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Biochemical changes in alfalfa injured by lesion nematode : Pratylenchus penetrans (Cobb, 1917) Filipjev & Schuurmans Stekhoven, 1941.

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BIOCHEMICAL CHANGES IN ALFALFA INJURED BY LESION NEMATODE, *PRATYLENCHUS PENETRANS* (COBB, 1917) FILIPJEV & SCHUURMANS STEKHOVEN, 1941.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I INTRODUCTION

The complex biochemical and physiological changes occurring within the host plant as a response to injury or invasion by pathogenic as well as non-pathogenic agents have been the subject of investigation by many plant pathologists, plant physiologists, biochemists and workers in other related fields. An important aspect of these studies which is of current interest, has been the determination of compounds or groups of compounds associated with injured or diseased plant tissues and the possible role that these compounds play in the host-parasite relationship.

The characteristic symptoms of browning and necrosis following invasion or infection by pathogenic organisms such as fungi, viruses and bacteria have been shown in many cases to be caused by enzymatic oxidation of phenolic compounds at the site of injury or diseased areas of plant tissues. Many diseased plants show increased synthesis of phenolic compounds and simultaneous increased activity of the polyphenolase system. Similar symptoms have been observed in response to mechanical wounding and treatment with chemicals, and in a few cases, to nematode feeding as well.

In a recent review Farkas and Kiraly (1962) cite many studies which have shown that infection or invasion of plant tissues stimulates plant cells, to synthesize relatively large concentration of phenolics which subsequently accumulate around and within injured or diseased tissues. Browning reactions have been associated with the oxidation of these phenolics by polyphenoloxidases which are released by

damaged cells.

Several workers have implicated phenolics as a possible mechanism of resistance in plant diseases (Farkas & Kiraly, 1962, Kúc, 1963, Uritani, 1961). These compounds have antibiotic action and inhibit growth of some pathogens (Link, et al., 1929, 1933, Johnson & Schaal, 1957, Kúc, 1957, Scot, et al., 1957, Suresh, et al., 1964). Many investigations have shown that resistant plants normally contain high concentrations of specific phenolis or are capable of producing these compounds in relatively large amounts compared to susceptible plants. There is, however some disagreement as to this role of phenolics in plant resistance (Cruickshank & Swain, 1956, Hulme & Edney, 1960). So far, no clear-cut designation can be made as to the real significance of these compounds in the biochemistry of plant diseases and their role in the host-parasite relationship.

This investigation was undertaken to determine whether or not phenolic compounds accumulate in alfalfa roots injured by the lesion nematodes, Pratylenchus penetrans (Cobb, 1917) Filipjev & Schuurmans Stekhoven, 1941, as indicated by symptoms of browning and necrosis, and if so, their implication in the host-parasite relationship.

II REVIEW OF LITERATURE

Literature reviewed during the course of this investigation dealt mainly with plant diseases caused by fungi, viruses and bacteria. However, in the following review no details of the above diseases are covered unless they are of particular interest and related to this study.

Phenols or phenolic substances are a large and diversified groups of compounds which are characterised by the presence of one or more phenolic hydroxy groups. One or two terms have been used interchangeably to refer to these compounds collectively.

In earlier literature, the term "tannin" was commonly used to refer to many groups of substances which were phenolic in character. This term as it is now used includes complex mixtures of compounds possessing astringent taste and the ability to tan leather. Tannins are divided into hydrolysable naturally occurring polymers, known as condensed tannins, catheeol tannins or phlobotannins and the hydrolysable tannins, also called tannic acid, gallotannin or ellagitannin. Red brown polymers derived from oxidation of leucoanthocyanidins are known as phlobaphenes or tannin-reds. All tannins contain polyhydroxy phenols or their derivatives. Alkali fusion and dry distillation of tannins give decomposition products that are principally phenolic in character such as catheeol, pyrogallol, phloroglucinol, resorcinol and hydroquinones and their corresponding acids.

Kúć (1963) used a general term 'polyphenol' to refer to all phenolic substances, including tannins. He included the following

groups of compounds in his definition of phenolics: flavonoids as anthocyanins, leucoanthocyanins and anthoxanthins; hydroxybenzoic acids; glycosides; sugar esters of phenolic acids; esters of hydroxycinnamic acid and coumarin derivatives.

Alston and Turner (1963) classified the major groups of phenolic substances into the following: simple phenols without side chains; simple phenols with one-, two-, or three-carbon side chains which occur as acids, aldehydes, ketones, or alcohols; depsides of simple phenols such as chlorogenic acid; polymers of simple phenols such as lignins; flavonoids; and coumarins.

The flavonoids are commonly occurring plant pigments consisting of several classes which are distinquished by an additional oxygen-heterocyclic ring and different patterns of distribution of the hydroxyl groups.

Coumarins are the unsaturated lactones of o-hydroxycinnamic acid.

The quinones are formed as a result of enzymatic oxidation of ortho-diphenols to corresponding ortho-quinones, which are further polymerized to colored products. This group includes a special class of ketones, the p-benzoquinones, in which the carbonyl groups are present as part of the ring.

The term "melanin" has been always associated with the dark-colored products formed upon oxidation of phenolic compounds. It is a polymer based on indole-5,6-quinones.

The idea that phenolics might be released at the site of parasitic attack by " protective enzymes " was first hypothesized by Cook, et al. (1911). Since then, many studies have shown that phenolic compounds

and their oxidation products accumulate in infected tissues of diseased plants (Farkas & Kiraly, 1962). Very few studies however, have been made specifically on phenolic accumulation in tissues infected by nematodes.

The mechanism involved in phenolic accumulation as a result of infection or parasitic attack has not been fully elucidated. Farkas and Kiraly (1962), in discussing the works of other investigators suggested two possibilities: (1) that phenolics are liberated from bound forms such as glycosides by increased activity of parasite enzymes or by host enzymes released through activity of the parasite, and (2) that host metabolism is diverted toward production of aromatic compounds.

According to Uritani (1961), polyphenol formation is induced by cellular stimulation as a result of incompatibility between the host plant and parasite. The pathogen may release some toxic metabolites and secrete enzymes that catalyze phenolic synthesis.

Kuć (1963) mentioned the possible metabolic pathways by which phenols may be synthesized: (1) Shikimic acid pathways, (2) acetate-malonate pathways or a combination of both pathways. Indirect evidence has been presented to show that parasitic infection caused increased activity of both of these metabolic pathways (Farkas & Kiraly, 1962).

The biochemical processes associated with lesion formation and browning reaction were first investigated by Dufrenoy (1936), and Humphrey and Dufrenoy (1944). Browning reaction has been attributed by many workers to enzymatic oxidation of polyphenols and tannins to quinones with subsequent polymerization to brown substances. Histological observation of necrosis in injured tissues by Uritani (1961)

led him to the following assumptions: (1) that polyphenolic compounds are oxidized by polyphenoloxidases which may exist in a latent state in intact plant tissues but are activated on exposure to pathogenic infection, (2) that oxidized polyphenols which are now quinones may be condensed to form polyquinoid structures or sometimes they may react with amino acids or proteins to form melanin substances.

Phenolics have long been associated with plant disease resistance. Farkas and Kiraly (1962), Kúć (1963) and Uritani (1961) discussed this role of phenolics. Phenolics have been observed to act as antibiotics or growth inhibitors of pathogens. The early work of Link and Walker (1929, 1933) showed that protocatechuic acid and catheeol were responsible for resistance of colored onions to the smut organism, Colletotrichum circinans. Johnson and Schaal (1957), Kúć, et al., (1956), Scot, et al.,(1957) and Suresh, et al., (1964) have all observed the inhibition of growth of various pathogens by different phenols.

Some fungus diseases in which phenolics were found to accumulate in and around diseased tissues of host plants were: mildews (Scot, et al., 1957), rusts (Kiraly and Farkas, 1962), wilts (Davies, et al., 1953), scabs (Barnes, 1960), rots (Uritani, 1962), and Phytophthora infections (Hughes, 1960).

Virus diseases have been also investigated. Geissman (1956) reported a two to five fold increase in phenolics in cherry leaves infected with rusty mottled virus and peach leaves with western x-virus. Johnson and Schaal (1957) isolated chlorogenic acid and other polyphenols from necrotic areas of potato tubers infected with aster yellows virus.

Simple mechanical injury and treatment with poisonous chemicals may also cause phenolic accumulation (Farkas & Kiraly, 1962).

In many studies, phenolics and their oxidation products have been identified and characterized as fluorescent compounds which occur in and around infected areas of plant tissues. Best (1936) identified the blue-fluorescing compound in lesions of tobacco plants infected with tomato spotted wilt virus as scopoletin (6-methoxy-7-hydroxycoumarin). The same fluorescent compound was isolated by Andreae (1948) in potato leaves and tubers infected with leaf roll virus. Hughes and Swain (1960) identified a blue-fluorescing zone surrounding areas of potato tubers infected with Phytophthora infestans as scopolin, a glycoside of scopoletin. Barnes and Williams (1960) observed the increased concentration of a fluorescent phenolic compound in diseased tissues of apple fruits and leaves attacked by Venturia inaequalis and Podosphaera leucotricha. They also observed a similar compound in mechanically injured apple tissues.

Romanowski, et al. (1962) detected chromatographically the presence of six fluorescent compounds in an extract of bean seedlings infected with Colletotrichum lindemuthianum, a pathogen which induces tissue necrosis. The fluorescent compounds were localized in the lesion and to a lesser extent, in adjacent uninfected tissues. He suspected that the fluorescent compounds were phenolics. Yu and Hampton (1964) found two additional fluorescent compounds in infected tissues of tobacco attacked by Colletotrichum destructivum, which were not detected in healthy tissues. Tobacco infected with tobacco mosaic virus has been found to contain two fluorescent compounds which are localized as a narrow band around the necrotic tissues (Hampton, et al., 1964).

There is some evidence that phenolic compounds accumulate in tissues invaded by nematodes. Pitcher, et al. (1960) found that phenolic compounds were a principal factor involved in root lesion formation and discoloration reactions in sterile apple roots infected with Pratylenchus penetrans. They observed epidermal cells of apple roots in the region of nematode attack to turn yellow three hours after inoculation with nematodes. Affected cells turned dark brown in later stages, with discoloration more intense and more rapid in the dermal and endodermal layers as compared to the cortical tissues. The nematodes moved away from the discolored areas a few days later. These authors associated the intensity of discoloration and necrosis to the concentration and distribution of phenolics in the root layers. They suggested that root necrosis was caused by toxic products which were released by nematode enzymes.

Townshend (1962, 1963) studied lesion formation and discoloration in strawberry roots infected by P. penetrans. He observed that the nematodes attacked only the cortical tissues. Different stages of the nematodes were found in amber-colored lesions but not in dark brown lesions where he observed heavy deposition of dark substances at the walls of the necrotic cells. He suspected that phenolic compounds were concentrated in the endodermis where intense discoloration occurred and associated intensity of discoloration with different levels of concentration of phenolics in various tissues. Townshend (1962, 1963) also observed similar discoloration and necrotic reactions in the roots of infected celery.

Wallace (1961) studied the nature and chemistry of discoloration in chrysanthemum leaves infested by the foliar nematode, Aphelenchoides

ritzema-bosi. He attributed leaf browning to the hydrolysis of chlorogenic and isochlorogenic acids to caffeic acid, which is subsequently oxidized to quinic acid and later polymerized into a brownish substance. According to him, the feeding activities of the nematodes on the leaf caused the host cells to release polyphenolic substrates which are acted upon by host polyphenoloxidases. He observed that resistant varieties of chrysanthemum reacted faster to nematode infection and that nematodes did not multiply in such browned areas. He found no differences, however, in the polyphenol contents between resistant and susceptible varieties.

Mountain and Patrick (1959) studied the pathogenicity of P. penetrans to sterile peach roots. They observed that discoloration occurred in epidermal cells within 90 minutes after inoculation with the nematodes. In this case, discoloration and necrosis were associated with the ability of the nematodes to secrete a B-glucosidase, which hydrolyzes amygdalin, a cyanophoric glycoside, thus releasing benzaldehyde and hydrogen cyanide, which are both highly phytotoxic.

Interesting observations have been made by several workers as to the effects of browning reaction on nematode activity. Wallace (1961) observed that fewer nematodes were found in chrysanthemum leaf tissues that brown rapidly upon infection by foliar nematode, compared with tissues that browned slowly but were able to support large population of nematodes. Pitcher, et al. (1960) and Townshend (1962, 1963) made similar observations with P. penetrans, where the nematodes were found to migrate away from dark-colored lesions.

III MATERIALS AND METHODS

Preparation of nematodes for inoculation. Specimens of P. penetrans were cultured aseptically in alfalfa callus tissue grown in nutrient agar containing 2,4-dichlorophenoxyacetic acid (2,4-D), following the method described by Krusberg (1960).

Nematodes to be used for inoculum were extracted from callus tissues aseptically. The callus tissues were placed on the surface of a cotton plug in a 15 ml conical centrifuge tube. The tube had previously been filled with water to the level of the cotton, capped with aluminum foil, and autoclaved. After 24 hours, when the nematodes had settled, the callus tissues and cotton were discarded, and the supernatant liquid poured off leaving a small amount of water at the bottom of the tube where the nematodes were concentrated. This portion was then poured into sterile small dishes for use as inoculum.

Preparation of alfalfa seedlings. Alfalfa seeds (Dupuits variety) were surface-sterilized for 15 minutes in sodium hypochlorite (prepared by mixing Chlorox bleach with an equal volume of water), rinsed several times in sterile distilled water and germinated in 2 per cent water agar in Petri dishes. After three to five days, the newly germinated seedlings were transplanted either individually into test tubes, or in groups of 10 to 20 into Petri dishes, each containing nutrient agar. Nutrient agar for growing seedlings had the same composition as that used for maintaining callus tissue except that 2,4-D was omitted. The seedlings were allowed to grow in the nutrient agar for two to three days before inoculation with nematodes.

Method of inoculation. About 20 to 30 nematodes were introduced into each test tube or 30 to 100 into each Petri dish by means of a sterile micropipette. Transfers of seedlings and inoculations were carried out in an ultraviolet light-sterilized chamber.

The inoculated seedlings were maintained in a plant growth room at 20° C . Plants were periodically examined for symptom formation. These inoculated seedlings served as source for extracts of lesion and uninfected tissues. Uninoculated seedlings were also allowed to grow in the plant growth room and these served as a source of extract of healthy tissues.

Mechanically - induced injury. Roots of week old alfalfa seedlings were punctured in several areas with a sterile microneedle and allowed to grow in nutrient agar in Petri dishes where development of symptoms was observed.

The influence on rate of browning by hydrolytic enzymes was observed when crude pectinases (Pectinol 59-L, Rohm & Haas), B-glucosidase (Almond emulsin) and horseradish peroxidases were added to punctured areas. Drops of a dilute solution of each enzyme were introduced on the surface of newly punctured roots of week old seedlings. Distilled water was used for control seedlings. Five test seedlings were used for each sample solution and these were allowed to grow in nutrient agar in Petri dishes. The rate of browning was observed at the following times: 1, 2, 4, 8, 12, 24, and 48 hours after addition of the solution. All the test plants were maintained in the plant growth room at 20° C .

Histochemical test. Portions of infected roots containing lesions were placed on a clean glass slide, covered with a coverslip and crushed lightly. Diazotized sulfanilic acid (DSA), a general histochemical reagent for phenolic compounds, was allowed to flow under the edge of the coverslip and observations of color changes of lesions were made. Punctured roots were tested in the same manner.

Cross - sections of living roots, 15 μ thick, were prepared from lesion area on a freezing microtome. Portions of roots with lesions were first embedded in 3 per cent partially cooled water agar in a Petri dish and allowed to gel in the refrigerator. The embedded lesion was trimmed and mounted in the freezing microtome. Sections were collected in a Petri dish containing water by means of a camel's hair brush and allowed to float in the water. Sections were transferred to a glass slide and stained with DSA as above. Color changes in the different regions of the root lesion were observed under a compound microscope. Free - hand sections were also made by means of a razor blade.

Histological studies. Paraffin sections of root lesions and punctured roots were prepared following standard procedures.

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 longitudinal 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 homogenates of lesions, uninfected adjacent tissues, uninoculated roots, oxidized roots and acid-hydrolyzed roots.

Lesions were cut and separated from adjacent uninfected tissues and each tissue was weighed separately. Approximately one or two g fresh weight of each tissue was dropped into small beakers containing two ml of boiling methanol, cooled and homogenized in a microhomogenizer for three to five minutes. The homogenates were well mixed and centrifuged for five minutes to remove cell fragments. The extract was concentrated to about 0.5 to 1 ml in a rotary evaporator under reduced pressure at 40° C . The concentrated extracts were stored at 0° C . Roots of uninoculated seedlings were cut into pieces, weighed and extracted as above.

Preparation of oxidized root homogenates. Two-gram samples of uninoculated roots were homogenized and then exposed at room temperature for different lengths of time. One sample of homogenates was exposed for each of the following times: 2, 4, 6, 8, 12, 24, and 48 hours. A crude extract of each sample was prepared as described above.

Acid-hydrolyzed root tissues. Roots from uninoculated alfalfa seedlings were homogenized and hydrolyzed in 2N Hydrochloric acid in a warm bath for two hours. A crude extract of the hydrolyzed roots was prepared as above.

Chromatographic methods. Thin layer and paper chromatography were used for analysis of the different extracts. Crude methanolic extracts were used without further purification.

Thin layer chromatography. The methods described by Truter (1963) were used in the preparation of the thin films, spotting of extracts and development pf the chromatoplates and chromatostrips. Detection of compounds was made with short wave-ultraviolet lamp, model Sl 2577 (253 mμ). The adsorbents used were Polyamide resin and Silica gel G. Polyamide layers were developed in methanol-acetone-water (MAW), 3:1:1 v/v/v and silica gel G in toluene-ethyl formate-formic acid (TEF), 5(4:1 v/v/v and benzene-methanol-acetic acid (BMA), 45:8:4 v/v/v.

Paper chromatography. Crude extracts were chromatographed on Whatman No. 1 filter 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), 4:1:5 v/v/v,organic phase.

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

(3) Ethyl acetate-acetic acid-water (EAW), 9:2:2 v/v/v, organic phase.

(4) Hydrochloric acid-acetic acid-water (HAW), 3:30:10 v/v/v.

(5) 6 per cent acetic acid.

(6) 10 per cent acetic acid.

(7) 15 per cent acetic acid.

(8) 70 per cent ethyl alcohol.

Two-way paper chromatography was done using the organic phase of BAW, 4:1:5 v/v/v and 15 per cent acetic acid as solvents. The chromatograms were air dried and examined under short wave ultraviolet lamp

with and without ammonia fumes. Rf values were measured.

The following color reagents were used for locating and identifying the compounds:

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

(2) Ferric chloride-ferricyanide reagent (Smith, 1960), a general reagent for phenols and tannins.

(3) Ammoniacal silver nitrate (Gage, et al., 1951) for detecting O-dihydroxy compounds.

(4) Tetrazotized benzidine (Smith, 1960), reagent for detecting phloroglucinol-resorcinol phenols.

(5) Tetrazotized o-dianisidine (Fast blue B salt, 1 per cent aqueous solution), reagent for locating most phenolics.

(6) Benedicts reagent (Block, et al., 1958), reagent for detecting reducing sugars.

(7) 0.3 per cent alcoholic ninhydrin (Block, et al., 1958), reagent for detecting amino acids.

Spectrophotometric method. Sections of the chromatograms containing the unidentified fluorescent compounds found only in the lesion extract were cut out and soaked for 12 to 24 hours in 95 per cent methanol. The absorption spectrum of the eluate was measured in a recording spectrophotometer (Bausch and Lomb Spectronic 505).

IV OBSERVATION AND RESULTS

Symptoms of injury.

Nematodes started to penetrate root tissues within three hours after inoculation, and after six to eight hours were found embedded in the cortical tissues of the roots. Small discolored areas one to two mm long were visible along the periphery of roots within 12 to 24 hours after inoculation. These discolored areas or lesions were at first yellow and in later stages became dark brown. Lesions gradually enlarged as nematodes moved, fed, and reproduced within cortical tissues. Coalescence of many small lesions caused a large portion of the root system to become discolored in appearance (Fig. 1A)

Roots punctured with a sterile microneedle showed symptoms similar to lesions caused by nematodes except that discoloration was limited to punctured areas and did not later spread. Discoloration was also visible within 12 to 24 hours after puncturing. The addition of pectinol, emulsin and peroxidases resulted in rapid discoloration of punctured areas with the development of a more intense brown color.

Histochemical tests with DSA.

The addition of DSA caused newly formed lesions to become intensely yellow to brown (Fig.1B). Cross-sections of fresh lesions stained with DSA showed discoloration in the cortical region (Fig.1C). Similar results were obtained with punctured roots (Fig. 1D).

Histological studies.

Paraffin sections of lesions revealed the presence of all stages of nematodes in the cortical region of alfalfa roots. Most cells directly adjacent to nematodes became deeply stained, with cell

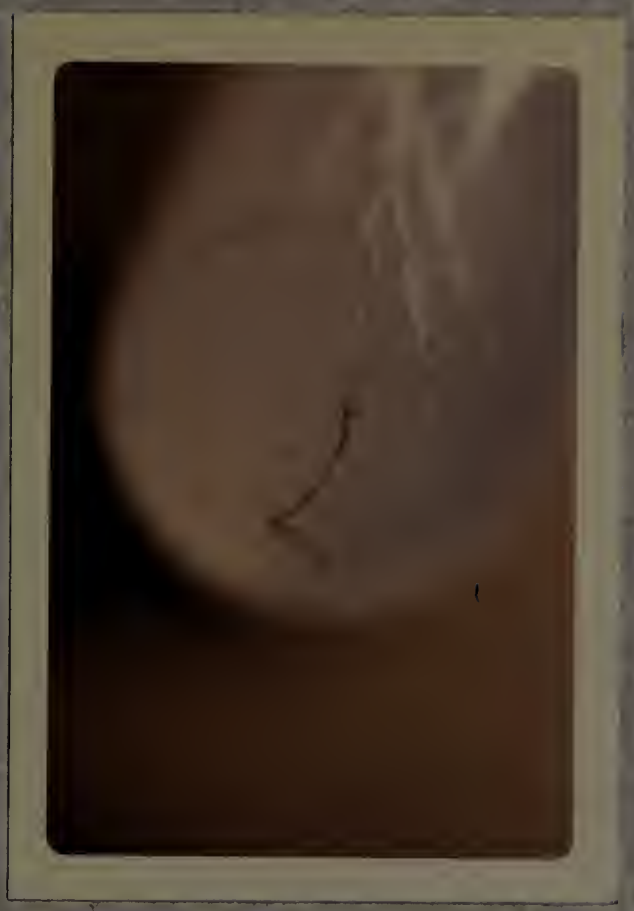
Figure 1. Symptoms of injury to Dupuits alfalfa roots.

A. Lesion caused by P. penetrans on alfalfa growing in nutrient agar three days after inoculation. X2.

B. Whole mount of P. penetrans lesion stained with DSA. Note nematode and eggs inside cortex. X25.

C. Free hand cross-section through lesion stained with DSA showing discoloration of cortex. X120.

D. Sterile microneedle puncture 48 hours after injury. Stained with DSA. X25.



A

B

C

D

walls deeply stained with safranin. Highly granular cytoplasm, stained blue, was prominent in most necrotic cells (Fig. 2 A). No nematodes were observed in the epidermis or in vascular tissues. In invaded areas, several cells in the cortex were often badly damaged, resulting in a cavity. The stem near the roots was also attacked by nematodes and showed symptoms similar to those found in roots.

Paraffin sections of punctured roots showed necrosis in the cortical region (Fig. 2 B). Some cells in this region were ripped and severely damaged due to the puncturing process. Widespread and intense discoloration was observed in punctured roots to which hydrolyzing enzymes had been added (Fig. 2 C). In general, punctured roots were very similar in appearance to those infected with nematodes.

Chromatographic analysis, paper chromatography.

Examination of the paper chromatograms of different extracts revealed five to six fluorescing compounds. The color of fluorescence varied from pale violet to bright blue. Larger and more intense spots were observed in lesion extracts as compared with healthy tissue extracts. An additional bright blue fluorescent compound was found in lesions which was not present in fresh uninoculated seedlings or in adjacent unaffected tissues (Fig. 3). The same fluorescing compound was found in extracts of punctured roots and oxidized uninoculated root tissues, but in much smaller amounts. This compound was not found in acid-hydrolyzed roots.

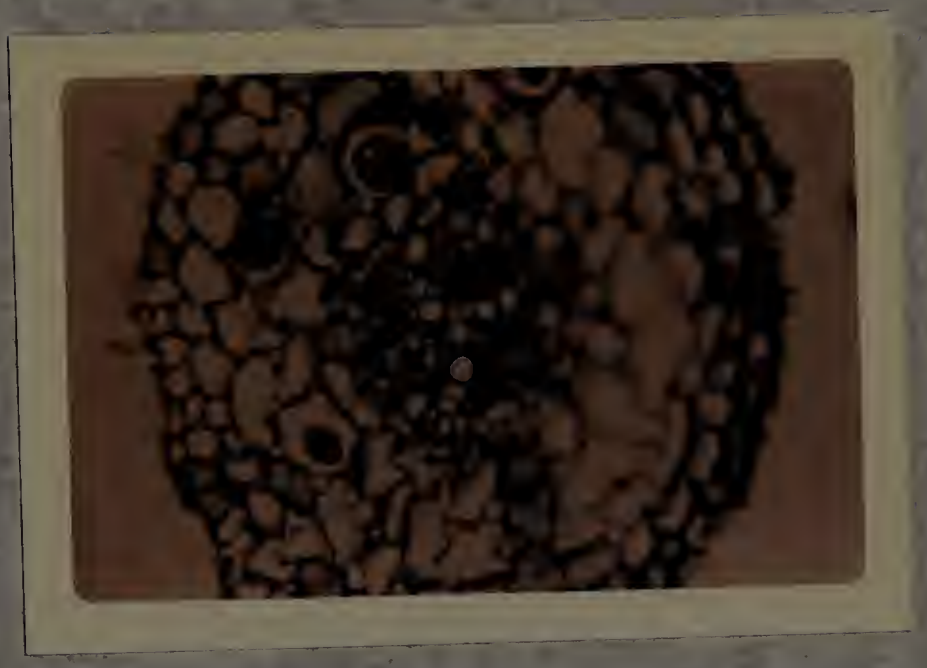
The fluorescent compound characteristic to lesions appeared in healthy tissues after four to eight hours exposure of homogenates at

Figure 2. Cross-section of Dupuits alfalfa roots injured in various ways. Paraffin section stained with safranin and fast green.

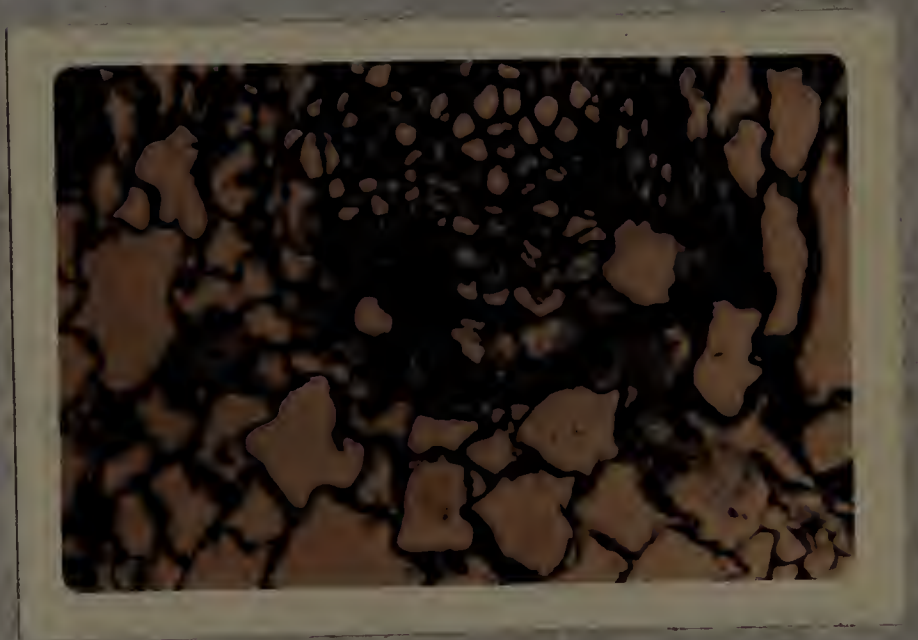
A. P. penetrans lesion seven days after inoculation. Note cross-section of nematodes in cortex and the discolored endodermis. X60.

B. Sterile microneedle puncture showing discolored endodermis three days after injury. X90.

C. Sterile microneedle puncture treated with Pectinol seven days after injury. X90.



A



B

C

Figure 3. Paper chromatogram illuminated with ultraviolet light showing the highly fluorescent spot characteristic of P. pentrans lesions. X1/6.

room temperature and could be detected as a very faint spots on the chromatogram. Oxidation of extracts for 12 hours resulted in a larger and more intense fluorescent spot.

A pinkish-brown fluorescent compound was detected in lesions but appeared only as faint trace in unaffected tissues. This compound was detected in chromatograms developed in organic solvents but not in aqueous solvents.

The Rf values of the different fluorescent compounds found in lesion and unaffected tissue extracts are shown in Table 1 . The Rf value of the bright blue-fluorescing compound was low in aqueous solvents and high in organic solvents.

Chromatographic analysis, thin layer chromatography.

Four to five fluorescing compounds were detected in extracts from different tissues using polyamide resin and which were developed in MAW, 3:1:1v/v/v. . An additional bright-blue fluorescent compound was present in the lesions but not in fresh uninoculated seedlings or in adjacent unaffected tissues, and which was presumed to be the same compound which appeared on paper. The Rf values of the fluorescent compounds are shown in Table 2 .

With silica gel G, five to six fluorescent compounds were also detected. The Rf values of these fluorescent compounds are shown in Table 3 .

Extracts of lesions were run against nine known phenolic mixtures on paper using several solvents. None of the Rf values of these authentic phenolics coincided with the Rf values of the fluorescent compounds.

Rf values of fluorescent compounds

Spots	Solvents							
	BAW (4:1:5)	BAW (4:1:1)	EAW (9:2:2)	HAW (3:30:10)	6 % acetic acid	10 % acetic acid	15 % acetic acid	70 % ethyl alcohol
1	.27	.03	.60	.92	.30	.81	.78	.63
2	.16	.05	.50	.26	.12	.10	.13	.57
3	.41	.06	.78	.47	.42	.45	.45	.49
4	.52	.13	.68	.36	.26	.20	.27	.74
5	.63	.44	.83	.77	.57	.63	.68	.80
6*	.79	.78	.89	.10	.03	.03	.05	.70
7*	.90	.90	.95	—	—	—	—	.84

Table 1. Paper chromatography of fluorescent compounds extracted from Dupuits alfalfa roots infected with Pratylenchus penetrans (Cobb).

Spots 1 to 5 were present in both lesion and unaffected tissue extracts. Spot 6*, a bright-blues fluorescent compound, was present only in lesion extracts. Spot 7*, a pinkish-brown fluorescent compound, was present in lesions, but appeared only as a faint trace in unaffected tissues.

___ no Rf values obtained.

Rf values of fluorescent compounds.

Spots	Rf
1*	.08
2	.14
3	.20
4	.33
5	.48

Table 2. Thin layer chromatography of fluorescent compounds extracted from Dupuits Alfalfa roots infected with P. penetrans.
Adsorbent: Polyamide resin
Solvent: MAW (3:1:1 v/v/v)

Spot 1*, a bright-blue fluorescent compound, was present only in lesion extracts. Spots 2 to 5, were present in both lesion and unaffected tissue extracts.

Rf values of fluorescent compounds

Spot	Solvents	
	TEF (5:4:1)	BBA (45:4:8)
1	.12	.15
2	.18	.05
3	.48	.59
4*	.57	.98
5	.62	.82
6*	___	.70

Table 3. Thin layer chromatography of fluorescent compounds extracted from Dupuits alfalfa roots infected with P. [illegible].
Adsorbent: Silica gel G

Spots 1 to 3 and 5 were present in both lesion and unaffected tissue extracts. Spot 4*, a bright-blue fluorescent compound, was present only in lesion extracts. Spot 6*, a pinkish-brown fluorescent compound, was present in lesions, but appeared only as a faint trace in unaffected tissues.

___ no Rf values obtained.

Two-dimensional chromatography showing the fluorescent compounds found in lesions is shown in Fig. 4 .

After exposure to ammonia fumes, the different compounds appeared more intensely fluorescent under ultraviolet light. Under visible light, the bright-blue- and pinkish-brown-fluorescing compounds appeared pale yellow color.

Both compounds also gave positive reactions to various color reagents used for characterizing phenolic compounds on paper. Pale yellow spots were obtained with DSA, blue spots with ferric chloride-ferricyanide, brownish-gray spots with ammoniacal silver nitrate, and brownish-pink spots with tetrazotized benzidine and tetrazotized o-dianisidine.

A negative test was obtained with Benedicts reagent which rules out the compounds being reducing sugars.

Ten unidentified amino acids were detected with ninhydrin reagent in lesion extracts. However, none of the Rf values of these amino acids coincided with those of the fluorescing compounds. The R f values of the different amino acids are shown in Table 4 .

Spectrophotometric readings.

Spectrograms of the bright-blue- and pinkish-brown-fluorescent compounds are shown in Fig. 5 . An absorbance peak around 260 mμ was obtained with the bright-blue fluorescent compound and 266 mμ with the pinkish-brown fluorescent compound.

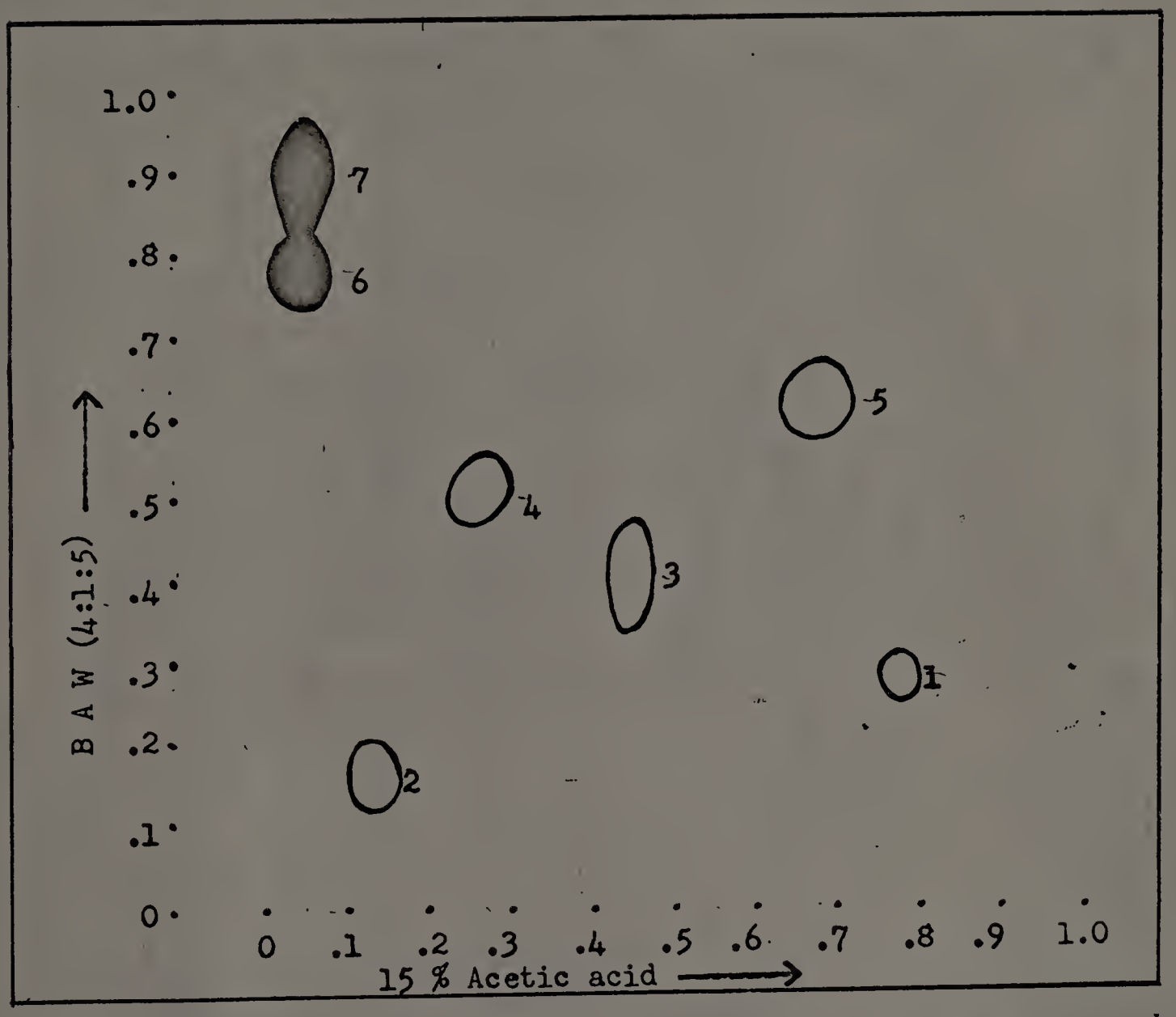


Figure 4. Two-dimensional paper chromatogram showing fluorescent spots from extracts of Dupuits alfalfa roots infected with Pratylenchus penetrans (Cobb).

Spots 1 to 5 were present in lesion and unaffected tissues. Spot 6 was found only in lesion. Spot 7 was found also in lesions, but appeared only as a faint trace in unaffected tissues.

Rf values of amino acids

Spot	Rf
1	.57
2	.43
3	.34
4	.26
5	.19
6	.17
7	.15
8	.12
9	.10
10	.05

Table 4. Paper chromatography of ten amino acids extracted from Dupuits alfalfa roots infected with P. penetrans.
Solvent: BAW (4:1:5 v/v/v)
Color reagent: 0.3% alcoholic ninhydrin.

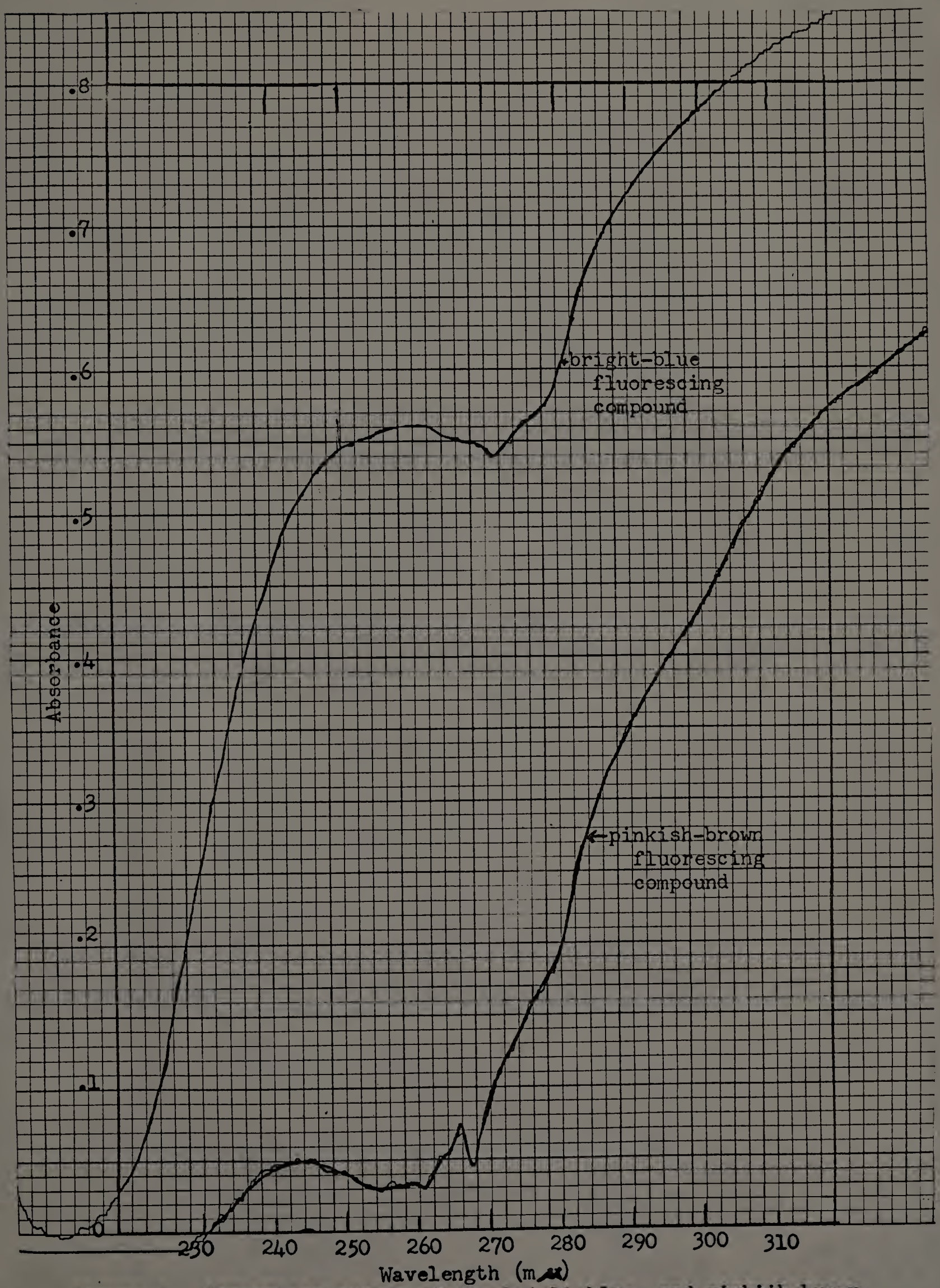


Figure 5. Absorption curves of the bright blue- and pinkish-brown-fluorescent compounds eluted from paper chromatograms.

V DISCUSSION AND CONCLUSION

Necrosis and cell destruction were the characteristic symptoms caused by Pratylenchus penetrans in roots of Dupuits alfalfa seedlings. The yellow discoloration which was visible 12 to 24 hours after inoculation can be attributed to biochemical changes occurring within the host plants as a response to nematode invasion. Discoloration was directly associated with nematode activity. Lesions caused by nematodes in roots did not appear to be different from those caused by mechanically-induced injury.

Necrotic reactions occurred mainly in cortical and epidermal tissues. The endodermis, which forms the inner face of the cortex was highly discolored and reactive to DSA. All stages of nematodes were found in cortical tissues but in no case did they penetrate the endodermis and vascular tissues. Uritani (1962) suspected that necrosis serves as a physical barrier to penetration by invading pathogen. Studies on the pathogenicity of P. penetrans by several workers (Pitcher, et al., 1960, Mountain & Patrick, 1959, Townshend, 1962, 1963) have shown that the endodermis is often the region which becomes most discolored. They suggest that the endodermis serves as a barrier to further penetration of nematodes into the vascular system. Discoloration has been attributed to the high concentration of phenolics in these tissues.

The bright-blue fluorescent compound which was present from extracts of lesions, punctured areas and oxidized uninoculated tissues was determined to be a phenolic compound by its reaction to different

color sprays. Another suspected phenolic compound found in the lesion extracts was a pinkish-brown fluorescent compound, which appeared as very faint trace in adjacent unaffected tissue extracts.

Acid-hydrolyzed root tissues did not yield these two phenolic compounds. However, the hydrolyzed tissues did turn brown which is an indication that polymerization occurred which resulted in the formation of brown-colored substances. These colored compounds were suspected to be polymers formed from oxidized phenols or melanin-like compounds which are generally not easily separated and identified on paper chromatograms.

Absorbance peaks around 260 to 266 m u, which were obtained with both phenolic compounds, are characteristic for many substituted phenols.

No definite conclusion can be made as to the mechanism involved in accumulation of these compounds in lesions or the biochemical processes associated with this reaction. Phenolic compounds are either synthesized in the lesion by the injured cells or perhaps released from preformed phenolics by increased activity of parasite enzymes. In either case, they are the principal factors responsible for browning and necrotic reaction. Injury to root cells during penetration and feeding of nematodes might stimulate injured cells to synthesize relatively large amounts of phenolics which serve as substrates for enzymatic oxidation by polyphenoloxidases released from injured plant cells. In most of the chromatograms examined, there were more of the fluorescent compounds in lesions than in healthy and unaffected tissues. This was indicated by larger and more intense fluorescent spots.

Discoloration due to subsequent polymerization of the oxidized products to brown substances has been found in a variety of plant dis-

seases (Farkas & Kiraly, 1962). This type of browning reaction was observed by Wallace (1961, a,b) in areas of chrysanthemun leaves infested by foliar nematodes. In this case, chlorogenic and isochlorogenic acids were the principal polyphenols oxidized.

The presence of these compounds in extracts of uninoculated root tissues may have been due to oxidation of phenolic substances released by damaged plant cells. Several hours exposure was necessary before the bright-blue fluorescent compound could be detected on paper chromatograms. Mechanical injury by puncturing the roots resulted in accumulation of the same compound in the punctured areas. The rapid and intense discoloration obtained after application of hydrolytic enzymes in punctured areas may be explained by the fact that these enzymes caused a rapid release of phenolic substrates and hastened the oxidation and polymerization of such compounds. Pectinol contains enzymes which are capable of hydrolyzing some beta-glycosides as well as pectinases which degrade cell wall materials. Peroxidases are widely distributed in plants and able to catalyze the oxidation and polymerization of phenols. Emulsin is an enzyme which hydrolyzes B-glucosides and is of special interst because a similar beta-glucosidase was observed by Mountain (1959) to be secreted by P. penetrans. Root cells of many plants contain glycosides which are normally non-toxic but which when hydrolyzed yield highly phytotoxic compounds which may caused death of root cells. Several nematode species have been shown to secrete high concentrations of hydrolytic enzymes, such as cellulases, which are capable of causing breakdown of cell walls with subsequent release of their contents (Krusberg, 1960b).

It can be concluded from this investigation that phenolic compounds

accumulated in infected root tissues of Dupuits alfalfa seedlings as a result of injury caused by P. penetrans. The reaction of root lesions in turning intensely yellow or brown with DSA is an indication of the presence of relatively high concentrations of phenolic compounds in injured areas of roots. Chromatographic analysis of different root extracts showed evidence of two fluorescing compounds, a bright-blue fluorescent compound, found only in lesion extracts and a pinkish-brown fluorescent compound, which was found mainly in the lesions but appeared only as a faint trace in unaffected tissue extracts. Both compounds, still unidentified, were confirmed to be phenolics by their reaction to color sprays. These phenolics were also suspected to cause necrosis to injured root cells as found by examination of histological sections of lesions.

The implication of these phenolic compounds in the host-parasite relationship was not fully determined. It is suggested that further investigations should be made on the biochemical changes occuring within the host tissues as a result of nematode infection and the effect of such changes on parasite metabolism.

VI SUMMARY

Host-parasite relationship of P. penetrans and Dupuits alfalfa were studied by inoculating plants grown in sterile nutrient agar with aseptic nematodes. P. penetrans started to penetrate the roots of alfalfa seedlings three hours after inoculation and were found embedded in the cortical tissues of the roots within six to eight hours. Yellow discolorations measuring one to two mm long were visible within 12 to 24 hours. They became dark brown in later stages. Similar symptoms were obtained in roots punctured with a sterile microneedle, except that discoloration was limited to the punctured areas.

The addition of pectinol, emulsin and peroxidases resulted in rapid discoloration of the punctured areas and caused a more intense brown color.

With addition of DSA, newly formed lesions became intensely yellow or brown indicating the presence of large amounts of phenolic compounds.

All stages of nematodes were found in the cortical region where necrosis occurred. Cell walls on the periphery of necrotic cells were stained red or brown and highly granular blue-stained cytoplasm was observed.

Several fluorescent compounds with color varying from pale violet to bright blue were present in extracts of lesions and unaffected tissues. An additional bright-blue fluorescing compound was present in extracts of lesions but not in extracts of fresh uninoculated and adjacent uninfected tissues. The same compound was found in extracts of punctured roots and oxidized uninoculated root tissues although in smaller amounts. A pinkish-brown fluorescing compound was also present

in the lesions, but appeared only as a faint trace in unaffected tissues.

The bright-blue fluorescent compound appeared in oxidized tissues after four to eight hours exposure of homogenates at room temperature, while a 12 hour exposure gave larger amounts approaching concentrations found in lesions. Larger and more intense fluorescent spots were found in lesion extracts as compared to healthy and unaffected tissues.

The Rf values of the bright-blue fluorescent compounds were low in aqueous solvents but high in organic solvents.

Various color sprays were used to detect and identify the bright-blue- and pinkish-brown fluorescent compounds. These compounds were determined as phenolic compounds by their color reactions.

The compound gave negative tests with Benedicts reagent and alcoholic ninhydrin reagent. Ten amino acids were detected in extracts of lesions, but were not identified.

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