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Research Article

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

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ABSTRACT
The root-knot nematode, *Meloidogyne incognita* is one of the most prevalent species of nematodes associated with unthriftiness of vegetables in India. Farmers experience chronic losses because of the high frequency of this nematode. 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 proteins and nucleic acids in the diseased and healthy root tissues. All the metabolites viz. DNA, RNA and proteins were found more in galled tissues as compared to healthy roots.

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

INTRODUCTION
Root-knot nematode, Meloidogyne spp act as a serious constraint to successful cultivation of various crops. It not only alters the normal anatomy, histochemistry and histophysiology of host but also induce changes in host-parasite interaction. 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, proteins, lipids, nucleic acids, lignins, cellulose and ascorbic acid 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 up to 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)Carnoy's Fluid***:** Absolute ethyl alcohol – 60 ml; Glacial acetic acid – 10 ml ; Chloroform – 30 ml Fixed at room temperature for 8 hours.
(b)10% neutral formalin: Commercial formalin – 10 ml; Distilled water – 30 ml. Fixed the material at 4°C for 24 hours, washed the tissue with running water for 24 hours to remove all traces of formalin.

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:

Localization of Total Proteins: Proteins were localized by mercuric bromophenol blue (MBB) method (Mazia *et al.,* 1953).

Preparation of Stain

Ten g of mercuric chloride was dissolved in 100 ml of 90% ethyl alcohol. To this l00mg of bromophenol blue (C.l. 20015 BDH, England) was added. This was filtered and stored.

Staining Procedure

Material fixed in neutral formalin was used.

(1) Deparaffinized the sections and brought down to water through a descending series of ethyl alcohol.
(2) Stained the sections in MBB for 15 minutes.
(3) Washed in 0.5% acetic acid for 20 minutes.
(4) Immersed in distilled water for 3 minutes.
(5) Dehydrated in 90% and absolute ethyl alcohol (2 dips each).
(6) Cleared in Xylene-2 changes.
(7) Mounted in DPX.

Effect – Proteins stained blue.

Control

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

Localization of Deoxyribosenucleic Acid (DNA)

DNA was localized by Feulgen method. Preparation of stain (Kallarackal, 1974) one g of basic fuchsin (C.I. 42525 E. Merck, Germany) was dissolved in 80 ml of boiling water. Cooled at 50° C and 10 ml of IN HCl and 2 g of Potassium metabisulphite were added. This was thoroughly mixed and kept in dark overnight. To this 1 g of activated charcoal was added to bleach the colour. This was mixed thoroughly, filtered into a brownish bottle and stored in a refrigerator.

Staining Procedure

Material fixed in Carnoy's fluid was used.

(1) Brought slides to 90% alcohol through a descending series of ethyl alcohol.
(2) Slides were dipped in a 0.5% celloidin solution. Celloidin coating was done to prevent the loss of sections during strong acid treatment. The celloidin solution was prepared by dissolving 0.5 g celloidin in 100 ml of alcohol-ether mixture (50 ml absolute ethyl alcohol added to 50 ml solvent ether).
(3) Allowed the slides to drain for a moment and placed in 90% ethyl alcohol to harden the celloidin.
(4) Brought down to water through 70% and 50% ethyl alcohol.
(5) Place in 5N HCl for 12 minutes at 35°C.
(6) Rinsed in distilled water.
(7) Stained for three hours.
(8) Rinsed in distilled water.
(9) Placed in 2% sodium bisulphite for 1.5 minutes.
(10) Washed in running water for 10 minutes.
(11) Dehydrated in descending ethyl alcohol series starting from 90% (2 dips in each).
(12) Cleared in Xylene (2 changes).
(13) Mounted in DPX.

Effect – DNA stained magenta.

Reaction

Warm Hel hydrolyses the purine-deoxyribose linkages, thus exposing free aldehyde groups. These groups formed a highly coloured complex with leucobasic fuchsin. The staining intensity depended on the concentration of DNA.

Control

Following treatments were given separately between steps 4 and 5 of the above staining schedule.

(A) Trichloroacetic acid extraction: Immersed the slides in 5% aqueous solution of trichloroacetic acid at 90°C for 15 minutes, washed three times, 10 minutes each in 70% ethanol, rinsed in distilled water.

(B) Perchloric acid extraction: Placed the slides in 10% aqueous solution of perchloric acid at 60°C for 15 minutes, neutralized by putting the slides in 1% sodium carbonate solution for 5 minutes. Washed in running water for 10 minutes and proceeded for staining (Erickson *et al.*, 1949).

Effect – Nuclei remained unstained.

Localization of Ribose Nucleic Acid (RNA)

RNA was localized by Pyronin-1staining (Tepper and Gifford, 1962).

Preparation of Stain

Two g of Pyronin^{-1} (C.I. 45005 E. Merck, Germany) was dissolved in 100 ml of distilled water. This was extracted in 20 ml lots in a separating funnel with 20 ml of chloroform. The extraction procedure was repeated thrice. All the stain solutions extracted by this procedure were pooled together and stored in a refrigerator.

Staining Procedure

Material fixed in Carnoy's fluid was used.

(l) Deparaffinized the slides and brought down the sections to water through a descending series of ethyl alcohol.

(2) Stained in Pyronin^{-1} for 2 minutes.

(3) Dipped thrice in distilled water.

(4) Back of the slides and uncovered areas were blotted with tissue paper.

(5) Placed in absolute ethyl alcohol-I for 30 seconds.

(6) Placed in absolute ethyl alcohol-II for 30 seconds.

(7) Placed in 1:1 ethyl alcohol and xylene for 30 seconds.

(8) Cleared in Xylene by giving two changes.

(9) Mounted in DPX.

Effect – RNA stained deep pink.

Reaction

Pyronin-1, being a basic cationic dye, bounded negatively charged phosphate anions of RNA molecule in aqueous solution. Property of cytoplasmic RNA to bind this basic dye was called pyroninophilia.

Control

(A) Trichloroacetic acid extraction: After deparaffinizing and hydrating the sections, placed the slides in 5% aqueous solution of trichloroacetic acid at 60° C for 10 minutes. Washed the slides in 70% ethanol three times, for 10 minutes each, followed by two rinses in distilled water. The slides were stained for RNA as usual.

Effect – Negative staining.

(*B) Perchloric acid treatment (Erickson et al., 1949):* After deparaffmizing the slides and hydrating the sections, placed the slides in.1N solution of perchloric acid at 4°C for 15 hours, neutralized by putting the slides in 1% sodium carbonate solution for 5 minutes and washed in running water for l0 minutes followed by usual pyronin^{-1} reaction.

Effect – No staining.

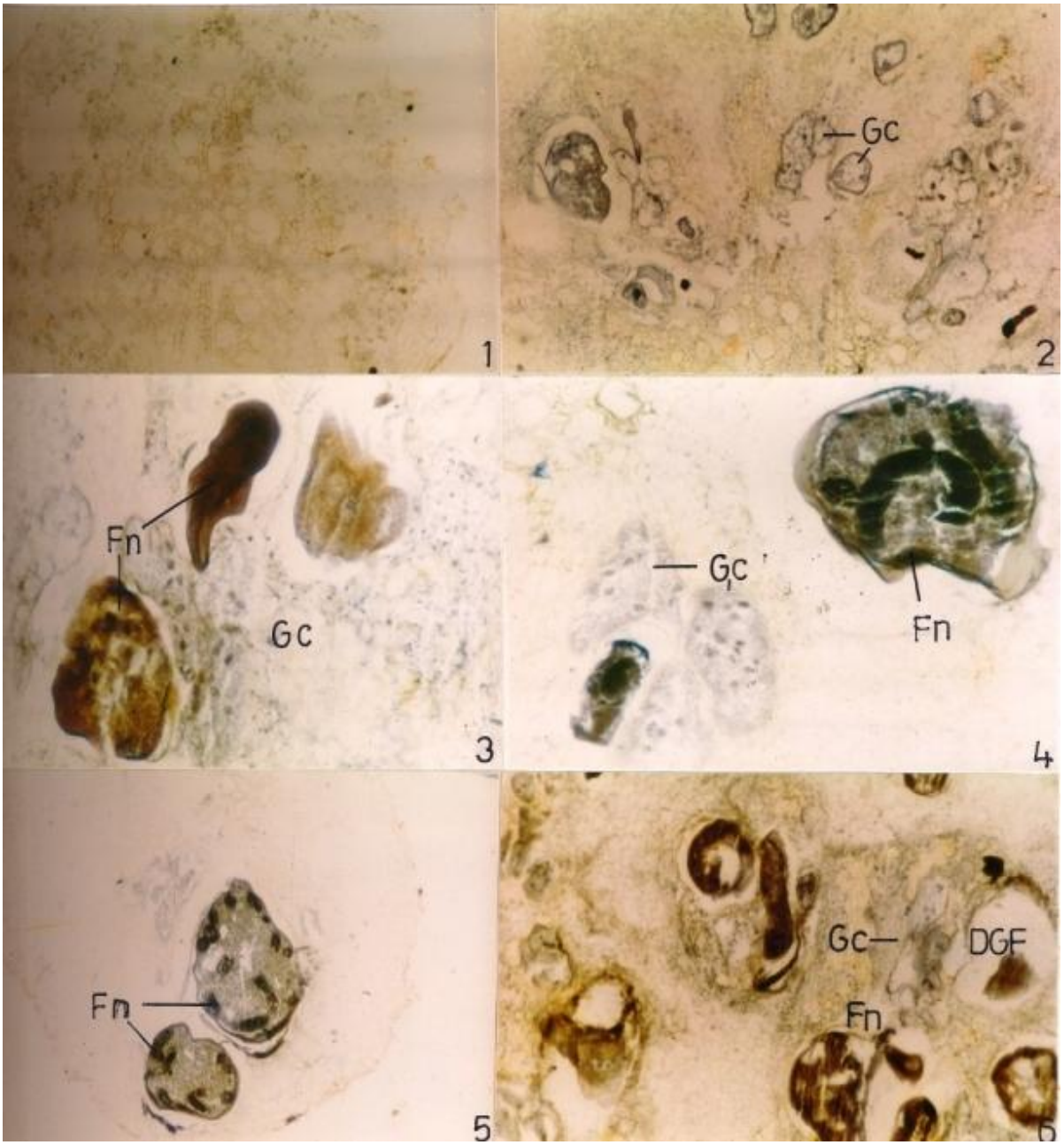
RESULTS

Total Proteins

Healthy Root: Cells showed a higher protein content in cytoplasm, nucleus and nucleolus. Phloem cells, cambial cells, lateral root primordia showed higher protein content than cortical parenchyma cells (Fig. 3.1).

Gall: Galls showed a higher amount of protein than healthy roots (Table 1). Proteins were abundant near the infection court. Nema bodies and eggs stained intensely for protein (Figure 1.3-6). Giant cells, nematode and hyperplastic parenchyma were the main sites of protein accumulation in galls (Figure 1.2-6). Giant cells showed a gradual increase of protein in their cytoplasm and nuclei, becoming maximum as nematode reached maturity.

All the developmental stages of the nematodes were rich in protein (Figure 1.3, 1.4). Egg matrix and egg masses showed a higher protein content. The parenchyma cells surrounding the giant cell complex and those lining the cavity containing female and egg mass showed high protein content.



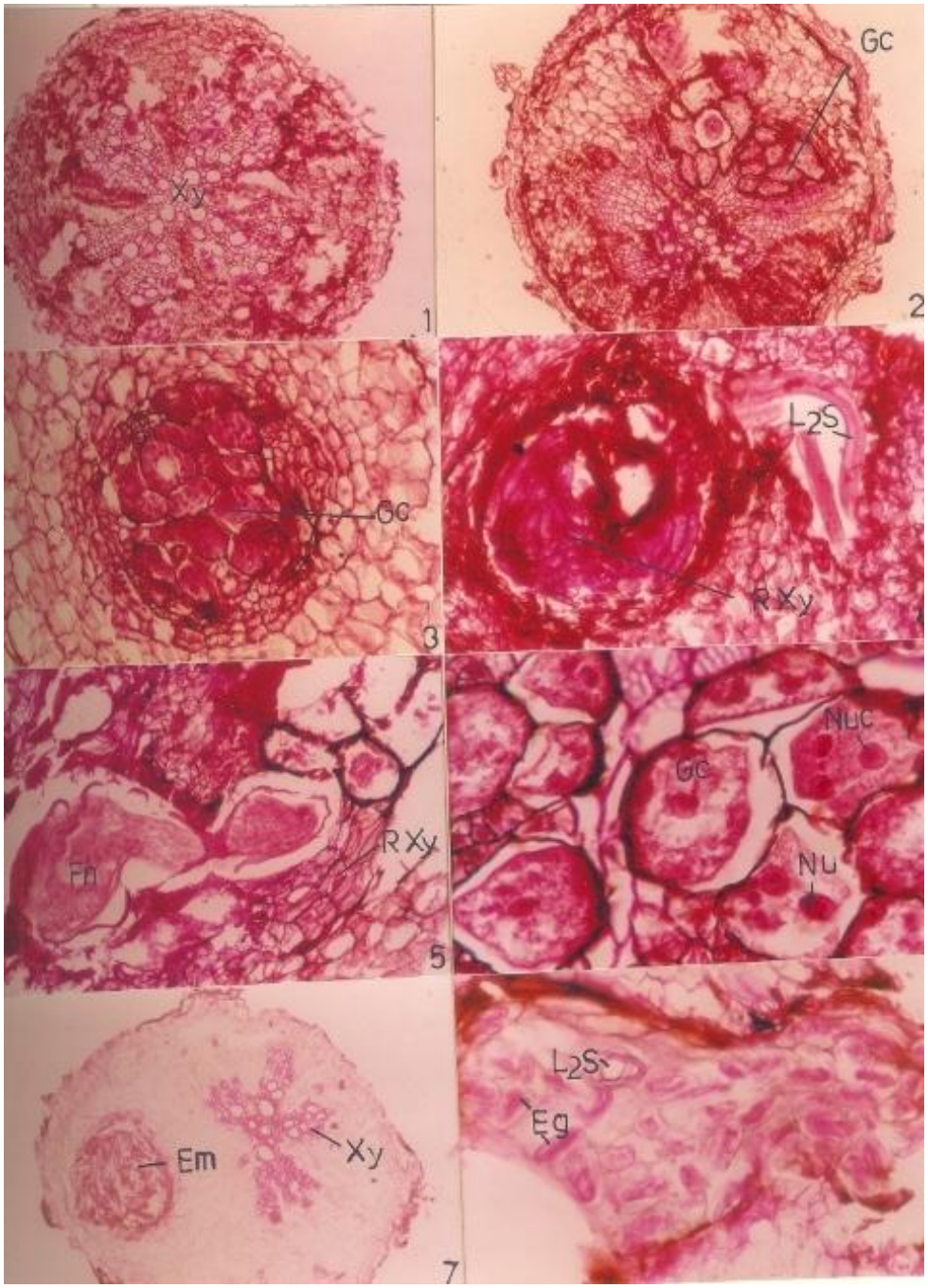


Figure 2: Localization of RNA in healthy and *Meloidogyne incognita* infected *Abelmoschus esculentus* (cv pusa sawani) roots. 1: T.S. healthy root, a portion,nuclei in the cells stained positively for RNA, xylem stained non-specifically 40x. **2:** T.S gall a, portion, RNA Localized of nuclei of giant cells, xylem stained non-specifically 40x. **3:** T.S. gall, a stele completely replaced by giant cell complex, nuclei of giant cell stained positively for R.N.A 100x. **4:** T.S. gall a. portion, nematode juvenile stained positively for R.N.A., reaction xylem stained non- specifically 100x. **5:** Same, female nematode stained positively for R.N.A, giant cell showing clumping of pyroniny positively nuclei.100x. **6:** Same, giant cell nuclei containing nucleoli stained positively for R.N.A 200x. **7:** T.S. gall, eggmass stained positively for DNA, xylem stained non- specifically. 40x. **8:** Same enlarged. 100x.

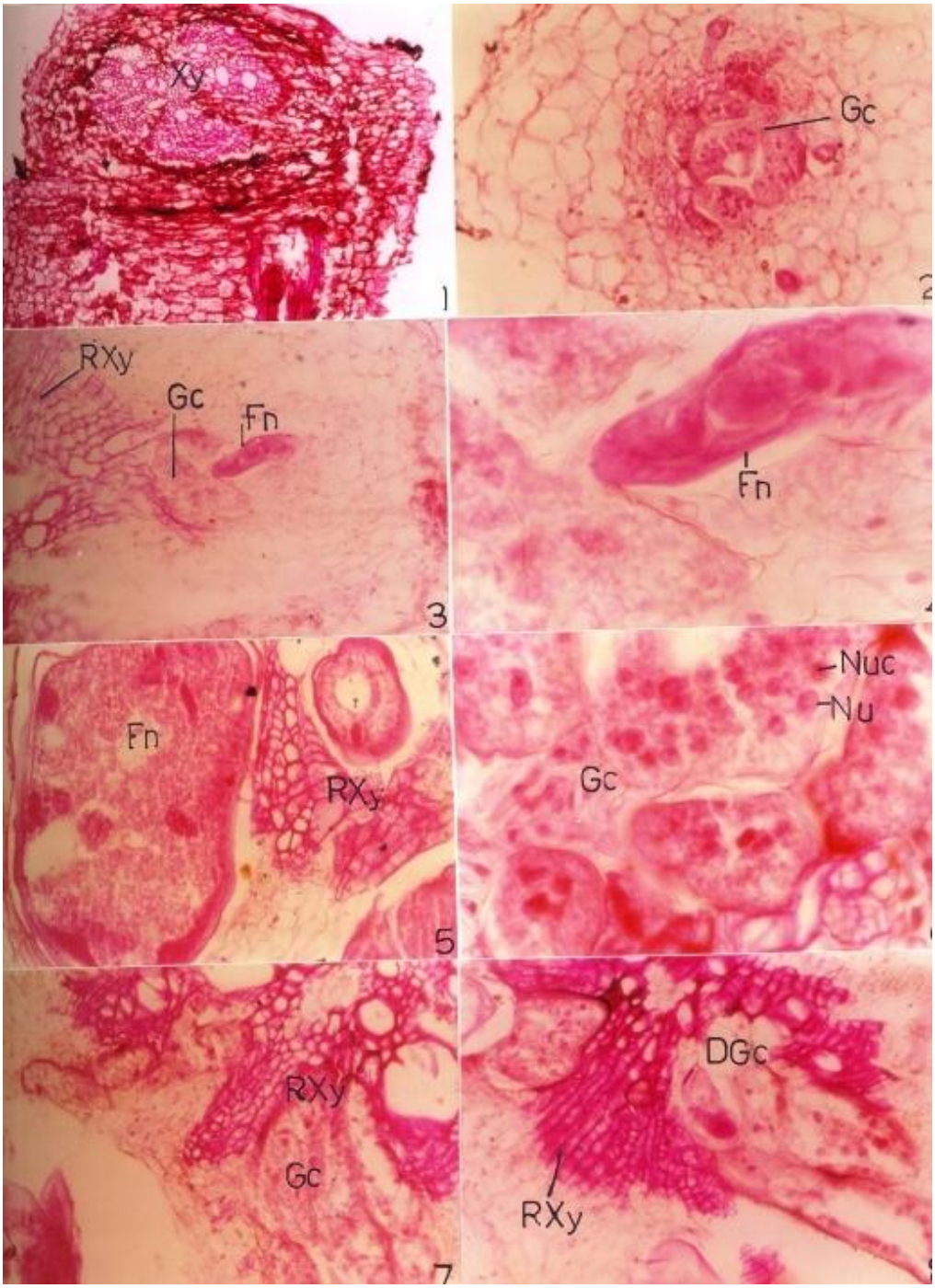


Figure 3: Localization of DNA in healthy and *Meloidogyne incognita* infected *Abelmoschus esculents* (cv Pusa sawani) roots. 1: T.S. healthy roots, the nuclei and cytoplasm stained positively for D.N.A., xylem elements stained non-specifically. 40x. **2:** T.S. gall, stele completely replaced by giant cell complex which stained positively. 100x. **3:** T.S. gall a portion, giant cells associated with third stage juvenile stained positively. 40x. **4:** Same, giant cell and nematode stained positively for DNA 200 x. **5:** Same nematode body stained positively for 100 x. **6:** Same DNA localized in nuclei of giant cell. 200 x. **7-8:** Same DNA localized in nuclei, reaction xylem stained non- specifically. 100x

Ribosenucleic Acid (RNA)

Healthy Root: Both nuclei and cytoplasm stained positively for RNA. The cell walls and xylem elements stained non-specifically. Nuclei of the cells of the lateral root primordia also stained strongly with pyronin Y (Figure 2.1).

Gall: RNA content was higher in galls than healthy roots (Table 1). The infection court was the main site for RNA localization. The giant cells and hypertrophied cells stained strongly (Figure. 2.2, 2.3, 2.6). All stages of nematodes also stained positively for RNA (Figure 2.4, 2.5).

The developing syncytium was rich in RNA and possessed hypertropied nuclei with large nucleoli (Fig. 4.6). The phloem and pericycle cells were also rich in RNA. More intense staining was observed in giant cells than the surrounding hypertrophied cells. Giant cells associated with mature stages of nematode showed more intense pyroninophilia than those associated with younger stages. Giant cells associated with mature females showed a prominently stained nucleolus, a granular cytoplasm and nucleoplasm which was uniformly stained. Walls of giant cells stained non-specifically.

Syncytium associated with dead or degenerating nematode showed less of RNA. Nuclei became smaller and nucleoli indistinct Cytoplasm was found to be less granular and vacuolate. The cell walls also stained feebly.

Control: RNA containing regions remained unstained.

Deoxyribosenucleic Acid (DNA)

Healthy Root: Nuclei in the root cells of all ages stained positive for DNA. Xylem stained non specifically. Cells of lateral root primordium showed large prominent Feulgen - positive nuclei (Figure 3).

Gall: The galled root had more DNA as compared to the healthy root (Table 1). Giant cell nuclei stained more intensely (Figure 3.2, 3.6-8). The hypertrophied parenchyma cells surrounding the giant cell complex also showed prominently stained nuclei. Walls and cytoplasm did not stain for DNA.

As the L_2S moulted, the syncytial nuclei showed clumping and fusion and a stronger staining by Feulgen reagent (Figure 3.3, 3.4). Syncytia, which were in contact with ovipositing females, contained smaller, lobed nuclei. The syncytial nuclei associated with spike - tail stage were maximum in size and multinucleolate. Besides, the positively stained nucleoli, small Feulgen - positive bodies were seen scattered throughout the nucleoplasm.

Giant cell walls, tracheidal elements and reaction xylem stained non-specifically. Nematode body of all stages stained feebly while eggs stained positive for DNA (Figure 3.7, 3.8).

Control: Nuclei remained unstained.

DISCUSSION

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

Total Proteins

The total protein content showed an increase in the infected roots as compared to the corresponding healthy roots. Increase in the protein content in the infected roots was also reported in tomato (Trivedi and Tiagi, 1980; Sharma *et al.*, 1989; and Bird 1974). Giant cells, nematode bodies and hyperplastic parenchyma around the giant cells were the main sites of protein accumulation. Rubinstein and Ovens, 1964) found a higher concentration of protein in giant cells and nema bodies in the root-knots of carrot and tomato, respectively. All stages of the nematodes stained positively for protein. Cuticle, eggs and eggmatrix showed a higher amount of protein supporting Bird and Rogers (1965) who reported cuticle to be lipoproteinaceous in nature. It was observed that the cells producing gelatinous matrix and the matrix itself stained positive for protein. Changes in carbohydrates and protein metabolism could be due to local nematode secretion of the waste products of their protein metabolism. This in turn caused an influx of free amino acid by host and also their local synthesis. However, according to Bird (1974) the histone-like

protein exuded by nematode egg production, needed, energy which was supplied by free protine, manufactured in the leaves and translocated to the site of nematode activity.

Nucleic Acids

Nucleic acid content of *M. incognita* infected okra galls increased as compared to the healthy roots of the same age. Such nematode induced nucleic acid increase was also reported (Ovens and Novotony, 1960; Shah and Raju, 1977 and Trivedi and Tiagi, 1980).

In galls, RNA and DNA were concentrated more in giant cells and the nema bodies. Kannan and Chandraguru (1981) reported 2-fold increase in nucleic acid content which accumulated in giant cells and nema bodies in *M. incognita* infected cucumber and tomato roots. Similarly, an increase in RNA by 87 per cent, DNA by 70 per cent and nucleotides by 2.9 per cent was reported galled tomato roots. The syncytial nuclei had 2-11 times more DNA and 4-11 times larger nuclear volume than those of normal cortical cells (Ovens and Novotony, 1960).

The syncytial nuclei associated with mature stages of the nematode were maximum in size and multinucleolate with small Feulgen-positive bodies scattered throughout the nucleoplasm in the present investigation. (Bird, 1961) also observed that *M. javanica* induced giant cells in tomato contained a large nucleolus in their nuclei and a number of feulgen positive bodies scattered irregularly along the nuclear envelope.

Giant cell initials around the head of L_2S were rich in RNA and possessed hypertrophied nuclei with large nucleoli. The amount of RNA was increased with the increasing age of giant cells. It was mainly localized in nucleoli, nucleoplasm and cytoplasm of giant cells, phloem and pericycle cells. It however, decreased when the giant cells degenerated. Walls of giant cells associated with L_3S stained intensely indicating RNA. Similar observations were made by Ovens and Novotony (1960), who reported that developing synctytium was a region of intense RNA and DNA biosynthesis. DNA synthesis was dependent upon a close association of the feeding nematode with the giant cell. Ovens and Novotony (1960) suggested that nematode feeding giant cells became a region of intense metabolic activity. They also reported that DNA synthesis was more in syncytium on which the nematode was feeding. This explained the phenomenon of differential staining of different giant cells of the same group.

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