HISTOCHEMISTRY AND ENZYME ACTIVITY OF RESISTANT AND SUSCEPTIBLE COTTON INFECTED BY MELOIDOGYNE INCOGNITA

by

Gregory Ross Noel

A Thesis Submitted to the Faculty of the

DEPARTMENT OF PLANT PATHOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE

In the Graduate College

THE UNIVERSITY OF ARIZONA

1 9 7 2

STATEMENT BY AUTHOR

This thesis has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Gregory R Noel

APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Michael A. McClure — 18 Sept 72

MICHAEL A. McCLURE — Date

Associate Professor of Plant Pathology

I wish to dedicate this thesis to my wife Jenny who is a blessing and a comfort to me. I would also like to dedicate this thesis to my parents Mr. and Mrs. Doyle E. Noel and to my Father- and Mother-in-Law, Mr. and Mrs. L. D. Scott, for their moral and financial help.

ACKNOWLEDGMENTS

I wish to express my appreciation and gratitude to Dr. Michael A. McClure for the help and guidance he has given me during the course of this study.

Appreciation is also given to Dr. R. L. Gilbertson for his assistance with the photography. Dr. Homer E. Bloss and Dr. Edward L. Nigh are to be thanked for their review of the manuscript.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS--Continued

LIST OF ILLUSTRATIONS--Continued

ABSTRACT

Acid phosphatase, 6-phosphogluconate dehydrogenase, peroxidase, proteinase, and shikimic dehydrogenase were histochemically localized in resistant Clevewilt (CW) and susceptible (M8) cotton infected and non-infected by Meloidogyne incognita. All enzymes except proteinase exhibited greater activity at infection sites than in adjacent non-infected tissue or in non-inoculated tissues. During the proteinase localization 12 days after inoculation nematodes undergoing lysis were found in the resistant CW.

Syncytial walls in CW and M8 cotton roots were studied histochemically and found to contain pectin, but no lipids or lignin.

Spectrophotometric assays of enzyme activity of acid phosphatase, 6-phosphogluconate dehydrogenase, and peroxidase were conducted 2, 6, and 10 days after inoculation. Acid phosphatase activity in non-infected roots of both CW and M8 declined during the 10 day period and was lower on all 3 days when compared to the infected roots. Activity of infected roots increased 6 days after inoculation but at 10 days had decreased below that found 2 days after inoculation. The activity of 6-phosphogluconate dehydrogenase decreased in both infected and non-infected, resistant and susceptible roots during the experimental period. The

greatest activity in all treatments was observed 2 days after inoculation and the least activity was observed 10 days after inoculation. The activity in the infected plants was greater than that of the non-infected 2, 6, and 10 days after inoculation. Peroxidase activity declined in the non-infected roots from 2 to 10 days after inoculation. The activity in infected roots increased during the 10 day period. Activity in infected roots was greater than that in non-infected roots at 2, 6, and 10 days after inoculation.

Isoenzymes of acid phosphatase, 6-phosphogluconate, and peroxidase were separated by polyacrylamide gel electrophoresis 6 days after inoculation. Gels were incubated in 3 different reaction mixtures to detect enzyme activity and were scanned on a scanning spectrophotometer. Acid phosphatase activity was greater in non-infected roots than in infected roots. Peroxidase activity was also greater in non-infected roots than in infected roots. Roots of infected CW had greater peroxidase activity than the infected M8. Activity of 6-phosphogluconate dehydrogenase was greater in the infected CW roots than in the infected M8 roots. Both infected treatments had more activity than the M8 non-infected.

Qualitative and quantitative differences in enzymatic activities of CW and M8 infected and non-infected roots were observed. These differences suggest a shift to

the Pentose Phosphate Pathway which might result in phenolic production and oxidation in CW roots.

INTRODUCTION AND LITERATURE REVIEW

The use of resistant varieties in plant disease control has long been recognized as a favorable method of disease control. This is especially true in light of current efforts to restrict or, in some cases, eliminate the use of pesticides. When the use of root-knot nematode (*Meloidogyne incognita* [Kofoid & White] Chit.) resistant cotton (*Gossypium hirsutum* L.) varieties now being developed is implemented the costs and inherent dangers of pesticide usage may be eliminated.

M. incognita is a serious problem in many cotton producing areas of the world. Although the resistance of cotton to this nematode has received much attention, the exact mechanism(s) of resistance is not known.

Brodie, Brinkerhoff, and Struble (4) studied the resistance of cotton to the root-knot nematode. They found no significant difference in penetration between resistant and susceptible cultivars 48 hrs after inoculation. They also found that larvae in the resistant variety either failed to grow or developed more slowly than those in the susceptible cultivar. Failure of nematodes to develop did not result in disintegration up to 30 days after inoculation. Infected roots of the resistant variety responded by becoming necrotic or slightly galled.

Later work by Minton (34) was in agreement with Brodie et al. (4). His histopathological evidence suggested two possible explanations for resistance: (a) hypersensitivity of the root tips resulted in necrosis which inhibited larval development, and (b) the failure of root cells to respond to the larvae which resulted in fewer galls. Resistance could no be attributed to morphological differences or to barriers which prevented penetration.

Dean and Struble (8) observed death and disappearance of M. incognita in sweet potatoes (Ipomoea batatas [L.] Lam.) resistant to the root-knot nematode and Riggs and Winstead (39) reported the death of root-knot larvae in the roots of resistant tomato (Lysoperscion esculentum [L.] Mill.).

Ellis (12) studied the resistance of cotton to M. incognita and found that resistance was not due to differences in attraction or penetration of larvae and that resistance and susceptibility were not transferable by grafting. He found that development of nematodes in resistant Clevewilt was retarded when compared to nematodes in the susceptible cultivar Deltapine Smooth Leaf. Nematodes did not egress from roots of the resistant Clevewilt at any time up to 16 days after inoculation, but at the end of that period there were fewer nematodes in the resistant cotton than in the susceptible. He also reported evidence that the nematodes died or were killed and

disintegrated in resistant tissue. Ellis concluded that resistance in Clevewilt was probably due to the presence of a toxin of a growth inhibitor which was inherent on a cellular level.

Subsequent work by McClure, Ellis, and Nigh (31) has shown that on Clevewilt, galls are formed which lack nematodes. Syncytial development generally was inhibited in the resistant cultivar as was development of the syncytial cell wall. In those cases where nematodes developed normally in the resistant cultivar, syncytia and syncytial cell walls were similar to those found in the susceptible cultivar. The walls of syncytia produced by ovipositing nematodes in both cultivars bore large protuberances similar in appearance to those found by Krusberg and Nielsen (23) in root-knot infected sweet potato. These authors found the protuberances to contain cellulose but not pectin. Dropkin and Nelson (10) studied the composition of the syncytial walls of several root-knot infected soybean (Glycine max [L.] Merr.) varieties and found that the cell walls contained pectin and cellulose, but no protein, starch, lignin, or suberin. The syncytial walls of tomato infected with Meloidogyne javanica (Treub) Chit. (2) and M. incognita (37) were also found to contain pectin and cellulose. Scheetz and Crittenden (41) working with soybeans resistant and susceptible to M. incognita reported results in agreement with those of Dropkin and Nelson (10).

The literature on enzyme histochemistry of nematode infected plants is limited. Myuge (35) reported an increase in peroxidase and cytochrome oxidase activity in the galled tissue of several plants infected by M. incognita. DeMott (9) observed strong glucose-6-phosphate and 6-phosphogluconate dehydrogenase activity in infected tissues, but little or no activity in noninfected portions of the same root. More recently, Endo and Veech (15) reported the histochemical localization of 5 dehydrogenases and 2 diaphorases in fresh sections of susceptible soybean roots infected by M. incognita. In another work by Veech and Endo (45), a study was made of the morphology and enzyme histochemistry in root-knot infected resistant and susceptible soybeans. Malate dehydrogenase, alkaline phosphatase, and cytochrome oxidase were identified histochemically in both cultivars.

Limited research has been completed on the quantitation of either enzyme activity of nematode infected plants or the activity of isoenzymes of nematode infected plants even though these phenomena are well documented in plants infected by other pathogens and plants diseased by nonparasitic agents (6, 17, 29, 33). Acedo and Rohde (1) found an increase in peroxidase activity of cabbage (Brassica oleracea capitata L.) infected by Pratylenchus penetrans (Cobb) Filipjev and Schuurmans Stekhoven. Huang, Lin, and Huang (20) found no qualitative differences between peroxidase isoenzymes of normal roots and root galls

incited by M. incognita. However, qualitative differences between isoenzymes of normal and galled stems were detected.

Qualitative differences in peroxidase isoenzymes of Wando peas (Pisum sativum L.) infected by Ditylenchus dipsaci (Kuhn) Filipjev were found by Hussey and Krusberg (21). They found an increase in peroxidase activity of plants infected by two D. dipsaci populations. The population which elicited a hypersensitive reaction caused a greater increase in peroxidase activity.

Myuge (36) advanced the concept of inhibitors in roots which respond to nematode secretions. If the enzyme inhibitor and the enzymatic secretions reach the proper balance syncytial development is initiated and the nematode develops normally. Complete inhibition would prevent nematode development whereas incomplete inhibition would allow limited syncytial development and limited nematode growth.

The literature previously cited indicates that resistance in cotton to M. incognita is due to either a pre-existing or a provoked biochemical mechanism. The death of nematodes which results in decreased numbers of nematodes in the resistant cultivar Clevewilt suggests either active enzymatic lysis or autolysis of nematodes which fail to mature. These nematodes may encounter a toxin inherent in resistant tissue or perhaps they are unable to promote the syncytial production necessary for establishing a parasitic

relationship. The lack of syncytial development may be caused by differences in the components essential for syncytial ontogeny or by a lack of stimulatory enzyme secretions (14).

Research Objectives

The objectives of this work were to study the resistance of cotton to M. incognita on an enzymatic and histochemical basis by the following methods:

1. Localization as defined by Veech and Endo (45) of acid phosphatase, peroxidase, 6-phosphogluconate dehydrogenase, shikimic dehydrogenase, and proteinase.
2. Spectrophotometric determination of enzymatic activity as defined by previous work (29, 43).
3. Determination of the activity of the isoenzymes of acid phosphatase, peroxidase, and 6-phosphogluconate dehydrogenase separated by polyacrylamide-gel electrophoresis as outlined by previous work (21, 32).
4. Analysis of the histochemical composition of syncytia to determine if histochemical differences existed which could explain the lack of syncytial development in the resistant variety.

MATERIALS AND METHODS

M. incognita larvae used in this work were derived from a single egg mass taken from an infected chile pepper (Capsicum frutescens [L.] var. longum Sandt.) growing in a field previously planted to cotton near Elfrida, Arizona. The nematodes were maintained on chile peppers in the greenhouse. Second stage larvae used as inoculum were obtained by the method of Lownsbery and Viglierchio (25). Larvae were collected every 24 hrs. After 2 lots were collected the nematodes were placed in a .013% solution of Aretan® (ethoxyethylmercuric chloride) (Plant Protection Ltd., Yaldim, Kent, England). After soaking for 4 hrs the nematodes were rinsed 4 times in sterile distilled water and the suspension was concentrated to either 1000 or 2000 nematodes/ml depending on the particular experiment to be conducted.

Seeds of both the resistant Clevewilt 6-3 (CW) and the susceptible (M8) cultivars were obtained as described previously (31). Prior to germination the seeds were soaked for 20 min in 0.5% $NaHClO_3$ then rinsed for a total of 70 min in sterile distilled water. They were then spread on autoclaved germination blotters and placed in incubators at temperatures that allowed for concurrent germination of the 2 cultivars. Clevewilt seeds were germinated at 28 C and the M8 at 29 C. After 48 hrs the seedlings were removed

from the blotters and the apices of 40 roots which were approximately 9 cm long were aligned along the upper edge of a 2.0 x 53 cm strip of Miracloth® (Chicopee Mills, Inc., New York). These were inoculated by a modification of the method of Chapman and Eason (5). The inoculation and subsequent growth of the seedlings were carried out in growth boxes filled with leached, autoclaved vermiculite (Fig. 1). After inoculation the boxes were placed in a growth chamber at 27 C with a photoperiod of 12 hrs.

Enzyme Localization

For the localization tests, 5000 nematodes were pipeted over 40 seedlings. Fresh longitudinal sections approximately 100 μ thick were obtained by holding the roots between 2 pieces of carrot and sectioning with a sliding microtome. Controls consisted of substrate deficient media and non-infected plants. All incubations were carried out in BPI dishes. After the roots were sectioned, they were placed in a holding medium of the buffer used for that particular localization demonstration for not more than 15 min.

The localization of acid phosphatase (AP) was a modification of an electrophoresis technique used by Meister (32). A stock substrate solution containing 5.0 mg sodium α naphthyl acid phosphate (SNAP) dissolved in 10.0 ml of 0.2 M acetate, pH 5.0 and a stock solution containing

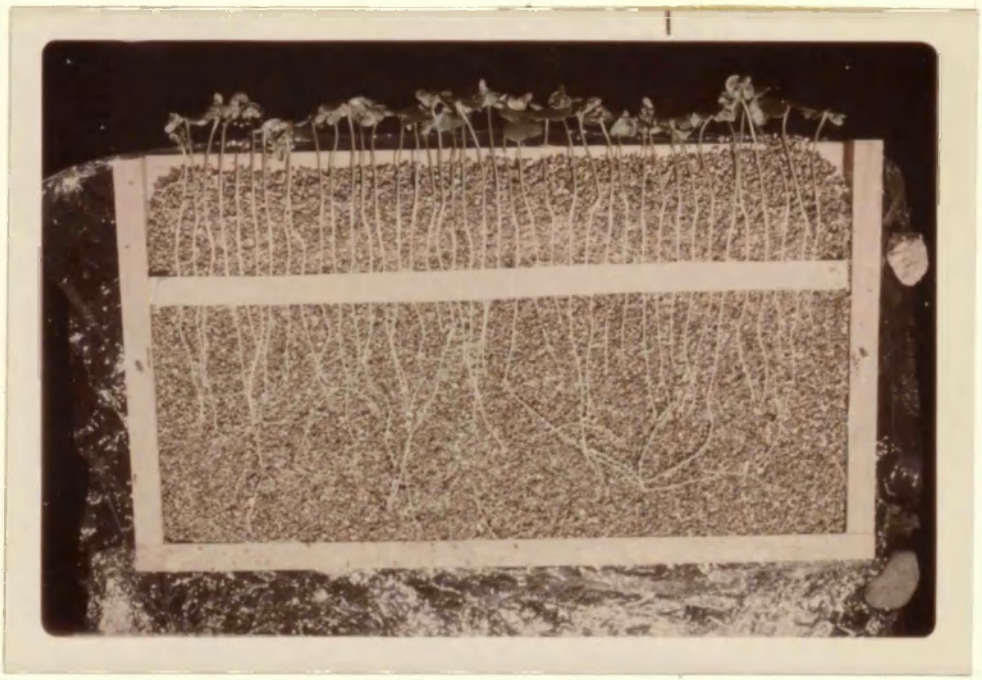

Fig. 1. Growth box with cotton seedlings 3 days after inoculation.

5.0 mg Fast Red TR salt (4-chloro-o-toluidine diazotate) in 10.0 ml of buffer were prepared before each localization demonstration. The sections were placed in 1.0 ml of the substrate for 15 min and then placed in 1.0 ml of the Fast Red solution for 30 min then mounted in the buffer for observation.

The method used for the peroxidase localization was described by Veech (44). Sections were incubated in 0.5 ml of 0.2% benzidine·2HCl in 1.0 ml of 0.2 M tris (hydroxymethyl) aminomethane-maleate buffer (Tris-M) pH 5.6 for 5 min. The sections were then transferred to 0.5 ml of 1.0% H_2O_2 in 0.5 ml of 0.1 M nickelous ammonium sulfate (NAS) for 1 min. The sections were then given two successive washings in NAS.

A modification of the electrophoretic demonstration of shikimic dehydrogenase (SDH) developed by Shaw and Prasad (42) was used for the demonstration of that enzyme. The reaction medium consisted of 9.0 ml of 0.2 M Tris-M, pH 7.2; 10.0 mg shikimic acid; 2.0 mg NADP; 2.0 mg nitroblue tetrazolium chloride (NBT); 0.5 mg phenozine methosulfate. The sections were placed in 1.0 ml of the medium for 40 to 60 minutes.

An adaptation from the electrophoresis work of Garner, Salisbury, and Graves (18) was used for the proteinase localization. Sections were placed in 1.0 ml of substrate medium which contained 2.8

mM-N-benzoyl-DL-arginine-β-naphthylamide HCl (BANA) in 0.2 M phosphate, pH 7.4 for 30 min and then transferred to 1.5 ml of 1 mg/ml fast garnet GBC salt in buffer (o-amino azotoluene, diazonium salt) for 10 min.

The localization of 6-phosphogluconate dehydrogenase (6-PGDH) was a modification of the technique described by Endo and Veech (15). The reaction medium was composed of 1.0 ml of 0.2 M Tris-M, pH 7.2; 1.0 ml of 1.0 mg/ml p nitro-blue tetrazolium chloride; 1.0 mg nicotinamide adenine dinucleotide phosphate (NADP); 0.1 ml of 4% MgCl; 3.4 mg of the trisodium salt of 6-phosphorgluconic acid (6-PG). The sections were placed in the medium for 25 min.

Syncytial Histochemistry

Tissue was placed in a fixative solution containing 50 ml ethanol, 5 ml glacial acetic acid, 10 ml formaldehyde, and 35 ml water. The material was then placed in a dessicator and evacuated for 24 hrs. Excised roots were mounted in carrot and sectioned at approximately 30 μ on a sliding microtome. Sudan Black B was used to stain for total lipids. Phloroglucinol and Ruthenium Red were utilized for staining lignin and pectins respectively (22).

Spectrophotometric Assays of Enzyme Activity

Peroxidase, 6-GDH, and AP activities were assayed at 2, 6, and 10 days after inoculation. The inoculum was standardized at 2000 nematodes/ml and 5.0 ml of the

suspension was pipeted onto 40 seedlings. Extracts to be used in the assays were prepared by freezing the desired number of 2.0 cm long roots in liquid nitrogen and pulverizing them with a mortar and pestle. After partial thawing, 2.0 ml of buffer were added with continued grinding. The grindate was pipeted into a syringe in which a folded 2.0 cm square piece of cheesecloth had previously been placed. The suspension was injected into a centrifuge tube containing the desired amount of buffer and centrifuged at 1500 g for 20 min at 6 C. Inoculated and non-inoculated plants of each variety were used. Controls consisted of autoclaved extracts.

Peroxidase activity was determined by the method of Loebenstein and Linsey (24). A Spectronic 20 (Bausch and Lomb) spectrophotometer was used to make a colorimetric determination of enzyme activity. The reagents were 1.0% H_2O_2 and pyrogallol reagent prepared immediately before use from fresh 0.5 M pyrogallol stock solution and 0.15 M phosphate, pH 6.0. After adding 10.0 ml of the stock solution and 12.5 ml of the buffer, the pyrogallol reagent was diluted 100 ml. The assay procedure was as follows: 5 roots were ground and centrifuged in a total volume of 5.0 ml; to 5.0 ml of the pyrogallol reagent in a colorimeter tube was added 1.0 ml of the extract; the tube was inverted to mix the contents, then inserted in the colorimeter; the galvanometer was adjusted to 0 optical density (OD) at a

wavelength of 420 nm; 1.0 ml of the 1.0% H_2O_2 was added to the tube; changes in OD were read after 90 sec.

Determination of 6-PGDH activity was patterned after previous work by Solymosy and Farkas (43). A grating spectrophotometer (Beckman DB-G) was used to measure the reduction of NADP at 340 nm. The root extract consisted of 10 roots in a total volume of 5.0 ml of 0.2 M Tris-M, pH 7.2. The reaction mixture contained 1.0 ml of extract; 1.0 ml of 6-PG at 15.0 mg/10.0 ml buffer; 0.1 ml of 4.0% $MgCl_2$; 1.0 ml of NADP at 15.0 mg/10.0 ml buffer. The change in OD was read after 8 minutes.

A grating spectrophotometer was used to make a colorimetric determination of AP activity. The change in absorbance at 420 nm was measured after 5 min. The root extract consisted of 15 roots in a total volume of 5.0 ml of 0.2 M acetate, pH 5.0. Th reaction mixture contained 1.0 ml of extract; 1.0 ml of SNAP at 10.0 mg/10.0 ml buffer; 1.0 ml of Fast Red TR salt at 10.0 mg/10.0 ml buffer.

Electrophoresis

Isoenzymes of peroxidase, 6-PGDH, and AP from infected and noninfected roots of both varieties were separated by polyacrylamide gel electrophoresis (7). Extracts from 40 roots were obtained in the same manner as for the spectrophotometric assays of activity except that homogenates were centrifuged at 18,400 g for 30 min at 6 C

in 2.0 ml M Tris-glycine buffer, pH 8.3. Extracts were diluted to equivalent amounts of protein as estimated by Lowry et al.'s (26) method using bovine albumin as a standard. Sucrose was added to make a 20% solution and 150 ml of extract containing 50 µg of protein were layered onto the stacking gel. The internal diameter of the glass column was 5.0 mm. A current of 2 ma/gel was applied and the protein was electrophoresed in an anionic system at 4 C in a 7.0% gel until the tracking dye had moved 60 mm at which time the gels were removed and incubated.

The incubation media were previously described by Meister (32). The reaction mixture for AP contained 4.0 mg SNAP in 9.0 ml of 0.2 M acetate buffer, pH 5.0. The stain contained 4.0 mg Fast Red TR salt dissolved in 10.0 ml buffer. After electrophoresis, the gels were held for 30 min in the acetate buffer and then incubated for 15 min in the reaction mixture after which time the gels were placed in the staining medium.

Peroxidase isoenzymes were located by incubating the gels in a solution containing 0.05% H_2O_2 and 0.5% guiacol.

Isoenzymes of 6-PGDH were located by adding 2.0 mg NBT and 0.5 mg PMS to the reaction mixture immediately before the gels were placed in the mixture. The reaction mixture contained 6.0 ml of 0.2 M Tris-M, pH 7.2, 30 mg trisodium-6-phosphogluconate, and 2.0 mg NADP. Gels were scanned on a scanning spectrophotometer (Giford 240).

RESULTS

Enzyme Localization

All enzymes tested were detected histochemically in healthy and inoculated roots with inoculated roots consistently having more intense color formation in the areas where the nematodes were present (Figs. 2, 3, 4, 5, 6). Proteinase, the only exception, was equally active in both the inoculated and healthy roots. Proteinase activity was indicated by a general overall reaction in sections of the four treatments. Sites of increased AP (Fig. 2), peroxidase (Fig. 3), SDH (Fig. 4), and 6-PGDH (Fig. 5) activities were readily demonstrated in cells along the nematode body and cells adjacent to the terminus and lip region. Not all sections showed localized activity about the nematodes. Since, however, it was not possible to produce serial sections, it is likely that certain areas showing localized activity were lost in cutting. Likewise, areas were found with increased localized activity without the presence of nematodes. These active sites were not found in healthy plants. The vascular tissues, especially the xylem, and lateral root primordia exhibited intense activity for peroxidase in both healthy and inoculated roots of both cultivars. SDH and 6-PGDH also exhibited activity in these tissues, but not in the magnitude of the peroxidase.

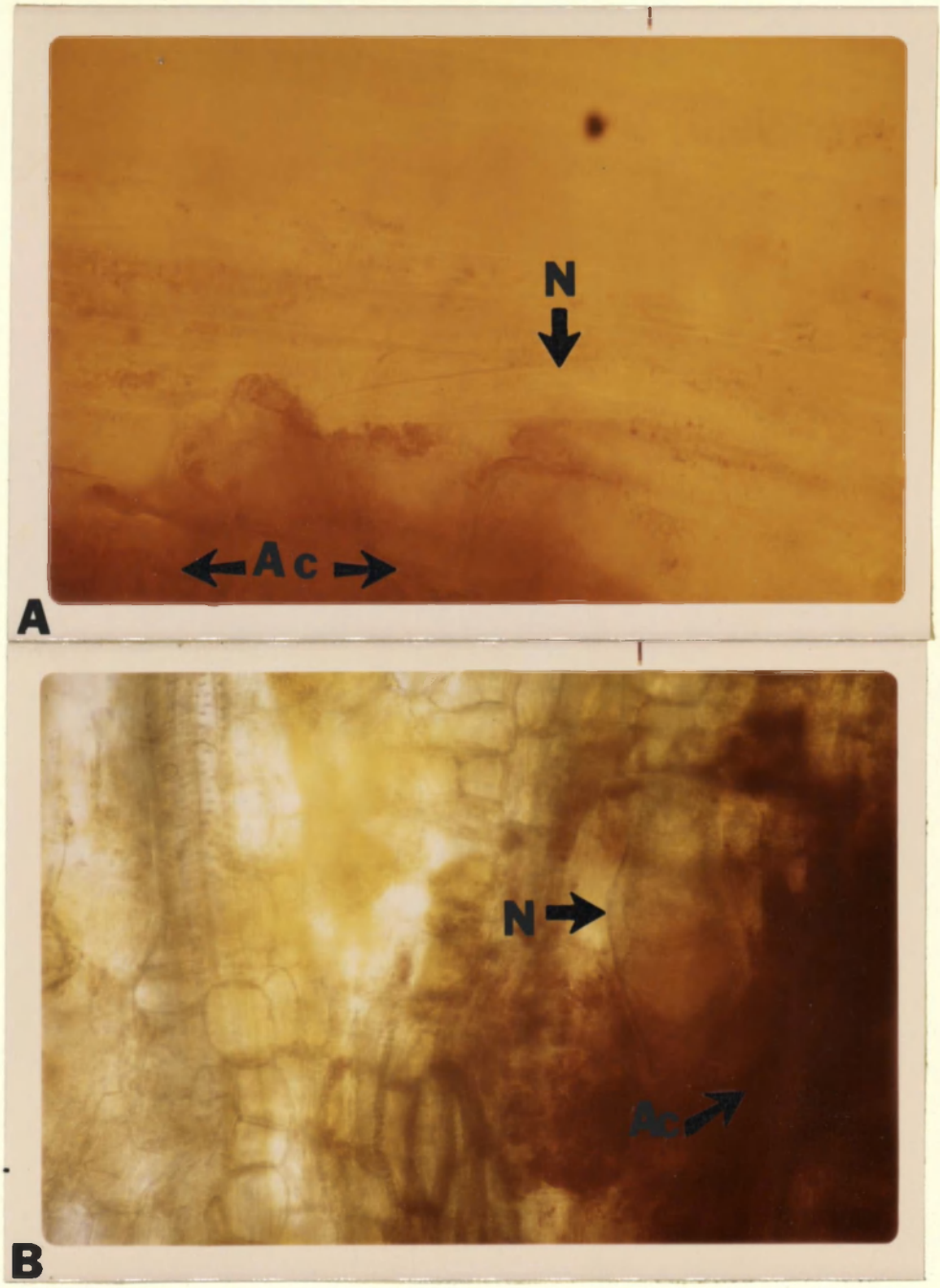

Fig. 2. Photomicrographs showing the histochemical localization of acid phosphatase in A CW and B M8 cotton roots infected by M. incognita -- A) Activity (Ac) near the head of a nematode (N) 3 days after inoculation. B) Intense activity (Ac) adjacent to a nematode (N) 8 days after inoculation.

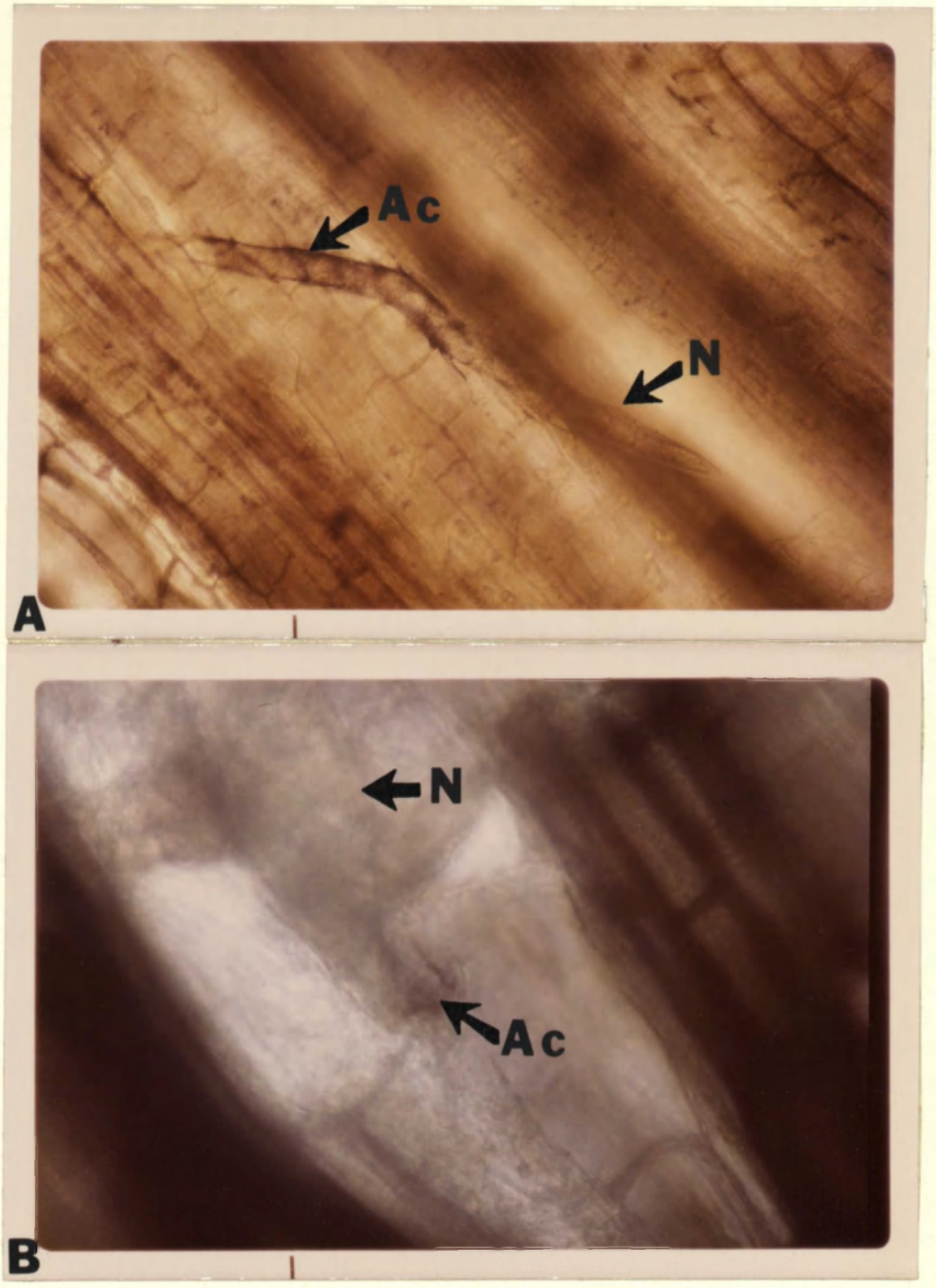

Fig. 3. Photomicrographs showing the histochemical localization of peroxidase in A CW and B M8 cotton roots infected by M. incognita -- A) Activity (Ac) surrounds posterior half of a 2nd stage larva (N) which is still in the cortex 4 days after inoculation. B) Slight activity (Ac) near the lip region of a nematode (N) 6 days after inoculation.

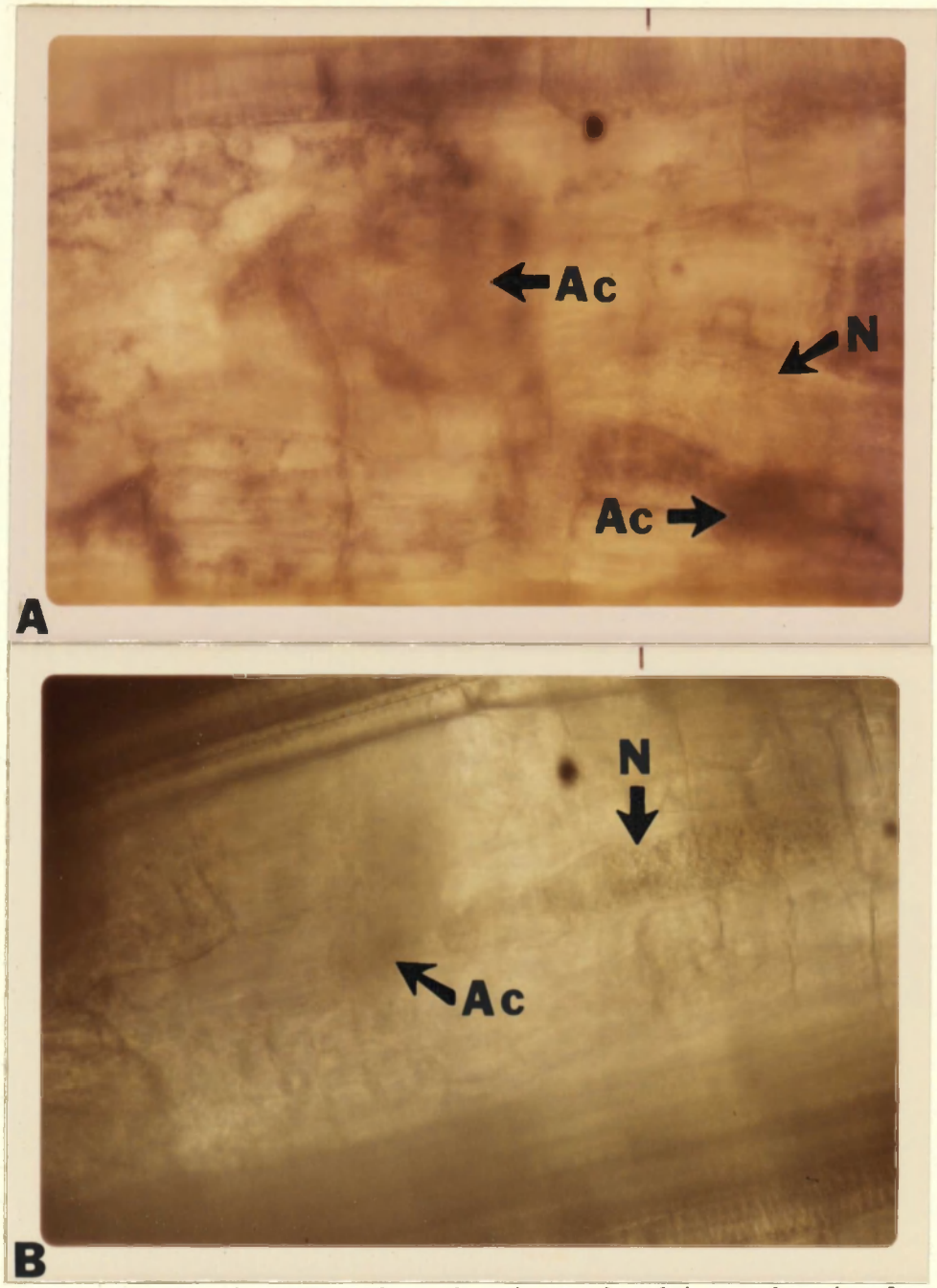

Fig. 4. Photomicrographs showing the histochemical localization of shikimic dehydrogenase in A CW and B M8 cotton roots infected by M. incognita -- A) Activity (Ac) extending from the lip region and region near the excretory pore of a 2nd stage larva (N) 5 days after inoculation. B) Activity (Ac) near the lip region of a 2nd stage larva (N) 5 days after inoculation.

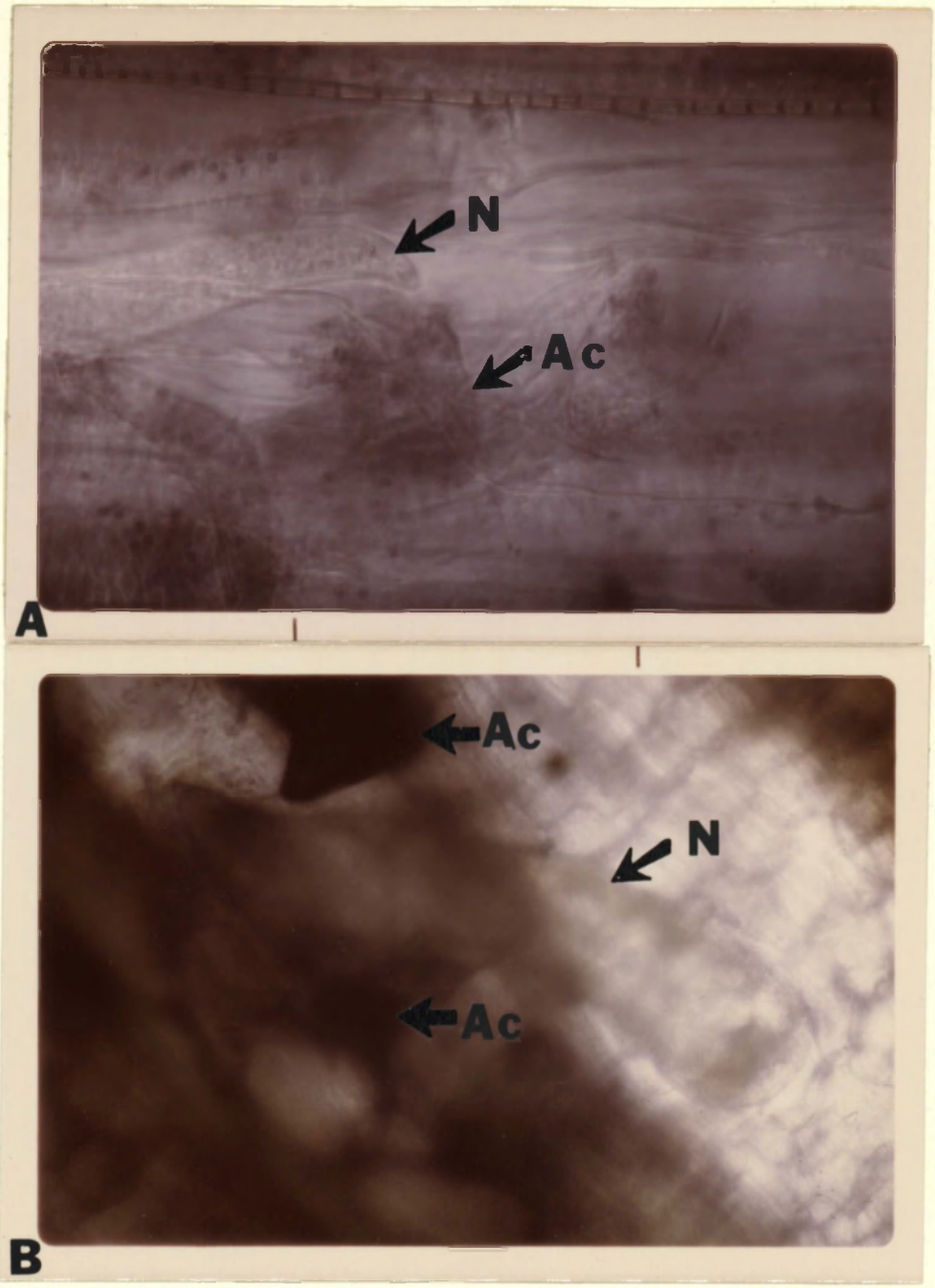

Fig. 5. Photomicrographs showing the histochemical localization of 6-phosphogluconate dehydrogenase in A CW and B M8 cotton roots infected by M. incognita -- A) Activity (Ac) extending from the lip region of a 2nd stage larva (N) 4 days after inoculation. B) Activity (Ac) surrounding the anterior portion of a nematode (N) 6 days after inoculation.

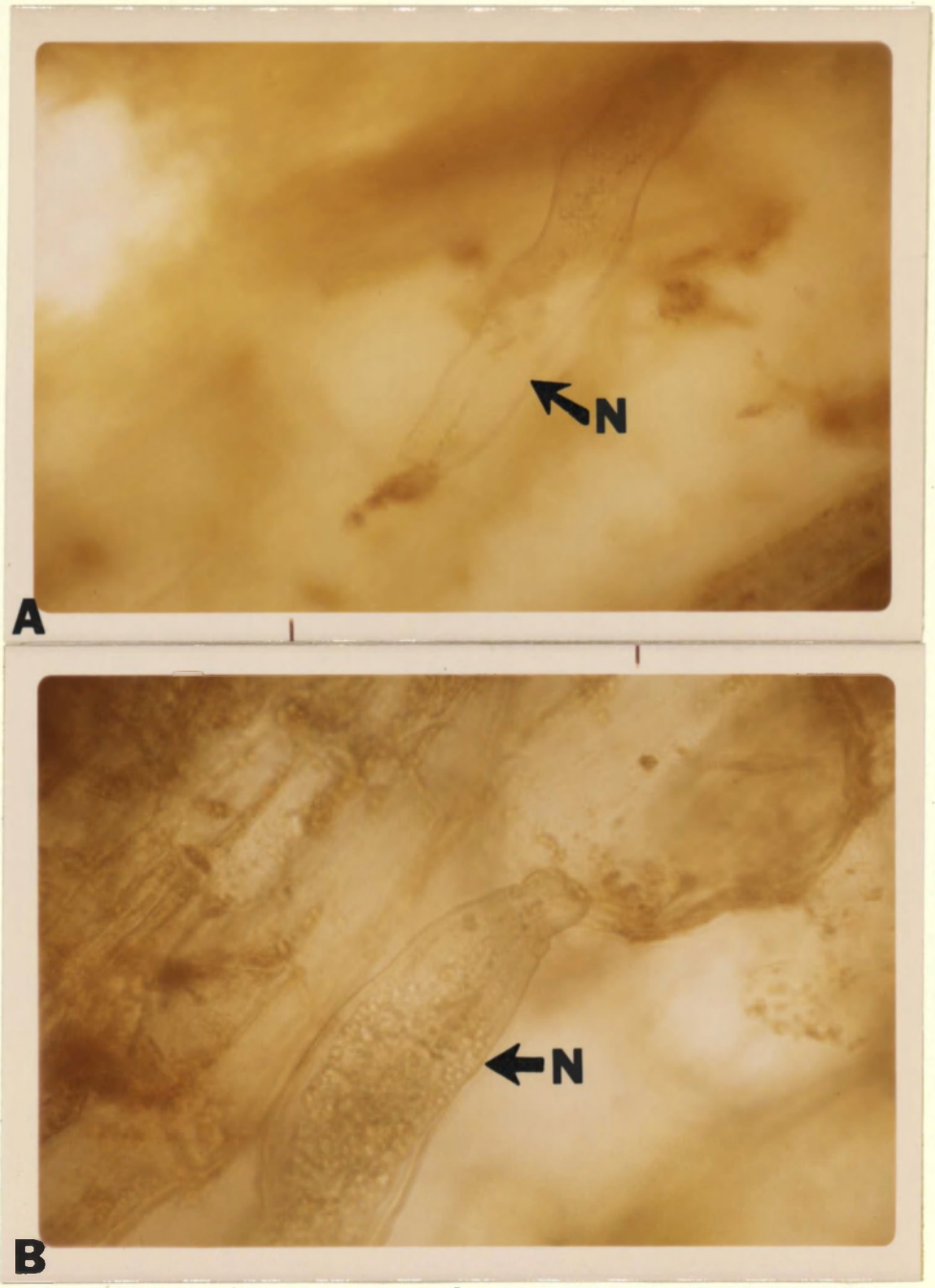

Fig. 6. Photomicrographs, A and B, showing the lysis of 2 different nematodes in CW roots infected by M. incognita 12 days after inoculation -- Photomicrographs show an unsuccessful attempt at the histochemical localization of proteinase activity near the nematodes. A) Lip region of the nematode (N) has been degraded and the body contents are not visible. B) No disruption of the nematodes (N) has occurred although the cuticle appears quite abnormal.

Uninoculated plants reacted more slowly than the inoculated plants. AP activity was more intense in the epidermal cells than in other cells of inoculated and healthy roots of both cultivars except for cells adjacent to the nematodes in both inoculated treatments. The color change in the inoculated and healthy plants took place at the same rate. The color formations indicative of the various reactions were blue for peroxidase, 6-PGDH, and SDH and red for AP and proteinase. Catalase activity was indicated by the formation of oxygen bubbles during the peroxidase localization. The gas formation was more intense in the inoculated treatments than in the healthy treatments. Nuclei extending from the lip region and paralleling the longitudinal axis of the nematode were found in the M8 cultivar 3 days after inoculation, but were not found in the resistant CW (Fig. 7). Nematodes in CW generally did not appear as well developed as nematodes in the M8. Nematodes undergoing lysis were found in the CW but not in the M8 12 days after inoculation (Fig. 6).

Syncytial Histochemistry

Total Lipids

The dark blue color indicative of the positive reaction of Sudan Black B was not found in the syncytial wall of either cultivar (Fig. 8). The cytoplasm of the syncytia in both cultivars stained slightly except in one section of CW syncytia in which the cytoplasm of a syncytium stained

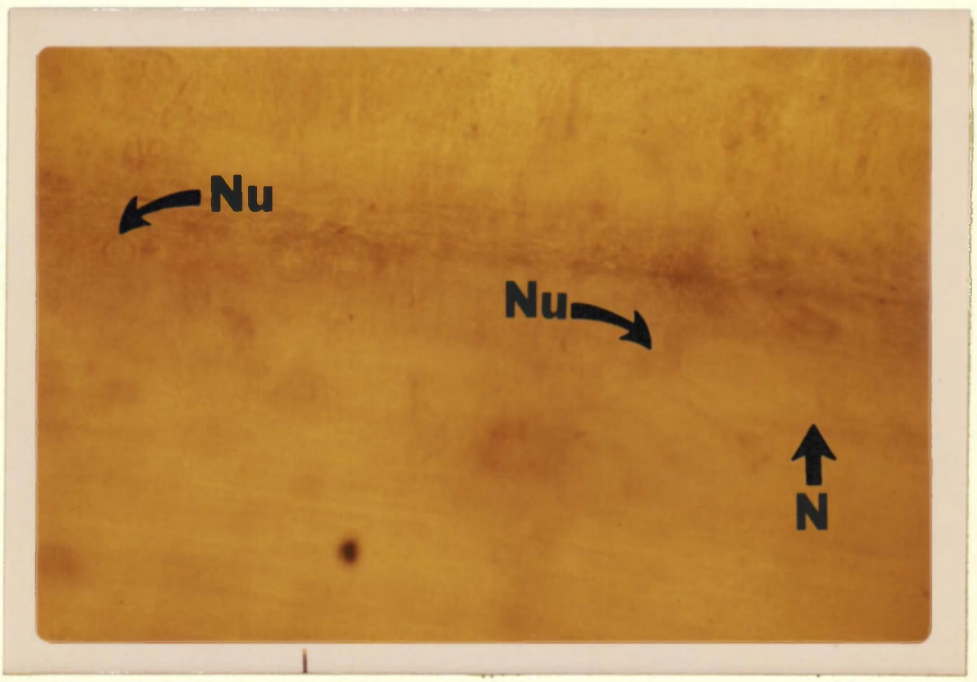

Fig. 7. Photomicrograph of nuclei (Nu) extending from the lip region of a nematode (N) in an M8 root 3 days after inoculation.

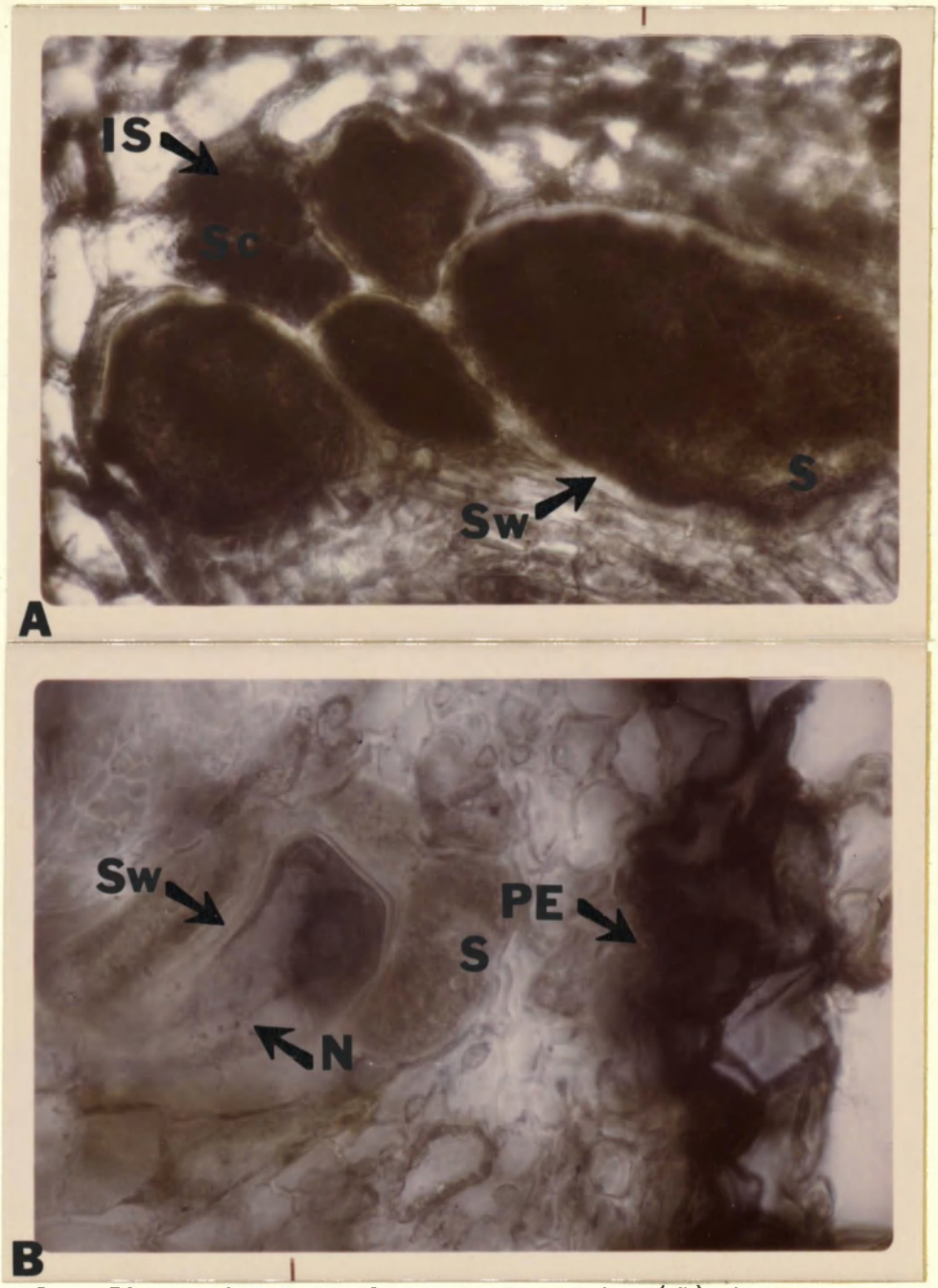

Fig. 8. Photomicrographs of syncytia (S) induced by M. incognita in A CW and B M8 cotton roots stained for lipids with Sudan Black B 24 days after inoculation -- A) Intense blue stain (IS) is of syncytial cytoplasm (Sc) and negative reaction of syncytial wall (Sw). The other syncytial cytoplasms stained slightly. B) A nematode (N) is surrounded by syncytia (S). Note the intense stain of the pericyclic-endodermal layer (PE) and the lack of stain in the syncytial wall (Sw). The syncytial cytoplasms stained slightly.

intensely (Fig. 8). In both cultivars the endodermal and pericyclic layers reacted positively. One area in the M8 roots where the phloem differentiates stained a dark blue. This area was several cells thick and separated the giant cell from the parenchyma cells in the central portion of the stele (Fig. 9). Sectioned nematodes reacted positively.

Lignin

Phloroglucinol was used to determine the presence of lignin. No reaction was found in the syncytial walls and cytoplasm of either cotton cultivar (Fig. 10). The xylem elements which characteristically contain large quantities of lignin stained a brilliant red-violet color.

Pectins

The syncytial walls but not the cytoplasm of both varieties stained a brilliant red with Ruthenium Red (Fig. 11). The wall protuberances in the syncytia of both varieties also reacted positively (Fig. 11). Walls of parenchyma cells and cells which had not undergone secondary thickening also stained positively.

Spectrophotometric Assays of Enzymatic Activity

Peroxidase

Peroxidase activity in homogenates of non-infected roots decreased slightly over the 10 day period after inoculation while the infected reaction was one of greatly

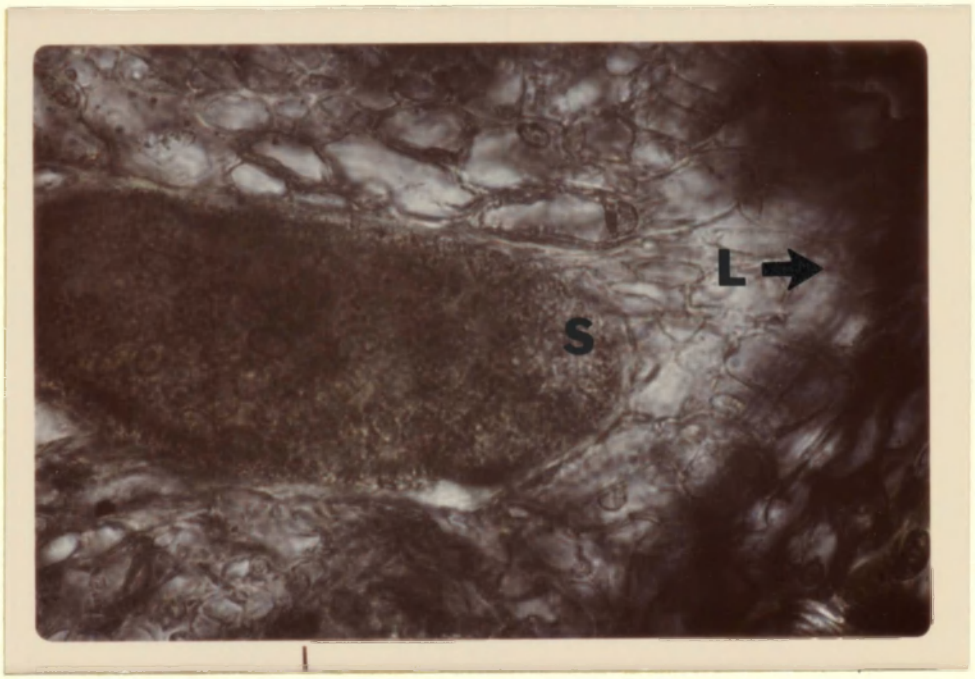

Fig. 9. Photomicrograph of a syncytium (S) induced by M. incognita in an M8 root -- The syncytium is separated from the central portion of the stele by a lipid layer (L).

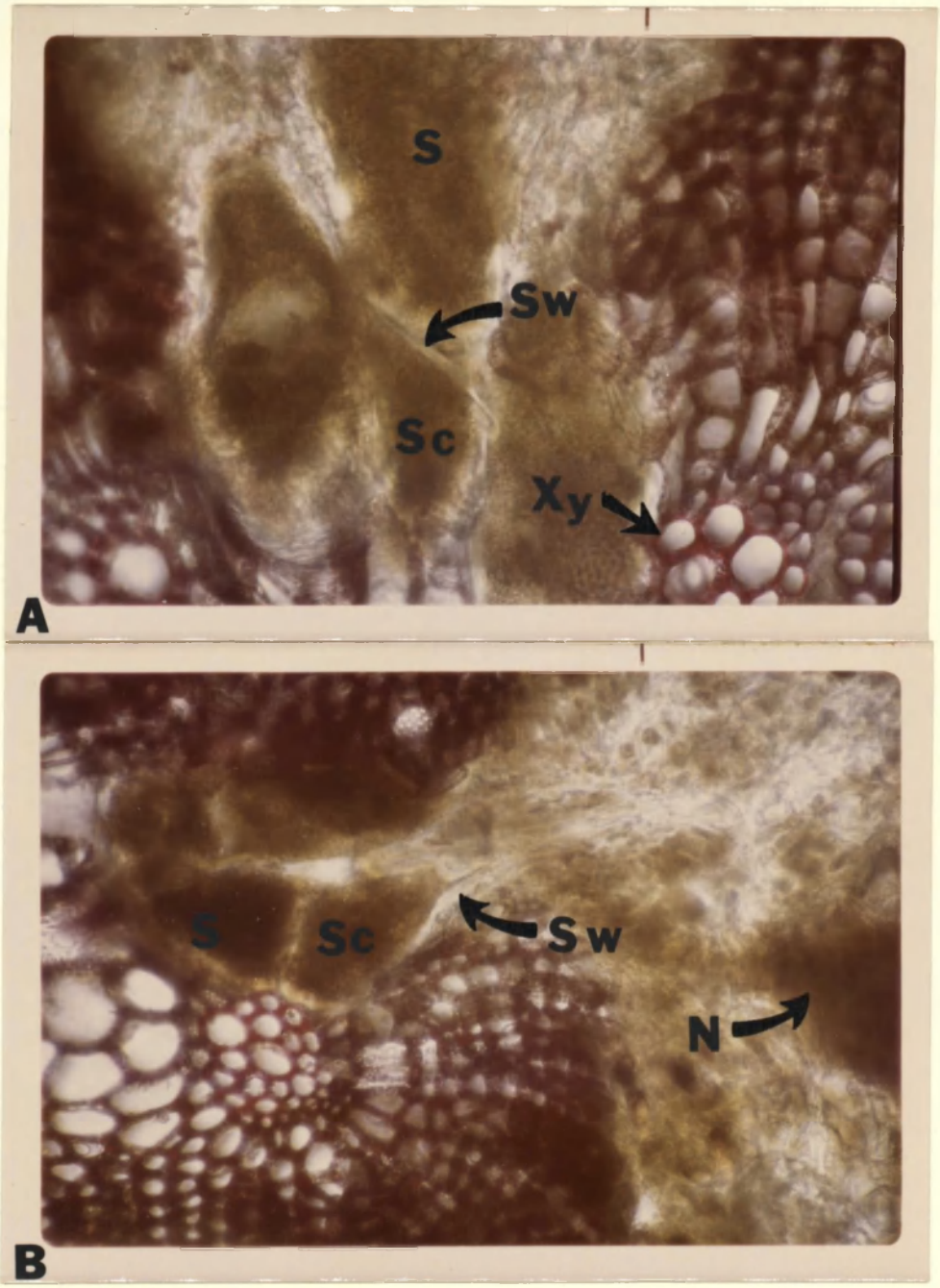

Fig. 10. Photomicrographs of syncytia (S) induced by M. incognita in A CW and B M8 cotton roots stained for lignin with phloroglucinol 24 days after inoculation -- A) Neither the syncytial wall (Sw) nor the syncytial cytoplasm (Sc) stained red. Note the intense stain of the xylem (Xy). B) Neither the syncytial wall (Sw) nor the syncytial cytoplasm (Sc) stained red. Note the intense stain of the xylem. A nematode (N) is visible.

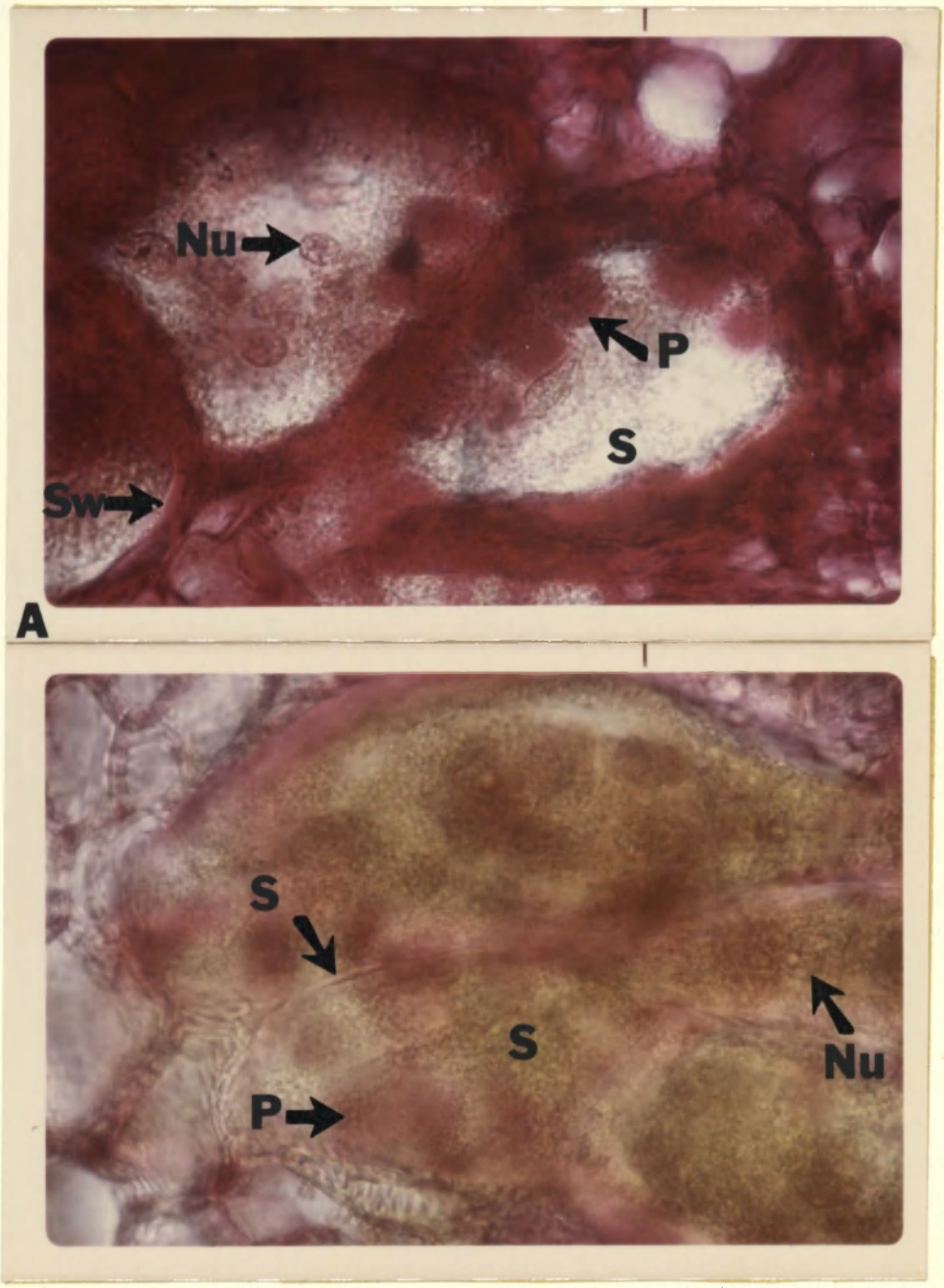

Fig. 11. Photomicrographs of syncytia (S) induced by M. incognita in A CW and B M8 cotton roots stained for pectin with Ruthenium Red 24 days after inoculation -- A) The syncytial wall (Sw) and the protuberances (P) stained red. Nuclei (Nu) are visible. B) The syncytial wall (Sw) and protuberances (P) stained red. Nuclei (Nu) are visible. The CW section stained darker because it was left in the staining medium longer than the M8 section.

increased activity during the same period (Fig. 12). The infected roots exhibited greater activity than non-infected roots at any time following inoculation. Activity in non-infected CW roots was greater than that in M8 non-infected roots on the second and sixth day after inoculation (Fig. 12), but on the tenth day activity was nearly the same (Fig. 12). The activity of the infected CW roots was higher than the infected M8 roots 2 days after inoculation (Fig. 12). However, on the sixth day after inoculation the change in activity was slightly greater in the infected M8 roots and on the tenth day the activity in the M8 infected roots was much greater than that in the infected CW roots (Fig. 12). At no time did the autoclaved controls exhibit any activity during the 90 sec assay period.

Acid Phosphatase

The change in activity of acid phosphatase was greater for the infected roots than the non-infected roots at all time intervals sampled (Fig. 13). Both the CW and M8 roots exhibited an increase in activity from 2 to 6 days with the activity on the tenth day being less than the activity 2 days after inoculation. The non-infected roots of both cultivars exhibited a decrease in activity over the 10 day period. The only exception was a slight increase in activity for the M8 roots between 2 and 6 days after inoculation (Fig. 13). The autoclaved extracts had a high degree

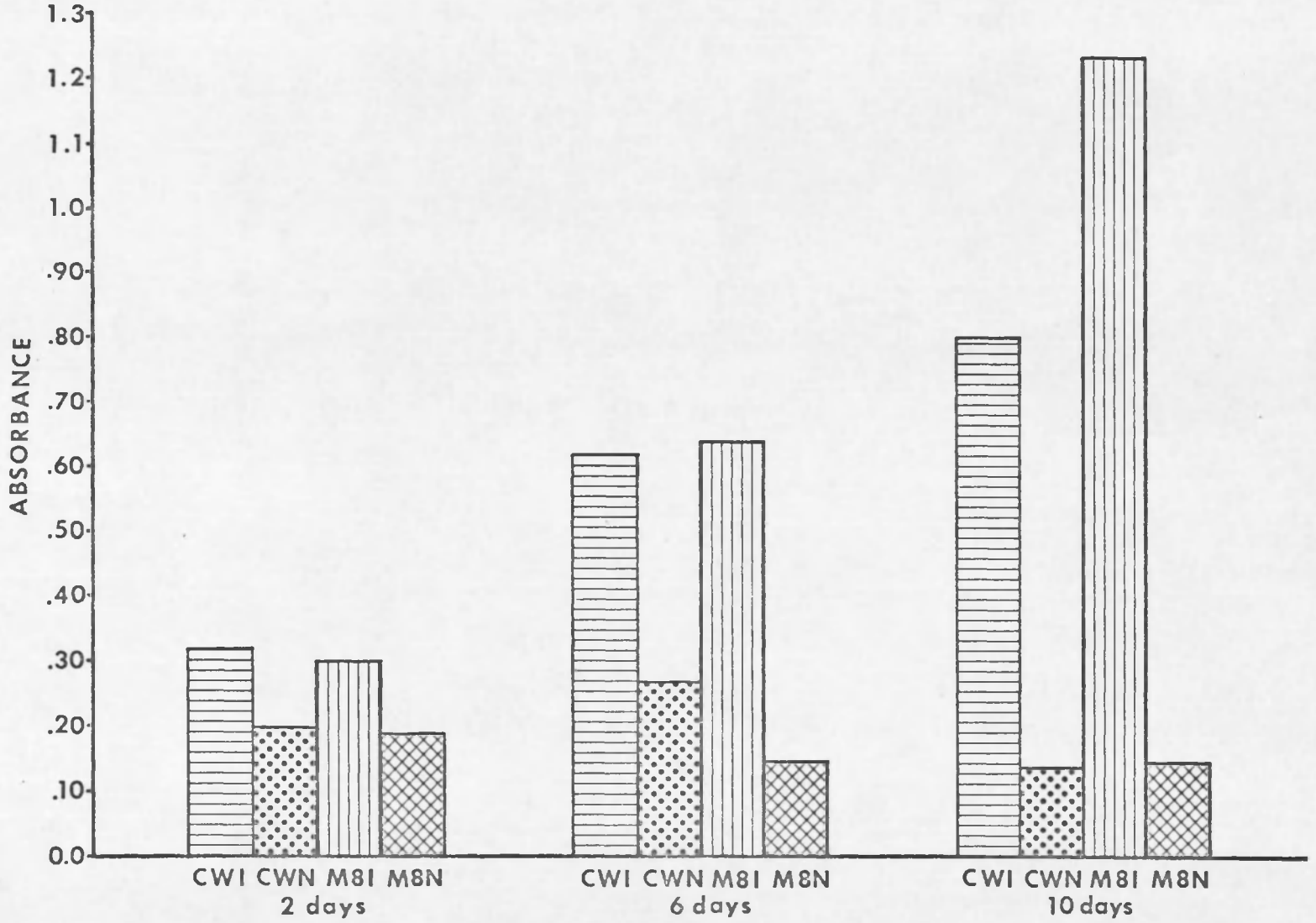

Fig. 12. Spectrophotometric determination of peroxidase activity 2, 6, and 10 days after inoculation -- Change in absorbance was measured at 420 nm at 90 sec. CWI = CW infected; CWN = CW non-infected; M8I = M8 infected; M8N = M8 non-infected.

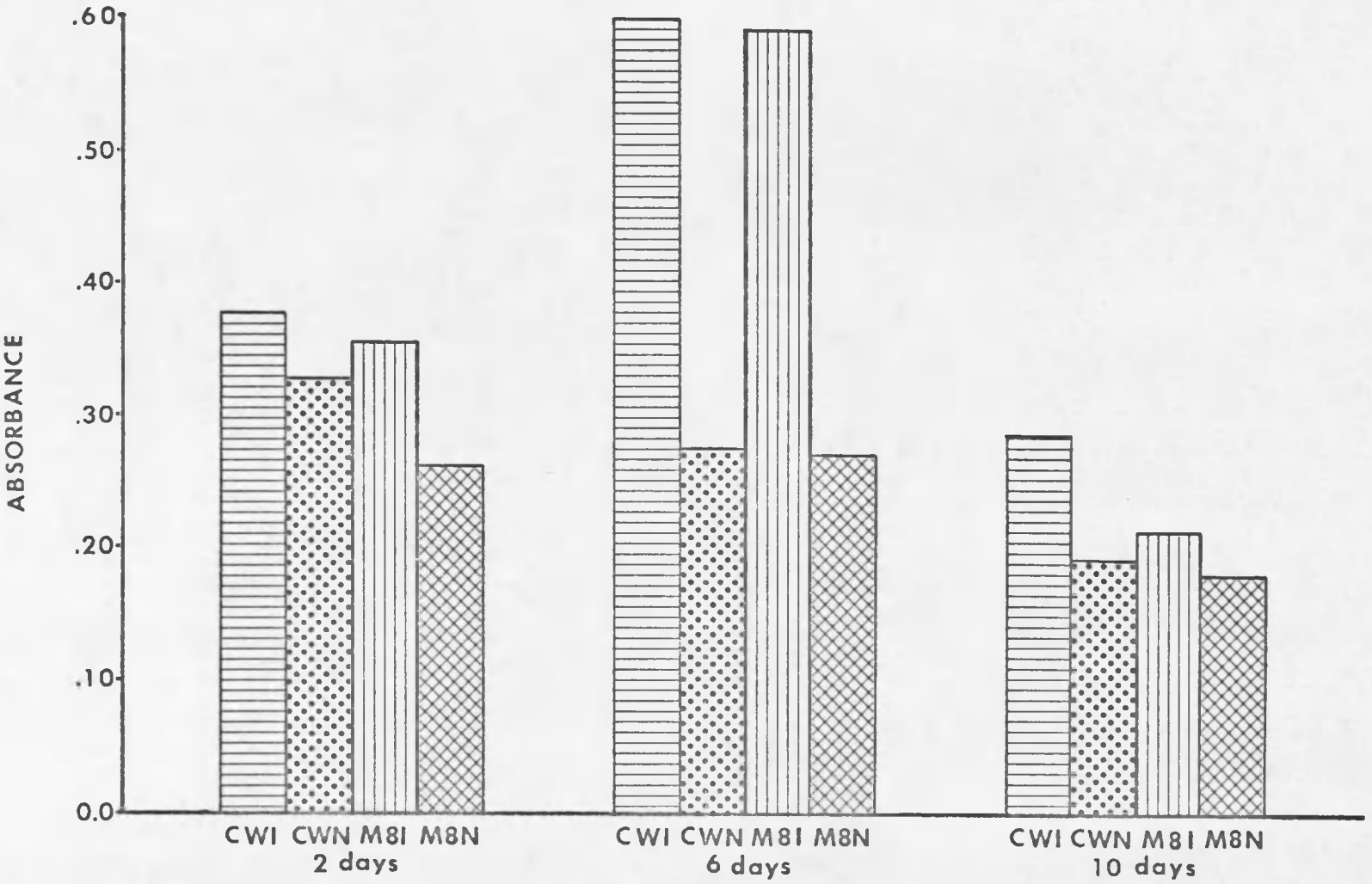

Fig. 13. Spectrophotometric determination of acid phosphatase activity 2, 6, and 10 days after inoculation -- Change in absorbance was measured at 420 nm after 8 min. CWI = CW infected; CWN = CW noninfected; M8I = infected; M8N = M8 non-infected.

of non-enzymatic activity throughout the 10 day period. This activity was always greater in the infected controls than in the non-infected controls with the M8 infected consistently having more activity than the CW infected.

6-Phosphogluconate Dehydrogenase

There was a decline in activity of non-infected roots from 2 to 10 days after inoculation (Fig. 14). At 10 days after inoculation there was slight activity in the M8 non-infected roots and no activity in the CW non-infected roots (Fig. 14). During the experimental period the activity in infected roots also declined but the decline in activity was not as great as that observed in the non-infected roots (Fig. 14). The autoclaved controls did not exhibit any activity.

Electrophoresis of Enzymes

Peroxidase

The densitometric readings of peroxidase isoenzymes separated by polyacrylamide gel electrophoresis 6 days after inoculation indicated several areas of density not visible in the photographs of the same gels (Figs. 15, 16, 17). Scans of the gel of M8 infected isoenzymes indicated a reduction in activity (Fig. 16). The largest number of shoulder peaks which indicated areas of density not found in the other treatments were found in the scan of the gel of M8

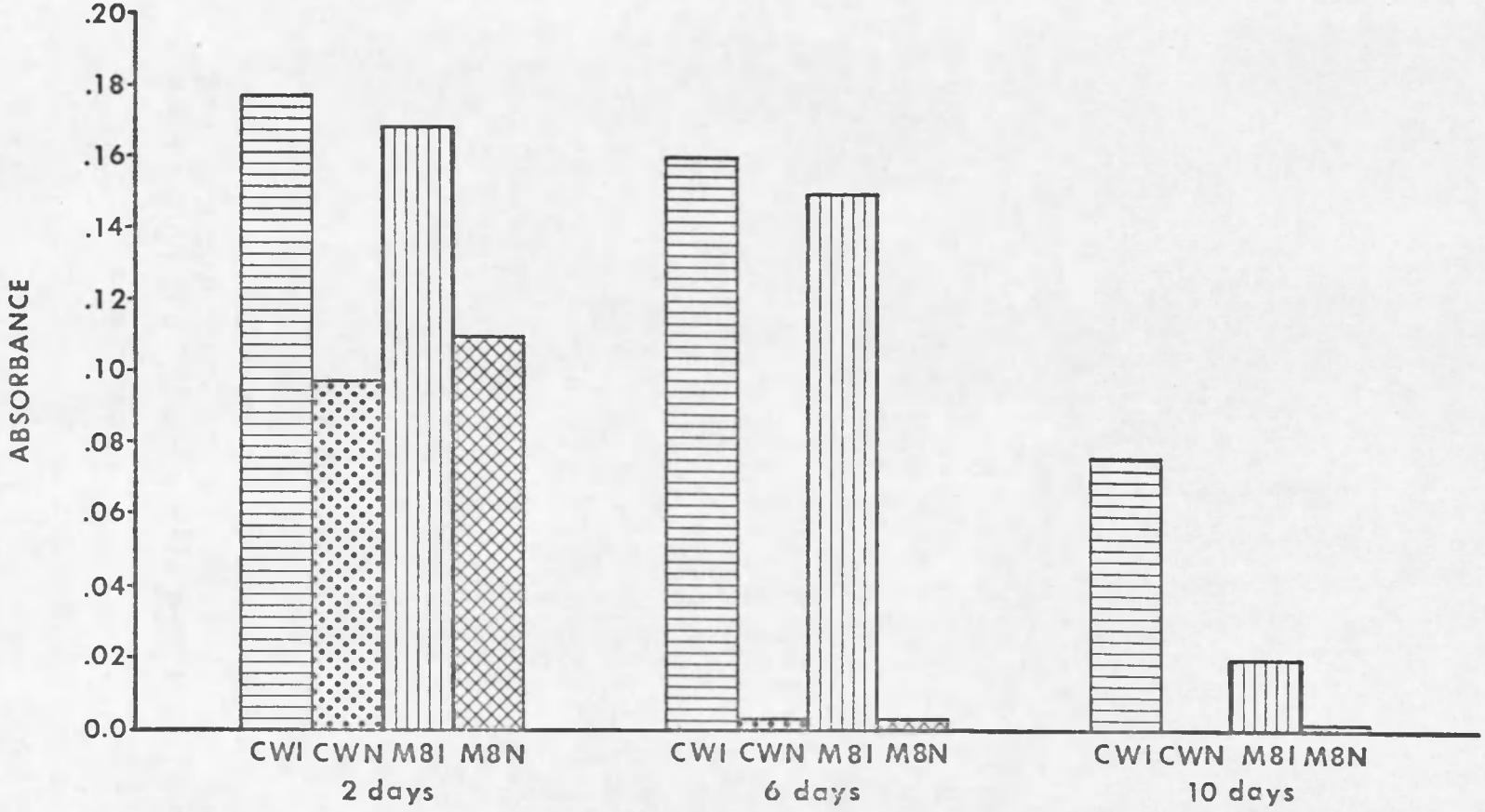

Fig. 14. Spectrophotometric determination of 6-phosphagluconate dehydrogenase activity 2, 6, and 10 days after inoculation -- Absorbance was measured at 340 nm after 8 min. CWI = CW infected; CWN = CW non-infected; M8I = M8 infected; M8N = M8 non-infected.

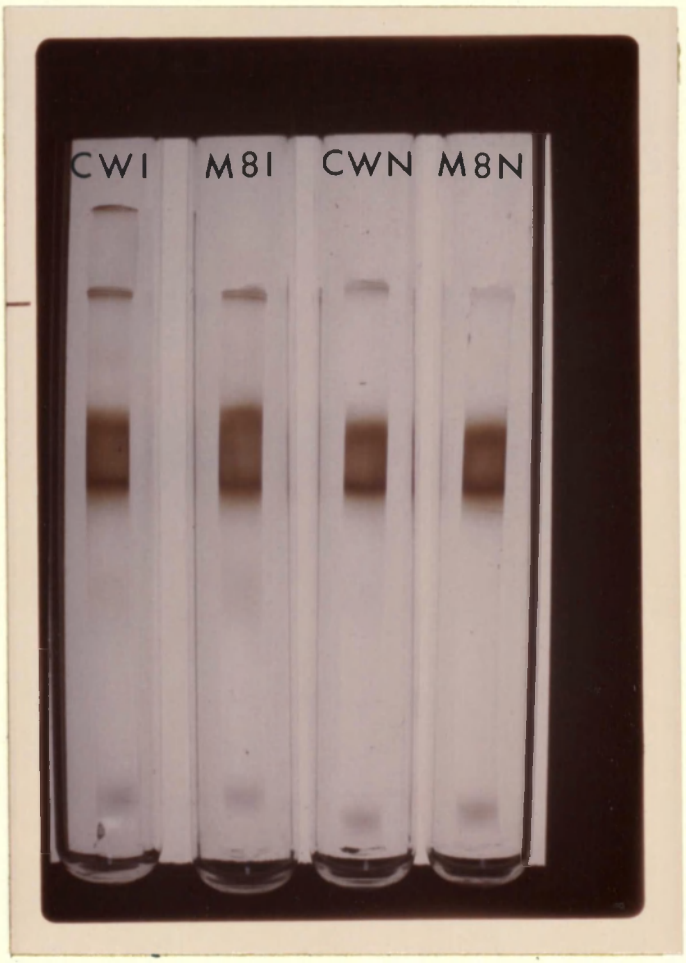

Fig. 15. Photograph of polyacrylamide gels stained for peroxidase activity of resistant and susceptible cotton non-infected and infected by M. incognita -- CWI = Clevewilt infected; CWN = Clevewilt non-infected; M8I = M8 infected; M8N = M8 non-infected.

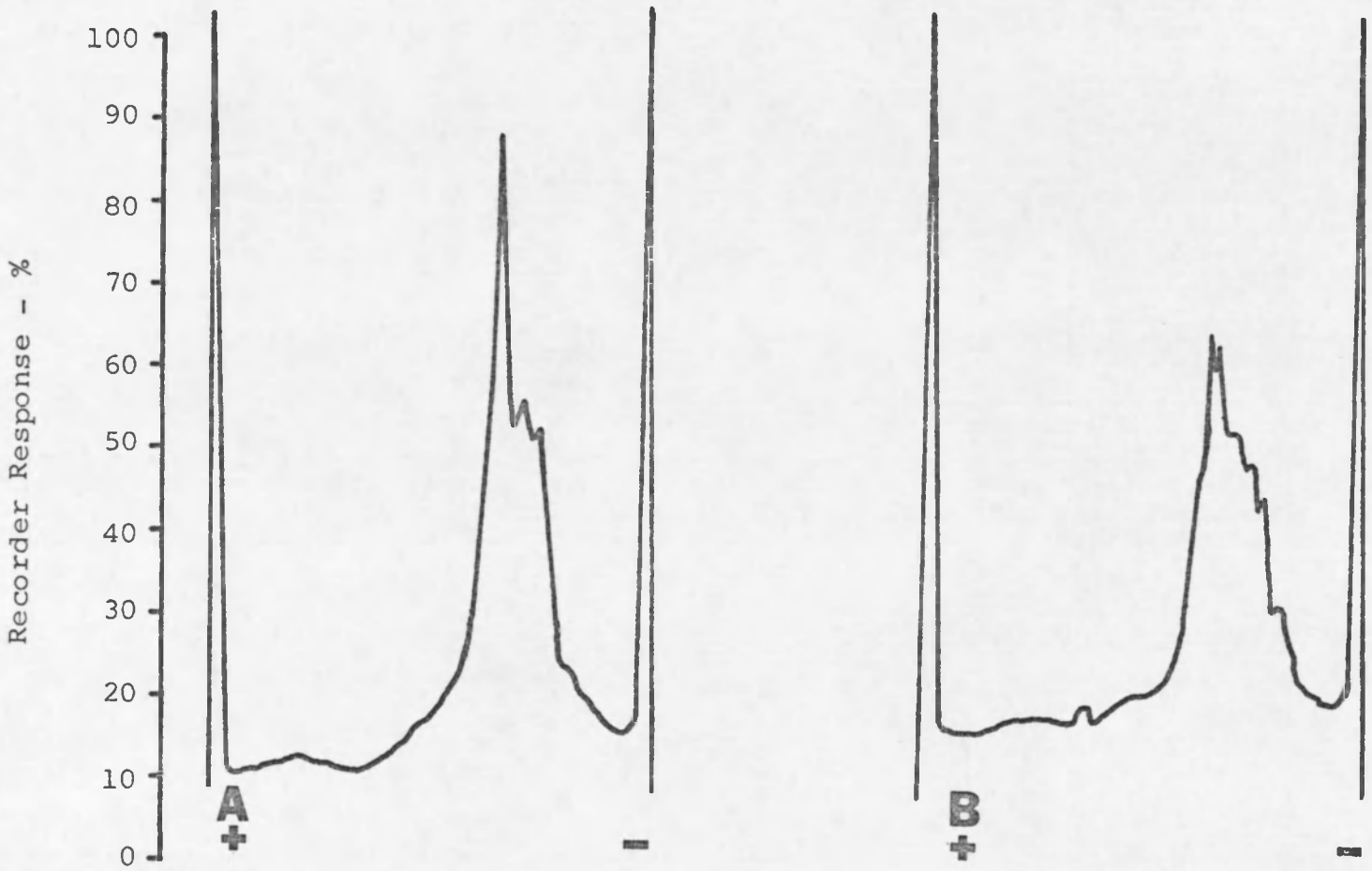

Fig. 16. Densitometric tracings of electrophoretic polyacrylamide gels stained for peroxidase activity; A) CW infected with M. incognita. B) M8 infected with M. incognita.

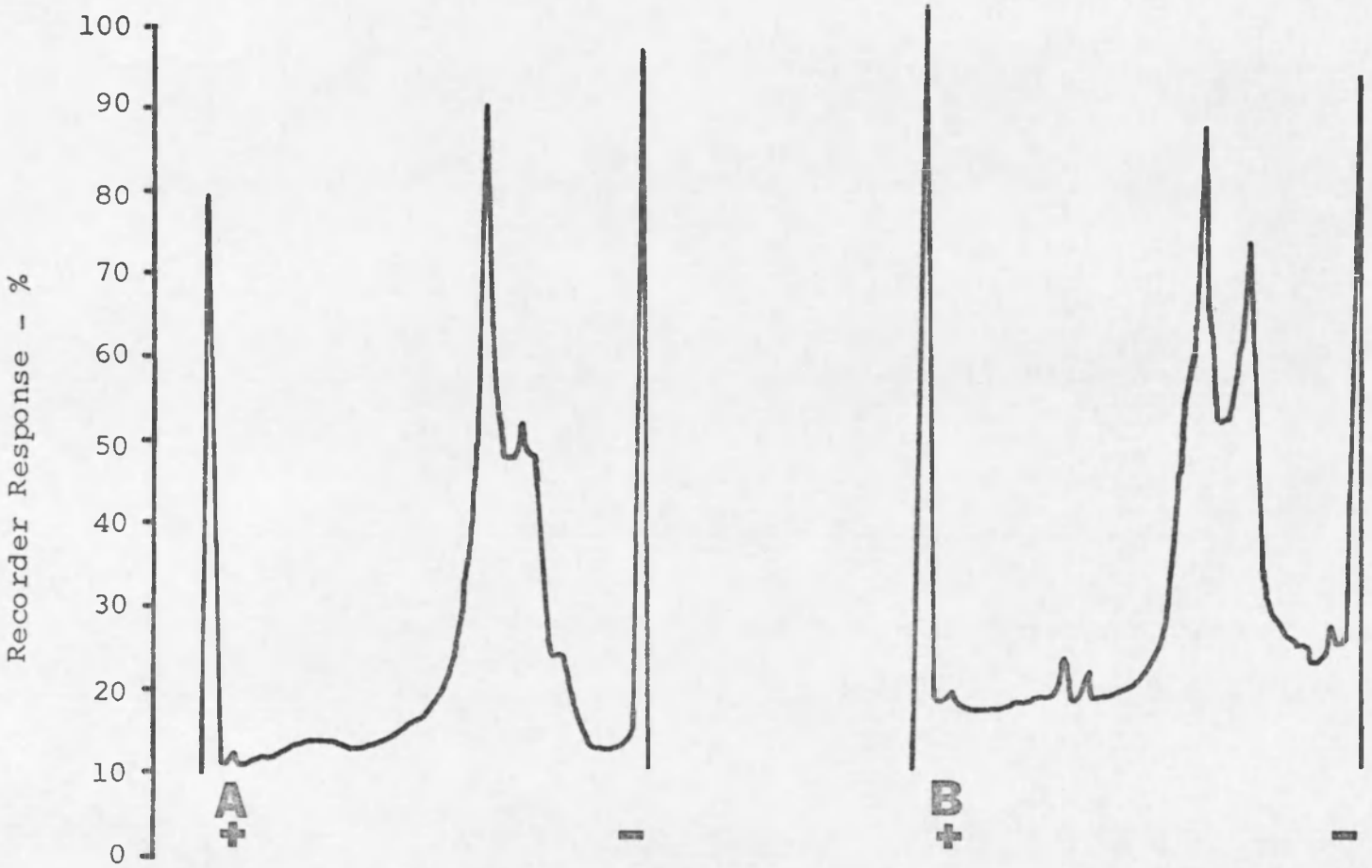

Fig. 17. Densitometric tracings of electrophoretic polyacrylamide gels stained for peroxidase activity; A) CW non-infected. B) M8 non-infected.

infected isoenzymes (Fig. 16). Densitometric readings of the M8 non-infected treatment indicated the greatest activity but the fewest indications of areas of density (Fig. 17). These data indicate a decrease in peroxidase but an increase in the number of isoenzymes in the infected M8 roots. Densitometric readings of CW infected and non-infected treatments indicated almost the same activity, but one area of density was found in the scan of the CW infected gel which was not present in the scan of the CW non-infected gel (Figs. 16, 17). Densitometric readings indicated that peroxidase activity in CW roots infected and non-infected was less than that in the M8 non-infected roots but greater than that in the M8 infected roots (Figs. 16, 17).

Acid Phosphatase

Densitometric readings and photographs of acid phosphatase activity indicated a reduction in activity in infected roots 6 days after inoculation when compared to non-infected roots of the same age (Figs. 18, 19, 20). CW non-infected roots exhibited greater acid phosphatase activity than did roots of the non-infected M8 (Fig. 20). The infected M8 had more activity than the infected CW (Fig. 19).

6-Phosphogluconate Dehydrogenase

Infected roots of both cultivars showed qualitative and quantitative differences in 6-PGDH isoenzymes when

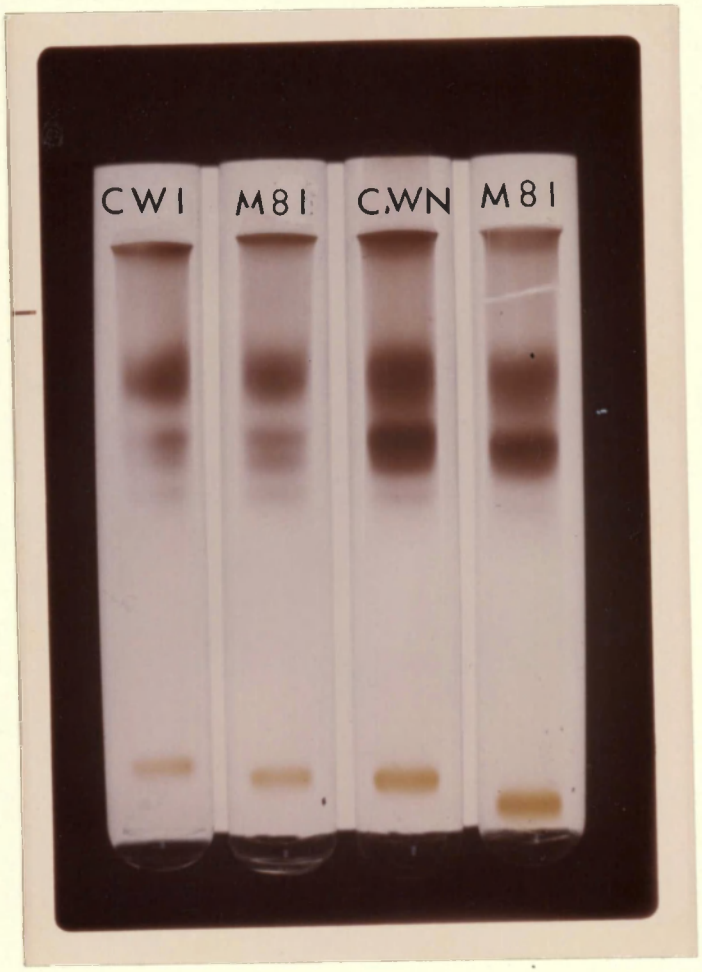

Fig. 18. Photograph of polyacrylamide gels stained for acid phosphatase activity of resistant and susceptible cotton non-infected and infected by M. incognita -- CWI = Clevewilt infected; CWN = Clevewilt non-infected; M8I = M8 infected; M8N = M8 non-infected.

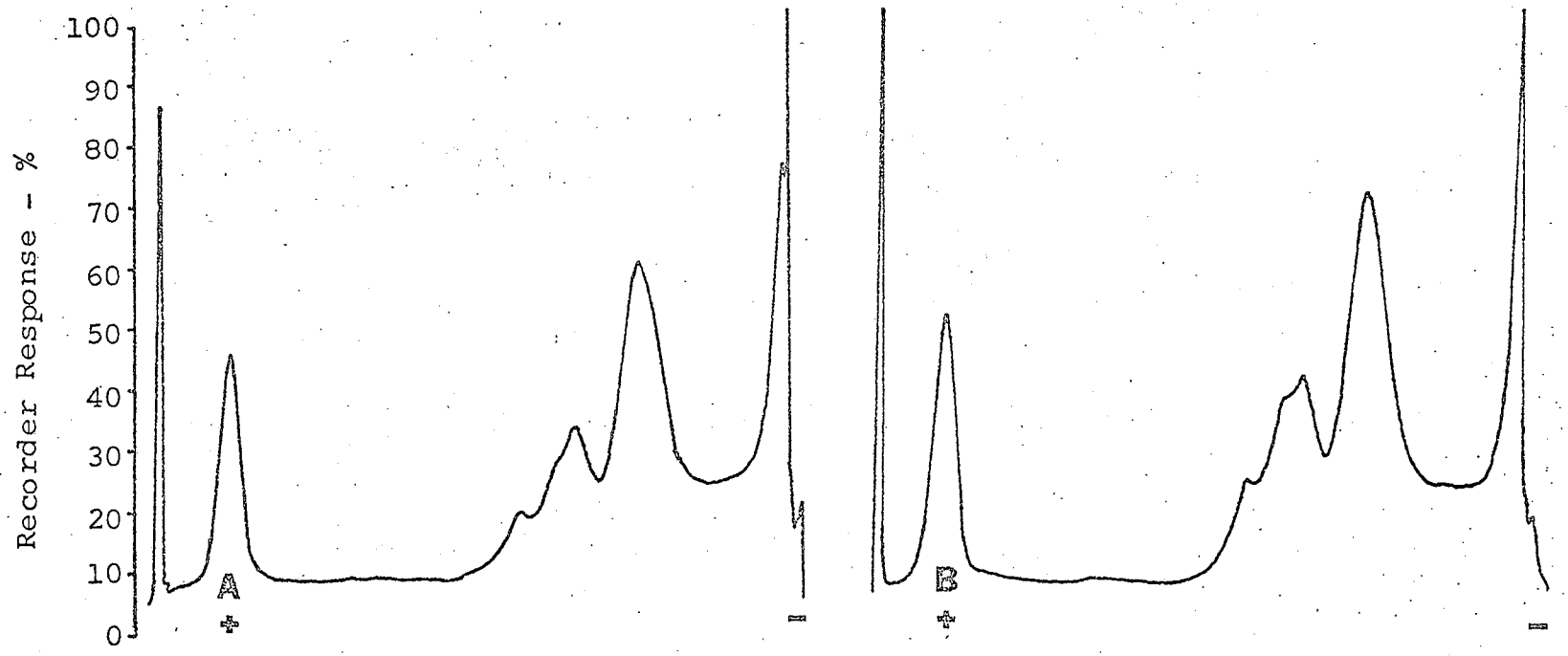

Fig. 19. Densitometric tracings of electrophoretic polyacrylamide gels stained for acid phosphatase activity; A) CW infected with M. incognita. B) M8 infected with M. incognita.

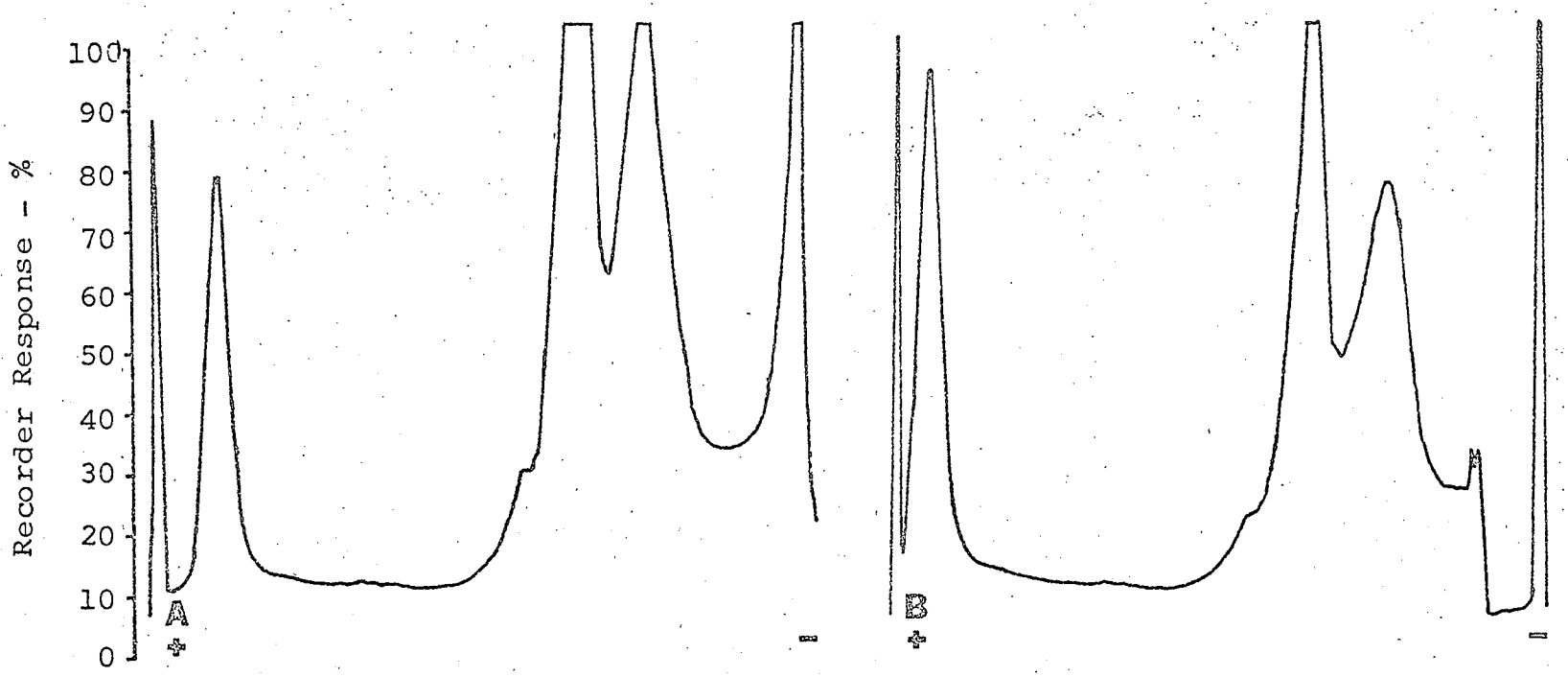

Fig. 20. Densitometric tracings of electrophoretic polyacrylamide gels stained for acid phosphatase activity; A) CW non-infected. B) M8 non-infected.

compared to the M8 non-infected 6 days after inoculation (Figs. 21, 22, 23). Non-infected CW was not available because of procedural difficulties. Densitometric scans and photographs of the gels of infected roots indicated the presence of an isoenzyme not found in non-infected M8 (Figs. 22, 23). The two sites of isoenzyme activity stained much darker in the CW than in the M8 infected (Fig. 21). The densitometric readings also indicated more activity of the 2 isoenzymes in the CW infected roots than in the M8 infected roots (Fig. 22). The shoulder on the smaller peak of the CW infected densitometric reading indicated a dense area not visible in the photograph (Figs. 21, 22).

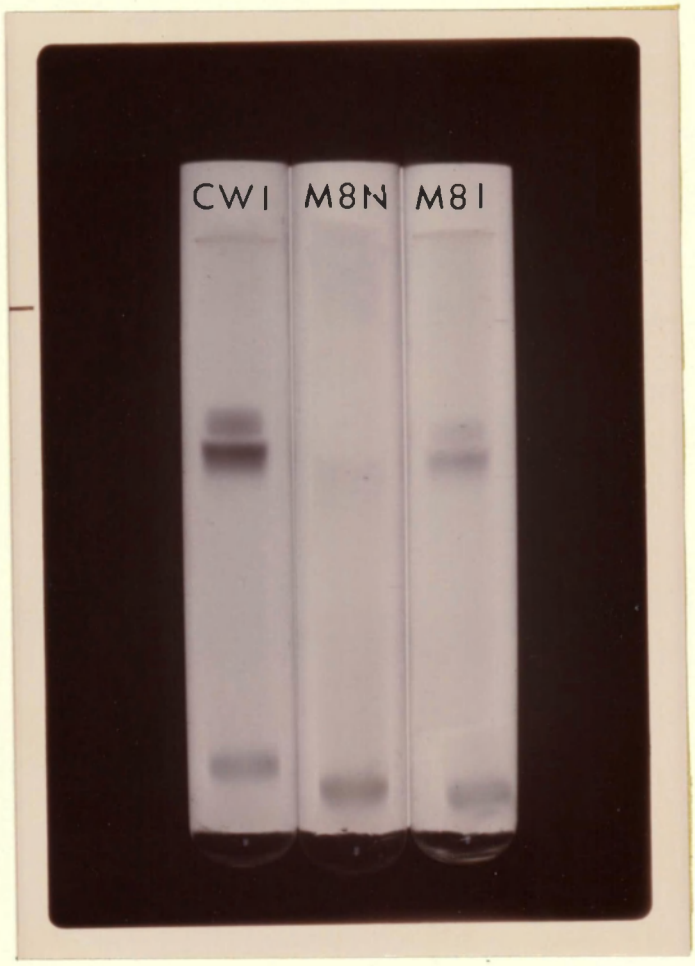

Fig. 21. Photograph of polyacrylamide gels stained for 6-phosphogluconate dehydrogenase activity of resistant and susceptible cotton non-infected and infected by M. incognita -- CWI = Clevewilt infected; CWN = Clevewilt non-infected; M8I = M8 infected; M8N = M8 non-infected.

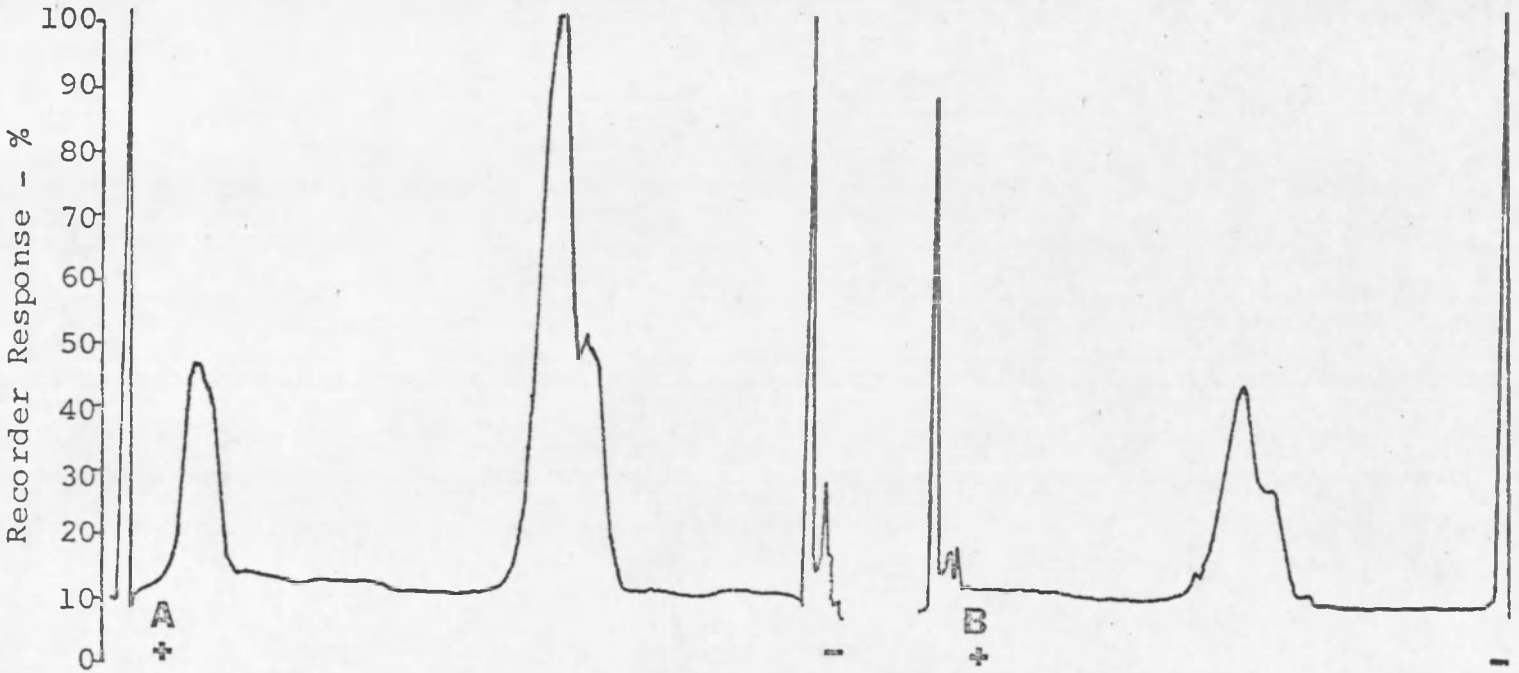

Fig. 22. Densitometric tracings of electrophoretic polyacrylamide gels stained for 6-phosphogluconate dehydrogenase activity -- A) CW infected with M. incognita. B) M8 infected with M. incognita.

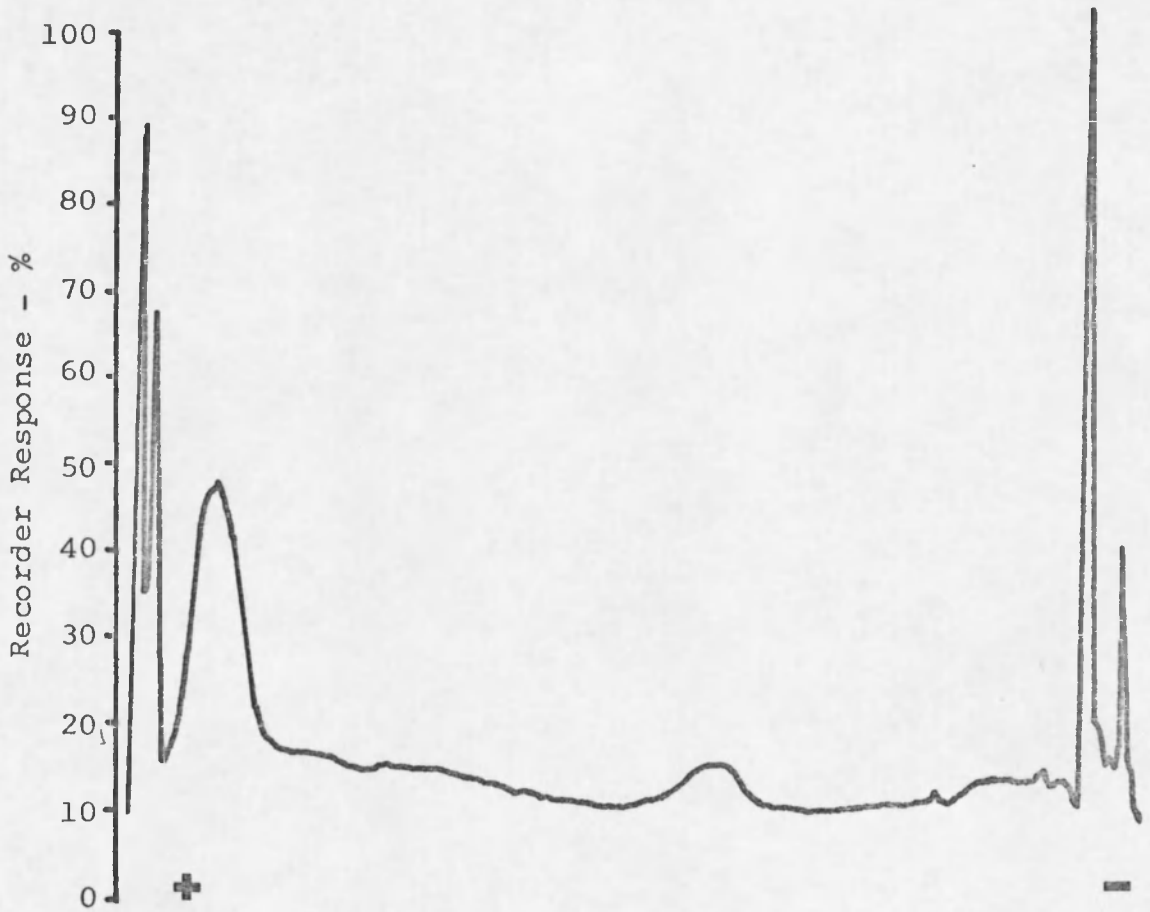

Fig. 23. Densitometric tracing of an electrophoretic polyacrylamide gel stained for 6-phosphogluconate activity in M8 non-infected roots.

DISCUSSION

This is the first report of the increased activities of enzymes at the feeding sites of cotton infected by M. incognita. The increase in activity was noted as early as 3 days after inoculation and was accompanied by the induction of syncytia. This work did not demonstrate differences in the activities of the enzymes localized at the feeding sites of M. incognita in the CW and M8 roots. Activities as indicated by chromophore formation were greater at all times in the infected roots of both cultivars when compared to the non-infected roots except for the proteinase activity. This study did confirm the earlier finding of Ellis (12) in which the development of nematodes in CW was retarded when compared to the development in M8. The gells without nematodes described previously by McClure et al. (31) also were observed. Associated with such galls was the discovery of nematodes undergoing lysis in the resistant Clevewilt.

Fixation of root material in the FAA and subsequent sectioning of the material mounted in segments of carrot root gave tissue with good cellular and nuclear detail. These methods were employed when the duplication of the methods of earlier workers (41) failed to yield sections in which lipid material had not been dissolved during fixation and alcohol dehydration for paraffin embedding. In contrast

to results published by Scheetz and Crittenden (41), the syncytial walls induced in cotton roots by M. incognita were not found to contain lipid materials. This observation is in agreement with the findings of other workers regarding the absence of lipid material in syncytial walls (2, 10, 37). The significance of the syncytial cytoplasm which stained intensely for lipid in the CW root can not be ascertained si ce only one was found in the course of several attempts. Although it could have been an artifact the condition of the nematode suggested a syncytium from which the nematode no longer fed. The occurrence of lipid material between the syncytium and the parenchyma tissue in the root may have been an artifact or could have been the beginning of a host response similar to that reported by Malo (28). More research is needed before this can be determined.

The lack of lignin in the syncytial cell wall and cytoplasm in both cultivars is in agreement with observations of root-knot induced syncytia in roots of other hosts (2, 10, 37).

The protuberances borne on syncytial walls of both the M8 and CW were found to contain pectin. This is in agreement with results obtained by other workers (2, 10, 37), but the protuberances of sweet potato, which most closely resemble in morphology those of cotton, did not stain positively for pectin (23).

A comparison of enzymatic activities by spectrophotometric and electrophoretic means indicated certain discrepancies in enzymatic activity of acid phosphatase, peroxidase, and 6-PGDH enzymes 6 days after inoculation. These discrepancies could be explained by the different methods used for sample preparation. Spectrophotometric measurements were based on samples of equal root length while electrophoretic separations were conducted on samples of equal protein content. Root-knot infected tissues are known to contain greater amounts of protein than comparable non-infected tissues (38) and the infected roots of both cultivars were heavier and larger in diameter than the non-infected roots with the M8 infected roots being quite larger and heavier than the CW infected roots. It would seem unlikely, therefore, that a comparison of enzymatic activity based on these 2 methods of sample preparation would result in the expression of equivalent activities.

The qualitative and quantitative increase in 6-PGDH in infected roots of both cultivars might indicate a shift in metabolism to the Pentose Phosphate Pathway (PPP). This method of glucose breakdown is known to supply NADPH, ribose-5-phosphate, and erythrose-4-phosphate in the non-infected plant (40). The production of one of these compounds might explain the increased activity in infected roots as well as the greater activity infected resistant

roots exhibit when compared to infected susceptible roots. Dwurazna and Weintraub (11) demonstrated a substantial shift in respiration of tobacco infected with potato virus X to the PPP. They found increased 6-PGDH and glucose-6-phosphate dehydrogenase activities spectrophotometrically and they reported a decrease in the C_6/C_1 ratio in infected tissues and the use of labeled glucose.

The decrease in AP activity in infected plants might also lend evidence to a metabolic shift to the PPP but the role of this enzyme in non-infected roots is not clear (27, 40). The high activity of the autoclaved controls might be due to the non-enzymatic oxidation of naphthyl derivatives which may occur naturally in roots.

As a result of the electrophoresis work, a peroxidase isoenzyme not found in the other treatments was visually demonstrated in the roots of infected M8. Several areas of greater density were also found by scanning spectrophotometrically. A decrease in activity of peroxidase in the M8 infected when compared to other treatments was also observed. An explanation of these observations might lie in he possible roles of indole compounds in root-knot infected tissues. Indole compounds have been shown to exist in higher concentrations in galls induced by root-knot nematodes (3, 46). Plant peroxidases are able to catalyze the oxidation of indole acetate (13). If indole concentrations could be shown to increase in infected M8 roots, then

the decrease in peroxidase activity might function in the increase of indole compounds since decreased peroxidase activity could indicate reduced oxidation of indole compounds. The greater peroxidase activity of CW infected roots when compared to M8 infected roots might function in the resistance of cotton to M. incognita by the oxidation of phenolic compounds (30); however, hypersensitive necrosis has not been observed in CW. The role of peroxidase enzymes and phenolic compounds in disease resistance of other plants to other pathogens is well established (16, 19).

Both qualitative and quantitative differences in enzymatic activities of CW and M8 infected and non-infected roots were observed. These differences suggest a shift to the PPP which might result in phenolic production and oxidation in the CW roots. Further studies utilizing labeled glucose for measurement of C_6/C_1 ratios and assays of the activities of other enzymes in the PPP would be useful. Assays of malate dehydrogenase and cytochrome oxidase should also be made in order to determine whether there is a general increase in respiration involving the Kreb's Cycle or whether the increase is due primarily to a shift to the PPP. Other useful work would be to compare the activity of polyphenoloxidase enzymes such as tyrosinase in infected and non-infected roots of both cultivars, since this would provide insight to the role of phenolics in the resistance of CW to M. incognita.

LITERATURE CITED

1. Acedo, J. R., and R. A. Rohde. 1971. Histochemical root pathology of Brassica oleracea capitatal L. infected by Pratylenchus penetrans (Cobb) Filipjev and Schuurmans Stekhoven (Nematoda: Tylenchidae). J. Nematol. 3:62-69.

2. Bird, A. F. 1961. The ultrastructure and histochemistry of a nematode-induced giant cell. J. Biophys. Biochem. Cytol. 11:701-715.

3. Bird, A. F. 1962. The inducement of giant cells by Meloidogyne javanica. Nematalogica 8:1-10.

4. Brodie, B. B., L. A. Brinkerhoff, and F. Ben Struble. 1960. Resistance to the root-knot nematode Meloidogyn incognita acrita in upland cotton seedlings. Phytopathology 50:673-677.

5. Chapman, R. A., and M. J. Eason. 1969. A technique for studying penetration of roots of plants by endoparasitic nematodes. J. Nematol. 1:279-280.

6. Curtis, C. R., and R. K. Howell. 1971. Increases in peroxidase isoenzyme activity in bean leaves exposed to low doses of ozone. Phytopathology 61: 1306-1307.

7. Davis, B. J. 1964. Disc Electrophoresis II. Methods and application to human serum proteins. Ann. N. Y. Acad. Sci. 121:404-427.

8. Dean, J. L., and F. B. Struble. 1953. Resistance and susceptibility to root-knot nematodes in tomato and sweet potato. Phytopathology 43:250.

9. DeMott, H. E. 1965. Observations on the utilization of the hexose menophosphate pathway in nematose-infected roots of tomato. Ph. D. Dissertation. Univ. Virginia. 46p.

10. Dropkin, V. H., and P. E. Nelson. 1960. The histopathology of root-knot nematode infections in soybeans. Phytopathology 50:442-447.

11. Dwurazna, M. M., and M. Weintraub. 1969. Respiratory pathways of tobacco leaves infected with potato virus X. Can. J. Bot. 47:731-736.

12. Ellis, K. C. 1970. Factors influencing the resistance of cotton to the root-knot nematode Meloidogyne incognita. Ph. D. Dissertation. Univ. Arizona. 61p.

13. Endo, B. Y. 1968. Indoleacetate oxidase activity of horseradish and other plant peroxidase isoenzymes. Plant and Cell Physiol. 9:333-341.

14. Endo, B. Y. 1971. Syncytia in host-parasite relationships. In Plant Parasitic Nematodes. Vol. II. Academic Press, New York. 347p.

15. Endo, B. Y., and J. A. Veech. 1969. The histochemical localization of oxidoreductive enzymes of soybeans infected with the root-knot nematode Meloidogyne incognita acrita. Phytopathology 59:418-425.

16. Farkas, G. L., and Z. Király. . Role of phenolic compounds in the physiology of plant diseases and disease resistance. Phytopath. Z. 44:105-150.

17. Farkas, G. L., L. Loverkovich, and Z. Klement. 1963. Increased activity of glucose-6-P-dehydrogenase in tobacco leaves affected by Pseudomenas tabaci. Naturwissenschaften 50:22-23.

18. Garner, D. L., G. W. Salisbury, and C. N. Graves. 1971. Electrophoretic fractionation of bovine acrosomal proteins and proteinases. Biol. Reprod. 4:93-100.

19. Goodman, R. N., Z. Király, and M. Zaitlin. 1967. The Biochemistry and Physiology of Infectious Plant Disease. Van Nostrand, Princeton, N. J. 354p.

20. Huang, C. S., L. H. Lin, and S. P. Huang. 1971. Changes in peroxidase isoenzymes in tomato galls induced by Meloidogyne incognita. Nematologica 17: 460-466.

21. Hussey, R. S., and L. T. Krusberg. 1970. Histopathology of and oxidative enzyme patterns in Wando peas infected with two populations of Ditylenchus dipsaci. Phytopathology 60:1818-1825.

22. Jensen, W. A. 1962. Botanical Histochemistry. W. H. Freeman and Co., San Francisco. 408p.

23. Krusberg, L. R., and L. W. Nielsen. 1958. Pathogenesis of root-knot nematodes to the Puerto Rico variety of sweetpotato. Phytopathology 48:30-39.

24. Loebenstein, G., and N. Linsey. 1961. Peroxidase activity in virus infected sweet potatoes. Phytopathology 51:533-537.

25. Lownsbery, B. F., and D. R. Viglierchio. 1961. Importance of response of Meloidogyne hapla to an agent from germinating tomato seeds. Phytopathology 51:219-221.

26. Lowry, O. H., N. J. Rosenbrough, A. L. Furr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 193:465-471.

27. Mahler, H. R., and E. H. Cordes. 1966. Biological Chemistry. Harper and Row, Publishers, New York. 872p.

28. Malo, S. E. 1965. Histological studies on the nature of resistance of "Okinawa" and "Nemaguard" peace root stocks to Meloidogyne javanica (Treub). Nematologica 11:43.

29. Maxwell, D. R., and D. F. Bateman. 1967. Changes in the activities of some exidases in extracts of Rhizoctania-infected bean hypocotyls in relation to lesion maturation. Phytopathology 57:132-136.

30. McClure, M. A. 1972. Personal communication. Professor of Plant Pathology, Univ. of Arizona.

31. McClure, M. A., K. C. Ellis, and E. L. Nigh. 1972. Resistance of cotton to the root-knot nematode, Meloidogyne incognita. J. Nematol. In press.

32. Meister, C. W. 1972. Enzyme composition of virus infected citrus tissues. Ph. D. Dissertation. Univ. Arizona. 112p.

33. Meister, C. W., R. M. Allen, and R. L. Caldwell. 1971. An electrophoretic examination of selected isoenzymes in stubborn diseased citrus. Phytopathology 61:903.

34. Minton, N. A. 1962. Factors influencing resistance of cotton to root-knot nematodes (Meloidogyne spp.). Phytopathology 52:272-278.

35. Myuge, S. G. 1956. The nutritional physiology of the gall nematode (Meloidogyne incognita). Dokl. Akad. Nauk SSSR 108:164-165.

36. Myuge, S. G. 1964. Plant parasitic nematodes. Feeding of Phytohelminths and their relationships with plants. Izdatel'stvo Kolos, Moskva. 47p.

37. Owens, R. G., and R. F. Bottino. 1966. Changes in host cell wall composition induced by root-knot nematodes. Contrib. Boyce Thompson Inst. 23:171-180.

38. Owens, R. G., and H. W. Specht. 1966. Biochemical alterations induced in host tissues by root-knot nematodes. Contrib. Boyce Thompson Inst. 23:181-198.

39. Riggs, R. D., and N. N. Winstead. 1959. Studies on resistance in tomato to root-knot nematodes and on the occurrence of pathogenic biotypes. Phytopathology 49:716-724.

40. Salisbury, F. B., and C. Ross. 1969. Plant Physiology. Wadsworth Publishing Co., Inc., Belmont, California. 747p.

41. Scheetz, R. W., and H. W. Crittenden. 1971. Histochemistry of resistant and susceptible soybean roots infected with the root-knot nematode Meloidogyne incognita acrita. Del. Agr. Expt. Sta. Bull. 384.

42. Shaw, C. R., and R. Prasad. 1970. Starch gel electrophoresis of enzymes--a compilation of recipes. Biochem. Genet. 4:297-320.

43. Solymosy, F., and G. L. Farkas. 1963. Metabolic characteristics at the enzymatic level of tobacco tissues exhibiting localized acquired resistance to viral infection. Virology 21:210-221.

44. Veech, J. A. 1969. Localization of peroxidase in infected tobaccos susceptible and resistant to black shank. Phytopathology 59:566-571.

45. Veech, J. A., and B. Y. Endo. 1970. Comparative morphology and enzyme histochemistry in root-knot resistant and susceptible soybeans. Phytopathology 60:886-902.

46. Yu, P. K., and D. R. Viglierchio. 1964. Plant growth substances and parasitic nematodes. I. Root-knot nematodes and tomato. Exptl. Parasit. 15:242-248.