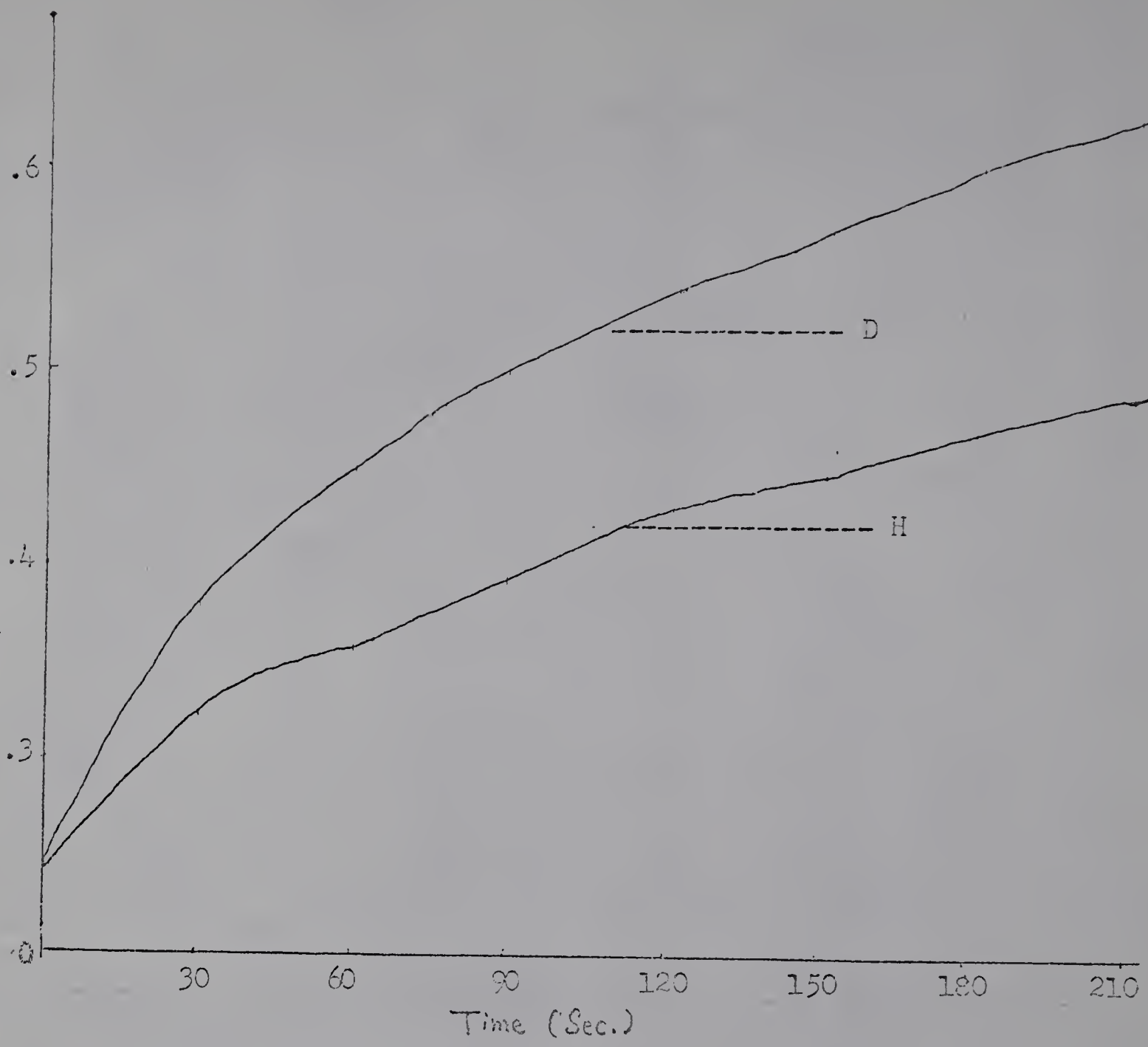

Fig. 13.--Polyphenol oxidase activity in healthy and infected B-5 tomato roots was measured spectrophotometrically at 400 mμ based on the rate of formation of a dark-colored polymeric compound from catechin.

D -- Infected by Pratylenchus penetrans.

H -- Healthy tissue.

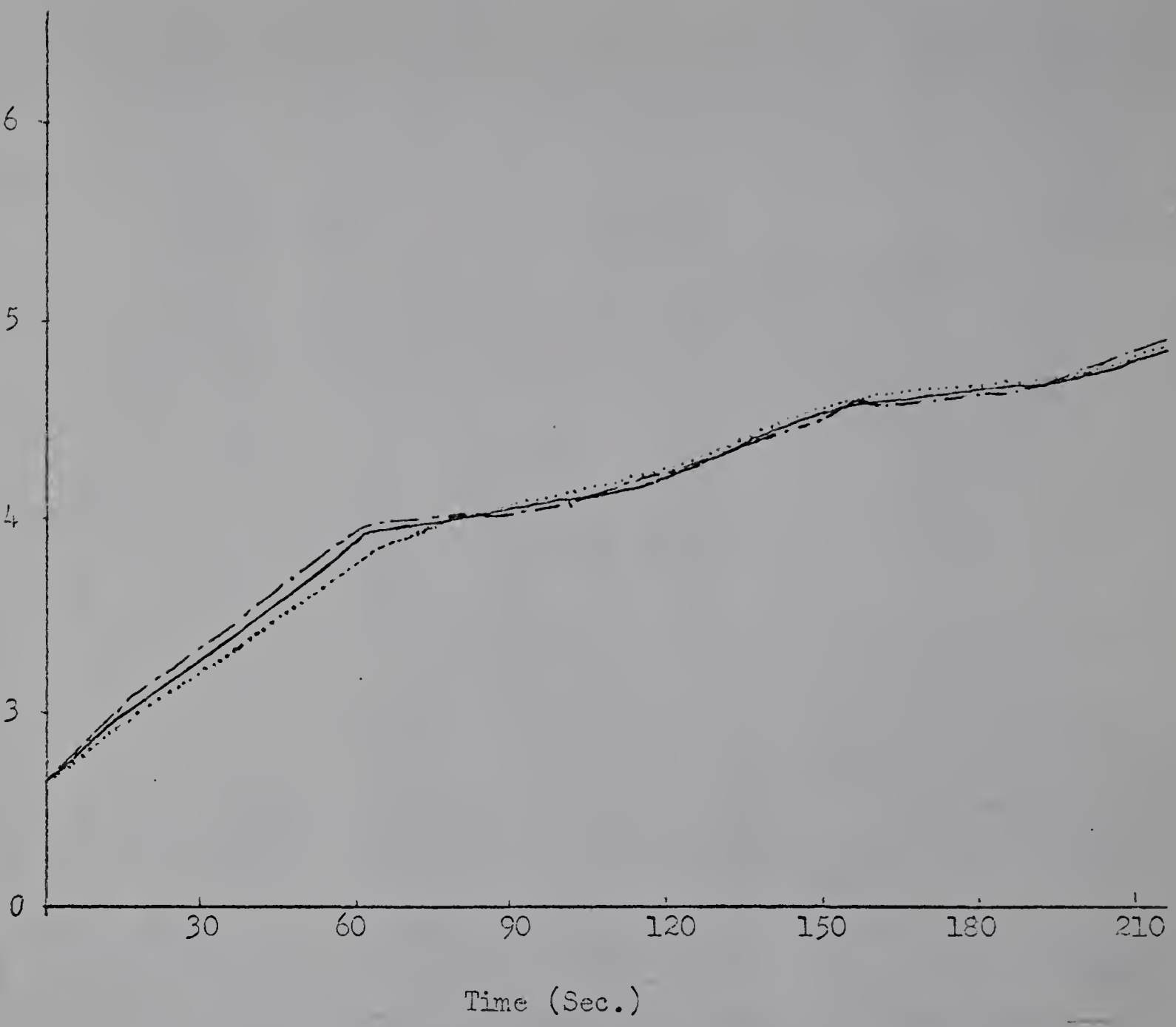

Fig. 14.--The activity of polyphenol oxidase in three varieties of healthy roots was measured spectrophotometrically at 400 mμ based on the rate of formation of a dark-colored polymeric compound from catechin.

_____ Nemared.

-.-.-.-.- Hawaii 7153.

--------- B-5.

TABLE 3.--Motility of lesion and root-knot nematodes after 24-hour treatment with chlorogenic acid

Nematodes	Concentration of chlorogenic acid (ppm.) 0	100	200	500	1000	2000
Pratylenchus penetrans						
Motile	20	15	12	15	12	12
Killed	0	0	1	0	0	0
Meloidogyne incognita acrita						
Motile	10	12	12	12	11	10
Killed	0	0	1	0	1	2

Each figure is the average of 3 replicate trials.

CHAPTER V

DISCUSSION AND CONCLUSIONS

Gall formation was the characteristic sympton on tomato roots of the susceptible B-5 variety infected with root-knot nematodes. Larval development and giant cell formation on a susceptible variety were not accompanied by any cell injury, and there was little or no accumulation of phenolic compounds (Fig. 2A and 2B).

In contrast to this, roots of resistant Nemared became discolored and necrotic when fed upon by root-knot larvae and large amounts of phenolic compounds accumulated in the injured area. Giant cells did not develop and the larvae died. If gall formation is considered as response to a secretion by the nematode, it is apparent that Nemared tomato reacts differently to this stimulus or else the stimulus is inactivated. Growth regulators are known to reach high levels in root-knot galls (Bird, 1962), and it is possible that a nematode secretion which causes growth regulators to accumulate in B-5 causes phenolic compounds to accumulate in Nemared. The phenolic compounds produced in Nemared may themselves prevent gall formation (Fig. 2C).

Early symptoms on resistant Nemared were similar to both nematode species. Root-knot injury is limited to a few cells, probably because the larvae move through the

cortex between cells and do not feed until they reach the area of the endodermis and pericycle. This area, however, is highly reactive in terms of phenolic compounds. Lesion nematode injury was much more extensive since cortical cells were broken down and the nematodes were much more active.

Varietal reactions to lesion nematodes were not apparent until five to six weeks after infection. In the B-5 variety, the lesion nematode penetrated into the stele of the roots, destroying the vascular elements; whereas in the Nemared variety, injury was confined to the cortex because nematodes were not able to pass through the endodermis.

Paper chromatography and spectrophotometry indicated only one major phenolic compound in healthy tomato roots, both before and after infection by root-knot and lesion nematodes. The major phenolic compound was identified as free chlorogenic acid.

An additional phenolic compound, which appeared on chromatograms of extracts of tomato roots infected with lesion nematodes, remains unidentified. On the basis of the incomplete information available, it appears to be an oxidized product of chlorogenic acid.

The hypothesis is proposed that the invasion by nematodes causes oxidation of chlorogenic acid to a quinone by the action of host polyphenol oxidase, resulting in the formation of brown-colored substances in the injured areas.

Different quantities of free chlorogenic acid were

found in the three varieties of healthy tomato roots. Nemared contained the highest concentration of free chlorogenic acid; the lowest concentration was found in the B-5 variety tomato roots. Furthermore, roots infected by nematodes contained a higher concentration of free chlorogenic acid than did healthy roots. These results suggest that injury to root cells during penetration and feeding by nematodes stimulates injured cells to accumulate relatively large amounts of phenolic compounds, although no information is available as to the source of these compounds. Higher polyphenol oxidase activity occurred after infection which may explain the browning reaction.

The varietal differences in amounts of free chlorogenic acid in the uninfected roots might serve as an explanation for resistance to injury by nematodes, particularly since the free chlorogenic acid concentration was found to increase after infection. It was observed, however, that 2000 ppm concentration of chlorogenic acid failed to kill the nematodes. Several possible explanations are suggested:

First, although free chlorogenic acid apparently does not affect nematodes, a derivative may be toxic.

Second, since histochemical tests showed that chlorogenic acid is concentrated in the endodermis, the concentration in individual cells may actually be much higher than 2000 ppm. The amount of chlorogenic acid which accumulates in an injured area may be enough to prevent root-knot larvae from developing or lesion nematodes from passing through the

endodermis.

A final possibility is that another compound, perhaps a phenol, is present in low amounts but is highly toxic.

It can be concluded that complex biochemical and physiological changes occurred within tomato roots as a response to the injury by lesion and root-knot nematodes. These changes are not yet understood, indeed probably are not even yet all discovered. The different amounts of chlorogenic acid in the three varieties of tomato roots and the accumulation of chlorogenic acid and its oxidized products in the host after inoculation with nematodes are presumed to be among the factors in the resistance of plant tissues to disease. The endodermis acted as a barrier for the invasion of nematodes, perhaps because it contained more chlorogenic acid than did the other tissues.

Tomato roots of the Nemared variety, which had a higher concentration of chlorogenic acid, had a defense against invasion by root-knot and lesion nematodes. The B-5 variety tomato roots, which were lower in concentration of chlorogenic acid, were readily penetrated by both nematode species even into the vascular cylinder and thereby suffered much more extensive injury. But whether the chlorogenic acid was responsible for the difference in susceptibility is not yet determined.

CHAPTER VI

SUMMARY

Host-parasite relationships of root-knot nematodes (M. incognita acrita) and lesion nematodes (P. penetrans) were compared on three varieties of tomato under axanic condition. The tomato varieties were genetically very similar except that Nemared was resistant to root-knot nematodes, Hawaii 7153 was moderately resistant, and B-5 was susceptible.

Root-knot nematode larvae induced typical galls containing giant cells in the roots of the B-5 variety. The few larvae which entered the Nemared plants became surrounded by necrotic cells and further development ceased.

The initial injury caused by lesion nematodes was similar on all varieties of tomato. Five to six weeks after infection, however, lesion nematodes penetrated into the stele of the B-5 roots, destroying the vascular elements; whereas in the Nemared variety, injury was confined to the cortex and the nematodes were not able to pass through the endodermis.

Chlorogenic acid was identified as the major phenolic compound in the tomato roots, both before and after infection with nematodes. Nemared contained the highest

concentration of chlorogenic acid and the lowest concentration was found in the B-5 variety tomato roots. Roots infected by nematodes contained a higher concentration of free chlorogenic acid than did healthy roots.

An additional phenolic compound which appeared on chromatograms of extracts of tomato roots infected with lesion nematodes remains unidentified. Incomplete evidence indicates that it is an oxidized product of chlorogenic acid.

Histochemical tests showed that chlorogenic acid is concentrated in the endodermis.

An hypothesis is proposed that invasion by nematodes causes oxidation of chlorogenic acid to a quinone by the action of host polyphenol oxidase, resulting in the formation of brown-colored substances in the injured areas. It is further proposed that high levels of chlorogenic acid or its derivatives are related to the observations that root-knot larvae are unable to develop and lesion nematodes are unable to penetrate the endodermis of this variety.

Exposure to a 2000 ppm solution of chlorogenic acid for 24 hours had no visible effect on the nematodes. Several possible explanations are suggested. A derivative oc chlorogenic acid rather than chlorogenic acid itself may be toxic or the concentration of chlorogenic acid in individual cells may be much higher than 2000 ppm. A final possibility is that another phenolic compound, present in low amounts, is highly toxic.

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