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Research Article

HISTOCHEMICAL LOCALIZATION OF CARBOHYDRATE IN HEALTHY AND *MELOIDOGYNE INCOGNITA*, INFECTED OKRA (*ABELMOSCHUS ESCULENTUS* (L.) MOENCH)

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ABSTRACT
Amongst the best known plant parasitic nematodes are the root knot nematodes, belonging to the genus *Meloidogyne*, which have a wide host range and are widely prevalent all over the world. These produce conspicuous galls on the roots and the infestations can be recognized easily in fields. It is very common and abundant in the sandy soils of Jaipur and adjoining areas. In Jaipur district, fields of okra an economically important vegetable crop were found infested with highly pathogenic *Meloidogyne incognita*. Histochemical tests were applied to localize total insoluble polysaccharides and cellulose in the diseased and healthy root tissues.

***Key Words*:** *Meloidogyne Incognita, Histochemical Localization, Root Knot Nematode, Okra, Metabolites*

INTRODUCTION
It is an accepted fact that pest including nematodes, can bring about disruption in the physiological equilibrium of the attacked plants. The plants, in turn, react in a number of ways to off-set these disturbances and the overall plant response determines, to a large extent, the success or failure of the interactions. For a better understanding of various histopathological changes that occurred as a result of nematode infection, *in situ* localization of various metabolites was helpful. Although, some pathogens used mechanical force to penetrate plant tissues but subsequent development of disease syndrome was dependent on histochemical alterations and biochemical reactions taking place between substances secreted by the pathogens and those already present or produced by the host as a response to the infection. Histochemical techniques were advantageous as they enabled *in situ* localization of various metabolites at the site of their synthesis or action. While information is available on morphological and biochemical changes that occur in plants invaded by endoparasitic nematodes, little work has been done with a histochemical technique. Since the anatomical area that is biochemically affected by such nematode infections may be quite small, it is imperative that histochemical techniques be employed, lest the effects of infection be lost by dilution from non-affected cells.

After histochemically studying the soybean roots infected by *Meloidogyne* sp., it was reported that giant cell walls contained cellulose and pectin but lacked lignin, suberin, starch or ninhydrin positive substances (Dropkin and Nelson, 1960). It was observed that *M. javanica* induced giant cell in tomato contained traces of carbohydrates and fats but it was particularly rich in protein and RNA. The large irregularly shaped nuclei contained a large nucleolus and a number of feulgen-positive bodies, scattered irregularly along the nuclear envelope (Bird, 1961).

It was reported that in ginger infected with root-knot nematode, giant cell nuclei and cytoplasm were rich in nucleic acids. Starch was absent in the giant cells and in the cells of the infected region of the rhizome. Giant cells showed the presence of minute protein granules. The outer side of the egg sac consisted of a thick layer of insoluble polysaccharides (Shah and Raju, 1977).

The nucleic acid changes in three tomato cultivars infected with *M. incognita* were estimated, the observations showed that the amount of DNA and RNA was higher in roots of inoculated plants as compared to healthy ones in all the three cultivars (Masood and Saxena, 1980).

In the present investigation histochemical tests were applied to localize total insoluble polysaccharides and cellulose in the diseased and healthy root tissues. It will prove helpful for a clear understanding of host parasite interaction.

MATERIALS AND METHODS

Raising of Seedlings

Seeds of okra (*Abelmoschus esculentus* L. moench) CV. Pusa sawani, highly susceptible to the root-knot disease, were used for experimental studies and histochemical localizations. The seeds were surface sterilized in 0.1% mercuric chloride for one minute and washed thrice with autoclaved distilled water. They were sown in autoclaved sandy-loam field soil. Fertilizer used as Hoagland's complete nutrient solution, 25 ml/pot once a week. Since okra was summer and rainy season crop, all the experiments were set between 15th March and 15th July at average temperature.

Preparation of Inoculum

The pure culture of *Meloidogyne incognita* Chitwood was maintained and multiplied on brinjal plants raised in autoclaved soil. Egg masses were isolated in sterile water and the eggs were allowed to incubate in a Baermann funnel for 48-72 hours. As the juveniles hatched out of the eggs, they passed through the double layers of tissue paper and collected in the tube below. The suspension was diluted with sterile water, stirred with a magnetic stirrer for obtaining a homogenous suspension; 5 ml of it contained the desired number of juvenils. Nematode inoculation was done when seedlings were 2 weeks old, by pipetting and pouring 5 ml of juvenile suspension in three holes made around the base of the seedlings, afterwards the holes were plugged with soil.

Nematode Counting

For counting the nematode one ml of juvenile suspension was pipette out in a multichambered nematode counting dish and the counting was done under a stereobinocular microscope. Mean of five such readings was taken and finally the total number of juveniles was calculated for the entire volume of the suspension.

Collection of Material

Seedlings of *Abelmoschus esculentus* L. moench were raised and inoculated as described earlier. For making histochemical studies infected and healthy plants were uprooted at an interval of a week upto 8 weeks after inoculation. Ninety days old plants were also uprooted and fixed in different fixatives.

Fixation

The material was fixed in following fixatives depending upon the metabolic product to be localized.
*(a)Formalin-acetic-alcohol (FAA):*70% ethyl alcohol – 60 ml ; Glacial acetic acid – 5 ml; Commercial formalin – 5 ml.

Adhesive

Haupt's adhesive (Jensen, 1962); Gelatin – 1 g; Distilled water-warm (90°C) – 100 ml; Mixture cooled at 30°C.
Glycerine – 15 ml; Phenol crystals – 2 g.
Filtered and stored at low temperature.
Celloidin :
Celloidin – 0.5 g; Solvent ether – 50 ml; Absolute ethyl alcohol – 50 ml.

Processing of Tissues

The fixed material was dehydrated through tertiary butyl alcohol (TBA) series, infiltered and embedded in paraffin wax. Serial transverse and longitudinal sections of the embedded material were cut at a thickness of 12µrotary microtome and mounted on clean slides with the help of Haupt's adhesive. For control of different histochemical tests, sections of the same material were used, thereby reducing the sample variations to the minimum.

Histochemical Techniques

The various histochemical techniques used for localization of different cell constituents were as follows :
(1) Localization of Total Insoluble Polysaccharides:
They were localized by periodic acid Schiff's (PAS) technique (Jensen, 1962).

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Preparation of Stain (de Tomasi, 1936)

Dissolved 0.5g of basic fuchsin (C.I. 42525 E. Merck, Germany) in 80 ml of boiling water. Cooled to 50·C and to this 10 ml of INHCI and 0.5 g of potassium metabisulphite were added. This was bleached overnight in dark and 1.0 g activated charcoal was added. After thorough shaking the solution was filtered and stored in a brownish bottle in a refrigerator.

Staining Procedure

Material fixed in FAA was used.

(1) Deparaffmized the sections in xylene.
(2) Passed through a series of ethyl alcohol and brought down to water.
(3) Incubated in 0.5% periodic acid for 15 minutes.
(4) Washed in running water for 10 minutes.
(5) Stained in Schiff's reagent for 12 minutes.
(6) Rinsed in distilled water.
(7) Placed in 2% sodium bisulphite for 1.5 minutes.
(8) Washed in running water for 10 minutes.
(9) Dehydrated in 50, 70, 90 and 100% ethyl alcohol series (2 dips in each).
(10) Cleared in Xylene-2 changes.
(11) Mounted in DPX.

Effect

Insoluble Polysaccharide grains stained magenta.

Reaction

Strong oxidant like periodic acid produced aldehyde groups by oxidation of 1 and 2 glycol ends which got selectively coupled and stained with leucobasic fuchsin in Schiff's reagent producing magenta colour. Omission of this step, therefore, did not provide aldehyde groups to stain with Schiff's reagent. Cell wall and starch took a deep stain, however, the intensity of magenta colour, when present in the cytoplasm, was directly proportional to the concentration of polysaccharide.

Control

(A) Acetylation:

(1) Dewaxed and hydrated sections were acetylated in a mixture of acetic anhydride and pyridine in 2:3 volumetric proportions, at 60°C for 18 hours.
(2) The tissue was washed in tap water and rinsed in distilled water.
(3) Followed the steps of regular PAS staining.

Effect - No staining.

Rationale

Acetylating the hydroxyl groups of polysaccharide rendered them inactive towards periodate oxidation.

(B) Omitting Periodate Oxidation:

Omission of the oxidation in periodic acid from PAS technique was used as a control.

Effect - No staining.

Localization of Cellulose

Cellulose was localized by IKI-H_2SO_4 method (Johansen, 1940).

Preparation of stain:

Added 1 g Iodine and 3 g potassium Iodide in 300 ml distilled water.

Procedure :

Stained and mounted hand cut sections of fresh tissue in IKl. From the side of the coverslip, added a drop of 65% sulphuric acid and observed.

Effect:

Gradually walls containing cellulose stained blue while lignin stained orange to yellow.

Control :

No control preparation was made because of the well known specificity of this stain.

RESULTS

Total Insoluble Polysaccharides

Healthy Root:

In young and old healthy roots, PAS - positive granules were rarely observed in cells. However, the cell walls and phloem tissue showed an intense reaction (Figure 1.1).

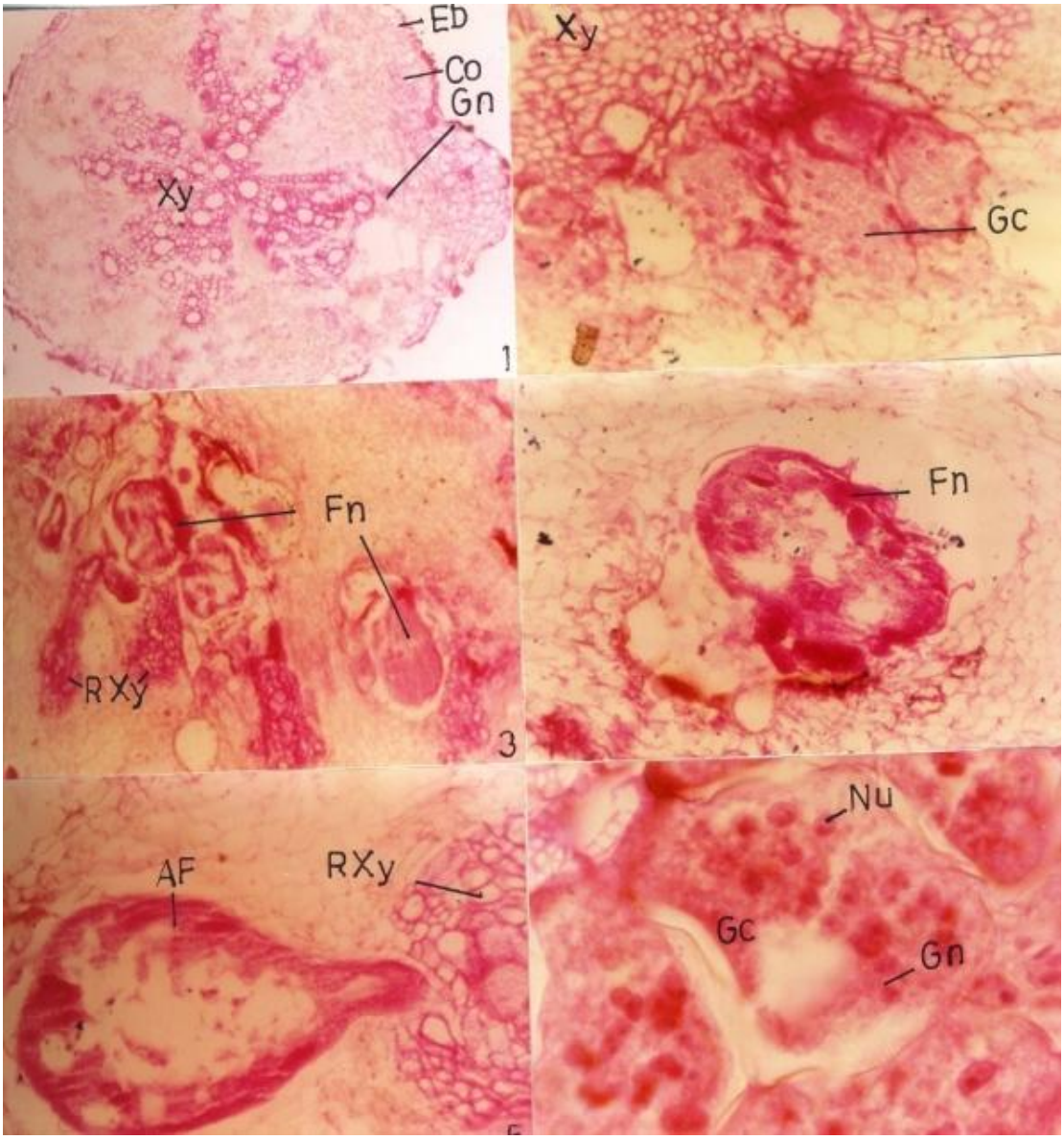

Figure 1: Localization of insoluble polysaccharides in healthy and *Meloidogyne incognita* infected *Abelmoschus esculentus* roots. (cv Pusa sawani). 1: T. S. healthy root a portion, P.A S. positive granules in the cell wall and phloem cells, showed intensive reaction. 40x. **2:** T. S. gall a portion, giant cell wall deeply stained, cytoplasm containing P.A.S. positive granules. 100 x. **3:** Same, nematode body and giant cells full of P.A.S. positive granules. 100x. **4:** Same, mature female full of P.A.S. positive granules.40x. **5:** Same, young female full of P.A.S. positive granules, reaction xylem elements stained nonspecifically 100x. **6:** Same, magnified giant cells, cytoplasm containing minute P.A.S. positive granules.40x

Figure 2: Localization of starch in healthy and *Meloidogyne incognita* infected *Abelmoschus esculentus* (cv Pusa sawani) roots. 1-2: T.S. healthy root, a portion, parenchymatous cells (phloem, pith and medullary rays)full of bluish black starch grains.40x. **3-4:** T.S. gall a portion, phloem cells, medullary ray cells and nematode body stained positively for starch .40x. **5-6:** Same, starch grains in parenchyma.400x

Gall:

PAS - positive granules were not observed in galls but the cell walls stained more 1ntensely than in healthy roots. Giant cell walls stained strongly and the nematode also showed a dense amount of PAS - positive granules (Figure 1.2-5, Table 1). The cytoplasm, nuclei and nucleoli of the giant cells were feebly stained (Figure 1.2, 1.6).

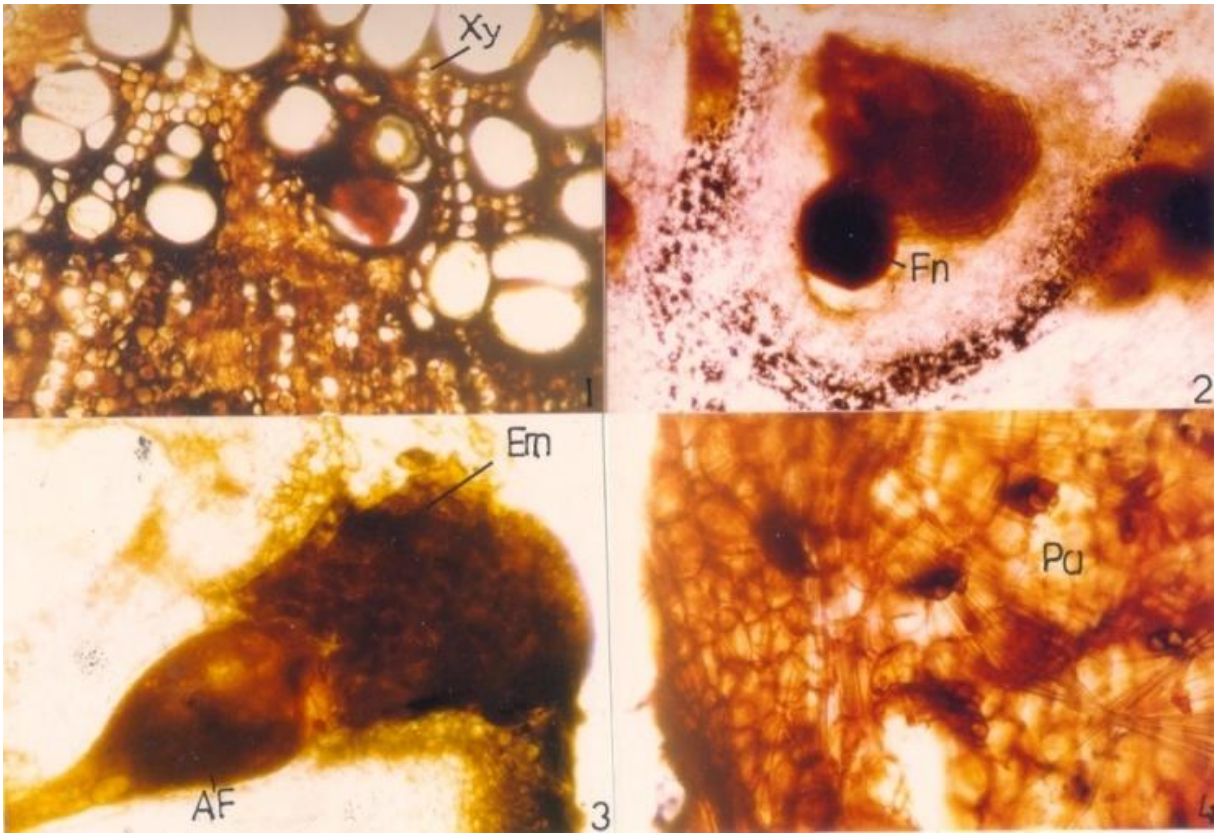

Figure 9: CELLULOSE localization of cellulose in healthy and *Meloidogyne incognita* infected *Abelmoschus esculentus* (cv Pusa sawani) roots. 1: T.S. healthy root , parenchyma cells lignified tissues stained positively for cellulose .100x. **2:** T.S. gall a portion , nematode and giant cell stained positively for cellulose. 100x. **3:** Same, egg laying female stained positively for cellulose .100x. **4:** Same, parenchymatous cells stained positively for cellulose .100x

The giant cells in association with L_2S or L_3S contained few grains. However, as the nematode reached maturity, associated giant cells showed thickening of walls due to PAS-positive substances. Fewer granules were seen in degenerating giant cells. Thick walls of hyperplastic cells took a darker stain.
The sedentary nematodes showed a gradual increase in number of PAS-positive granules reaching a maximum in ovi positing females (Figure 1.5) and in eggs. The gelatinous matrix enclosing eggs stained positively for PAS.
Control:
Insoluble polysaccharide grains were unstained

Starch
Healthy Roots:
Bluish black starch grains were observed with the IKI - test and were localized in the parenchyma; mainly in the phloem region, cells of the medullary rays and pith (Figure 2.1, 2.2, 2.4).
Gall:
Nematodes and eggs were rich in starch. Parenchyma cells surrounding the nematode cavity had abundant starch granules (Figure 2.3, 2.5, 2.6).

Cellulose

Healthy Root:

The walls of parenchyma cells in the root stained a deep violet blue with IKI – H_2SO_4 test and the lignified tissues of the xylem took a yellow stain (Figure 9.1).

Gall:

The giant cell wall, walls of parenchyma cells surrounding the reaction xylem and giant cells and those lining the cavity formed enclosing the nematodes or eggs stained positively for cellulose (Figure 9.2-4). The reaction xylem elements stained yellowish which was indicative of lignin (Figure 9.2).

DISCUSSION

The results revealed that excepting starch and carbohydrate, all other metabolites viz., proteins, nucleic acid, lipids, lignin, cellulose and ascorbic acid were found to healthy roots.

Carbohydrate

Syncytial walls with its protuberances stained strongly with PAS reagent in *M. incognita* infected okra roots. Giant cell wall contained all the usual polysaccharide , contents of cell wall except lignin. The walls of degenerating syncytia stained feebly indicating a change in its chemical composition.

The sedentary nematodes showed a higher level of insoluble polysaccharides, maximum being in ovipositing females. Bird and Saurer (1967) reported that within 2 or 3 days of entering the host, sub-ventral oesophageal gland became strongly PAS-positive and consisted of neutral mucopolysaccharide whereas in pre-parasitic larva, only the posterior region gave PAS-positive reaction. The eggs and egg matrix, produced by females in okra plants, were found to be rich in insoluble polysaccharides as observed. Bird and Rogers, (1965) observed that the cells of the ovipositing females which produced gelatinous matrix contained acid mucopolysaccharides.

Histochemically starch, as tested by IKI technique, was lower in diseased roots as compared to healthy ones. The giant cells and the cells around them did not contain starch. Absence of starch in giant cells and cells around them might be due to secretion of amylase by nematode that led to the hydrolysis of starch. It seemed that soluble polysaccharide produce d by the hydrolysis of starch were absorbed by the giant cells. They were probably further broke down to monosaccharides or still smaller molecules. These compounds were finally drawn by the nematode in their process of feeding. Increase in hydrolytic enzymes at the feeding site in root-knot susceptible and resistant soybeans was reported by Veech and Endo (1970).

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