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Research Article

HISTOCHEMICAL LOCALIZATION OF LIPIDS, SECONDARY METABOLITES AND LIGNIN IN HEALTHY AND *MELOIDOGYNE INCOGNITA*, INFECTED OKRA (*ABELMOSCHUS ESCULENTUS* (L.) MOENCH)

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ABSTRACT
The root knot nematodes, belonging to the genus *Meloidogyne*, which have a wide host range, are the deadliest enemy of vegetable crops and a pest of crucial economic importance. 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 lipids, lignins and ascorbic acid in the diseased and healthy root tissues. The metabolites viz. lipids and lignins were found more in galled tissues as compared to healthy roots.

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

INTRODUCTION

Human kind depends in a myriad ways on plant and plant products. The quest for new plant products and new methods of using them to satisfy the emerging needs is an ever expanding enterprise. Unfortunately, these plants become the victim of many disease causing micro-organisms including the plant parasitic nematodes which 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 lipids, 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 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) Silver nitrate reagent: Silver nitrate crystals – 5 g, Distilled water – 34 ml; Absolute ethyl alcohol – 66 ml; Glacial acetic acid – 5 ml.

The pH of this reagent was maintained between 2 and 2.5. Stored the reagent in amber coloured bottle at 4°C.

(b) Copper sulphate solution: Copper sulphate crystals – 5 g; Distilled water – 100 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.

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

Localization of Total Lipids

Lipids were localized by sudan III (Gomori (1952) and sudan IV (Jensen (1962).

Preparation of Sudan III/IV

Saturated solutions of Sudan IIIJIV in 70% ethyl alcohol were prepared (Sudan III.BDH, C.I. 26100; Sudan IV S.D's Pr No. 44070).

Staining Procedure

*(*1) Stained free-hand sections of fresh material in Sudan III/IV for 15 minutes.
(2) Differentiated in 50% alcohol for 1 minute.
(3) Mounted in Sudan III/IV solution.
Effect – Total lipids seen as red globules.

Reaction

Sudan dyes were more soluble in lipids than the solvent in which they were dissolved, the stain therefore, accumulated in lipids.

Control

(A) Immersed the slides in acetone at 60°C for 1 hour. This extracted all lipids except phospholipid. Stained as usual.
(B) Immersed in pyridine at 60°C for 1 hour and washed in running water stained as usual.
Effect – Lipoidal region did not stain.

Localization of Lignin

Lignin was localized by phloroglucinol-HCl test (Johansen, 1940).

Preparation of Stain

Added 1 g of phloroglucinol (Loba Chemie) in 100 ml of 95% ethanol.

Staining Procedure

(1) Placed free-hand sections of fresh galls and normal roots in phloroglucinol solution in a cavity slide till some of the solution, evaporated.
(2) Added a few drops of hydrochloric acid.
(3) Mounted the sections in glycerine jelly.
Effect – Lignin containing region stained reddish.
Control – No control preparation was made because of the well known specificity of this stain.

Localization of Ascorbic Acid

Ascorbic acid was localized by the methods given by Chayen (1953) and Chinoy (1969).

Procedure

(1) Galls and healthy roots were fixed in silver nitrate reagent, stored in a dark bottle, for a week at 41°C. The material was washed repeatedly with ammonical alcohol (5 ml liquid ammonia added to 95 ml of 70% ethanol) till white precipitate of silver hydroxide disappeared. The ammonia washed off unreduced silver nitrate from the tissue.
(2) The material was dehydrated in TBA series, infiltered and embedded in paraffin wax.
(3) The slides were dewaxed and hydrated.
(4) Dehydrated in upgraded ethanol series.
(5) Cleared in Xylene and mounted in DPX.
Effect – Brownish black granules appeared at the site of ascorbic acid.

Rationale

The fixation was achieved by alcohol and acid part of the fixative whereas, the localization of ascorbic acid was achieved by the formation of silver grains from silver nitrate by specifically caused reduction by

ascorbic acid. This reduction was stoichiometric, ie, the exposed metallic silver grains were in direct proportion to the endogenous amount of ascorbic acid.

Control

Oxidation of endogenous ascorbic acid:

Treated the unfixed material with 5% Copper sulphate solution for 24 hours and fixed in silver nitrate reagent, followed the test procedure.

Effect – No brownish-black granules appeared.

RESULTS

Lipids

Healthy Root:

Lipid globules took a red colour when the tissue was stained with Sudan III. The globules were localized in parenchyma cells of the root. The outer cortical parenchyma showed more lipid globules than other regions (Figure 1.1).

Gall:

Galls showed a greater amount of lipoidal bodies, compared to healthy tissue. They were concentrated more in the eggs, nema bodies (Figure 1.2-7) and in the cells surrounding the cavity formed due to the nematodes. In giant cells and nema bodies, their number increased with their development. Lipids were present in the cytoplasm of cortical cells and medullary rays (Figure 1.5, 1.6, 1.8).

Nematodes of all developmental stages showed the accumulation of lipid (Figure 1.2-7). The cuticle of the female, eggmatrix and eggmasses were all found to be rich in lipid.

Control:

Lipoidal region did not stain.

Lignin

Healthy Root:

Development of reddish colour with phloroglucinol -Hcl test indicated the presence of lignified tissues. Both the primary and secondary xylem in healthy roots showed a positive test for lignin (Figure 2.1, 2.2).

Gall:

Lignin was present in higher amount in galled roots owing to the presence of secondary xylem and a large amount of reaction xylem (Figure 2.3, 2.4). The cork cells and cells around the nematode or egg masses cavity showed the presence of lignin (Figure 2.3, 2.4). Giant cell walls remained unstained. Necrotic cells took a deeper stain.

Ascorbic Acid

Healthy Root:

A few brownish-black or tan coloured granules of silver, representing ascorbic acid were found in cortical and epidermal cells. In root with secondary growth granules of ascorbic acid were localized abundantly (Figure 3.1).

Gall:

The galls comparatively had more ascorbic acid than the healthy roots (Table 1). The cuticle stained dark tan and epidermal cells contained a few small granules. They were also localized in the giant cells and in the cells surrounding them, in phloem parenchyma, surrounding the reaction xylem elements, in the nema bodies of all stages and in the eggs (Figure 3.2-8). Giant cells associated with female nematodes showed increased amount of ascorbic acid, compared to giant cells associated with younger stages of nematodes. Parenchyma cells lining the cavity containing female or eggmass were also full of ascorbic acid granules (Figure 3.2-5; Table 1).

Control:

The sections remained unstained.

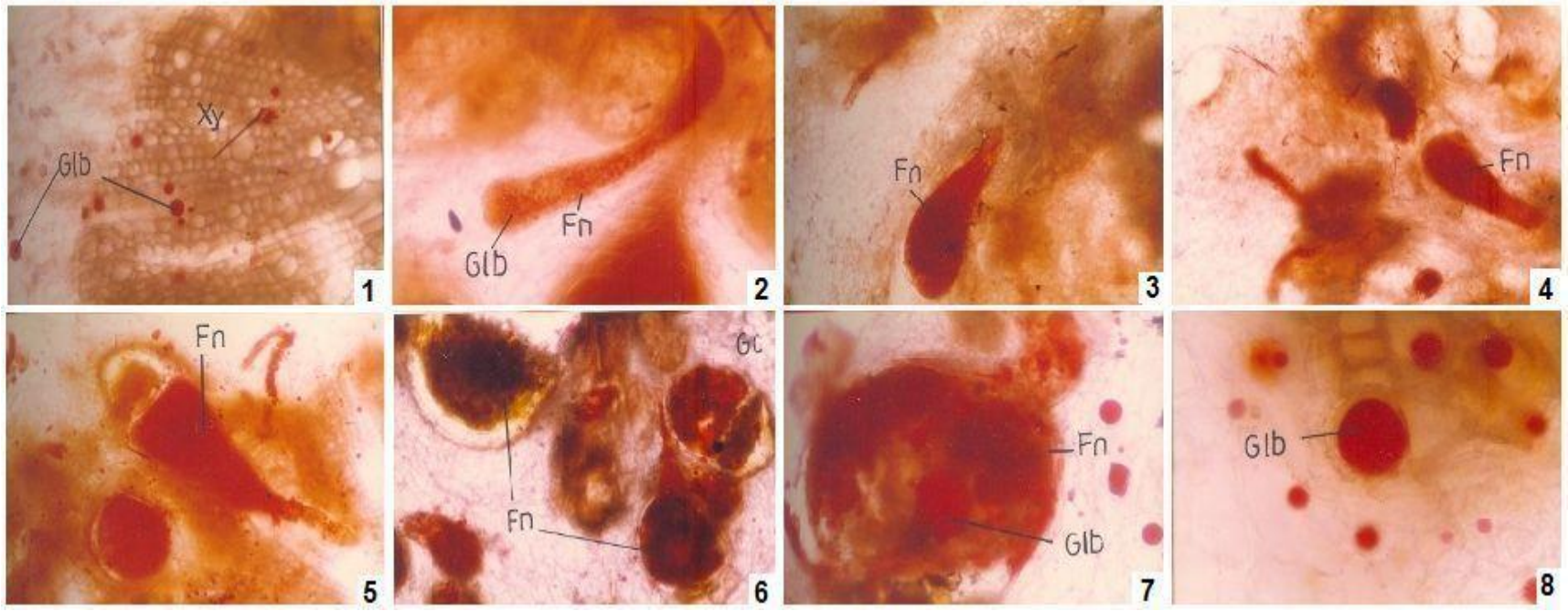

Figure 1: Localization of lipid substances in healthy and *Meloidogyne incognita* infected *Abelmoschus esculentus* (cv Pusa sawani) roots. 1: T.S. healthy root a portion, lipid globules present in secondary root.40x. **2-7:**T.S. gall a portion, showing nematode bodies full of lipid globules.100x. **8:** Same, magnified lipid globules. 400 x.

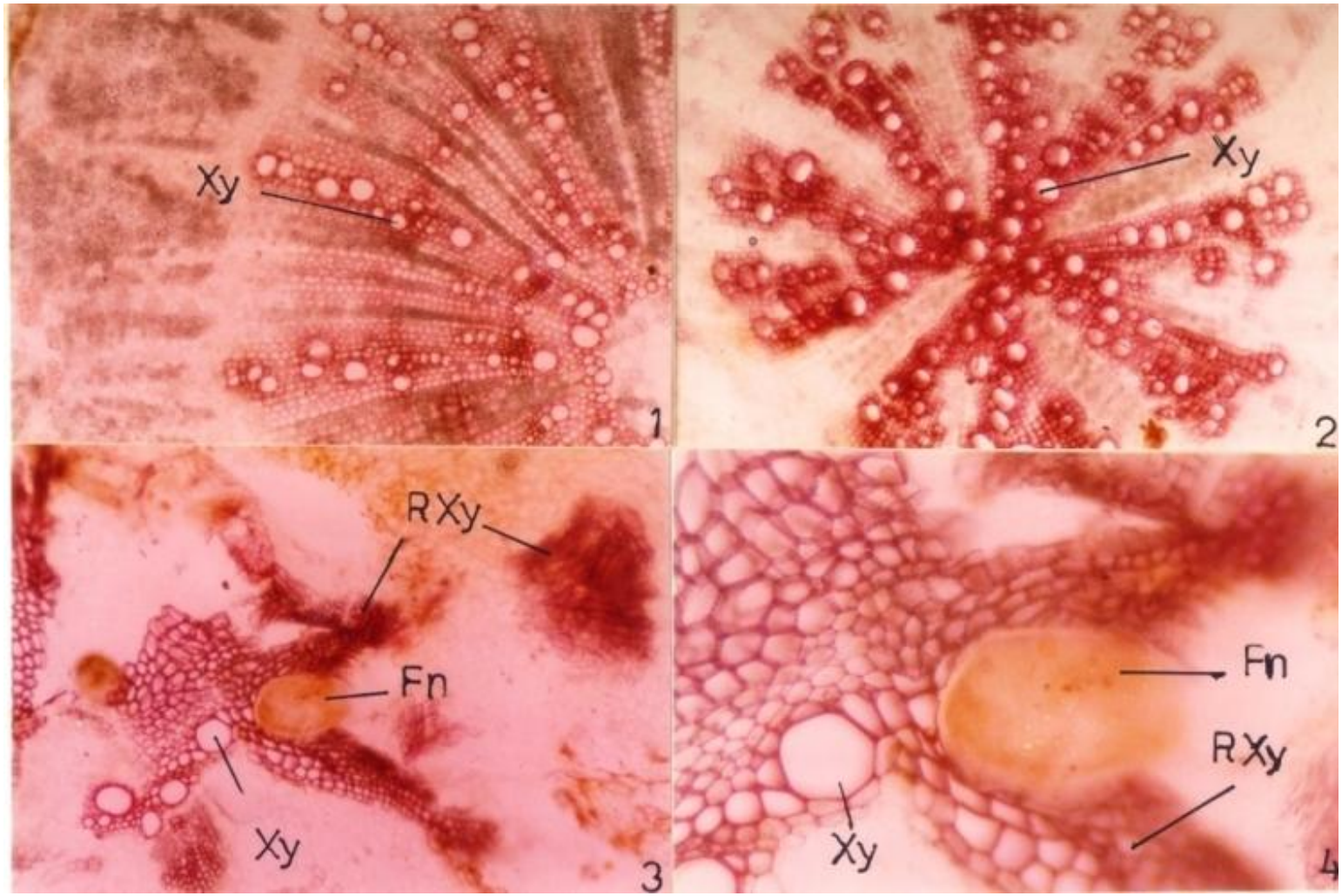

Figure 2: Localization of lignin in healthy and *Meloidogyne incognita* infected *Abelmoschus esculentus* (cv Pusa sawani) roots. 1-2: T.S. healthy root a portion, xylem stained positively for lignin, nematode body unstained.40x. **3-4:** T.S. gall a portion, xylem and reaction xylem stained positively for lignin, nematode body unstained. 40x, 100x.

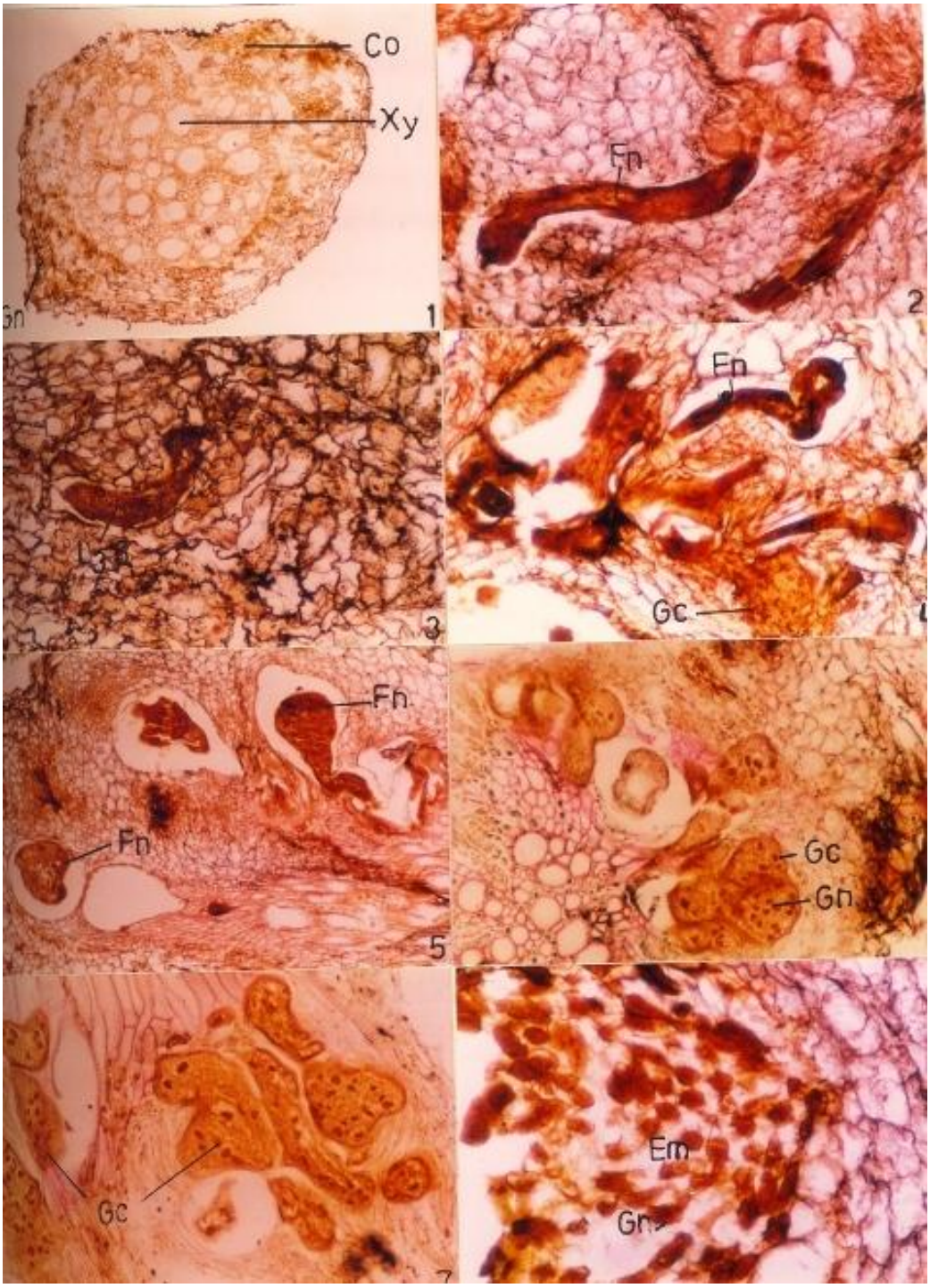

Figure 3: localized of absorbed acid healthy and *Meloidogyne* <u>*incognita*</u> infected *Abelmoschus esculentus* roots (cv Pusa sawani). 1: T.S. healthy root, brownish black or tan coloured granules of ascorbic acid localized abundantly. 40x. **2:** T.S. gall a portion, nematode body, giant cells and adjacent parenchyma cells full of ascorbic acid. 100x. **3-5:** Same, nematode bodies and giant cells full of ascorbic acid. 100x, 40x. **6-7:** Same, tan colour granules of silver in giant cells. 40x, 100x. **8:** Same, eggmass stained positively for ascorbic acid. 100x

DISCUSSION

Lipids

Lipids increased in galls as compared to corresponding healthy roots of okra. They were concentrated more in nema bodies, eggs, giant cells and hyperplastic parenchyma around the giant cells. The amount of lipids increased with the development of giant cells and the nematode. Bird (1961) noted traces of fat in syncytial cytoplasm and presence of fat besides RNA and feulgen-positive granules in hypertrophied nucleolus. Kannan and Chandraguru (1981) reported gradual increase in lipid content of infected roots of cowpea and *Dolichos* with the advancement of age. The mature females stained dark red on account of high lipid content. This accumulation of lipids was supposed to be the storage material for the nematode. Sugars and proteins were utilized to a greater extent than the fats during the adult stage in the life history of root-knot nematode because survival and infectivity of second stage larvae were known to be dependent on the food stored in the eggs (Van Gundy *et al.,* (1967)).

Gelatinous matrix and eggs were found to be rich in lipids as reported by Bird and Rogers (1965) and Trivedi and Tiagi (1983).

Lignin

Galls contained a higher amount of lignin compared to the healthy roots, its concentration in normal and abnormal xylem, necrotic cells and cells around the nematode cavity. Giebel (1970) observed that necroses formed in roots of *Heterodera rostochiensis* resistant potatoes had lignin-like characteristics. The strongest reaction of lignin was observed in cells adjacent to nematode head and its excretory pore. This indicated that substances secreted by the nematode were involved in the process of lignification.

Bird (1961) also supported the above view when he reported that syncytial walls contained all the normal polysaccharides components of cell wall but it was without lignin.

The process of lignification did not occur until IAA action ceased. If this was true, the IAA-oxidase system which was active in resistant plants favoured lignin synthesis (Giebel, 1974).

Ascorbic Acid

More amount of ascorbic acid was found in galled roots as compared to healthy ones. Increased ascorbic acid content in galled roots (Trivedi and Tiagi, 1980 and Sharma and Trivedi, 1989) had been reported. In the present study ascorbic acid was mainly localized in the nematode body and giant cells. In old galls, parenchyma cells adjacent to the giant cells and those lining the cavity containing females and egg-masses were also full of ascorbic acid granules. Zones of greatest cell activity were found to harbour high concentration of this vitamin. Kannan (1968) found that high ascorbic acid content in tomato root-knot was correlated with reduction in the amount of total sugars. In plants *in vivo* synthesis of ascorbic acid was achieved by the mobilization of sugars through the alternate pathways of carbohydrate metabolism. The data indicated that the development of cyanide-resistant respiration was conditioned by the presence of ascorbic acid in the cells. Thus, ascorbic acid should be considered as a factor of a primary importance in the biological defence mechanism of plants and animals (Arrigoni, 1979). Arrigoni *et al.,* (1979) demonstrated that a decrease in ascorbic acid in plants induced a reduction in their resistance to root-knot nematode. The concentration of ascorbic acid in plant roots was decreased by the root application of aqueous solution of lycorine, an alkaloid. In the roots of tomato cv Brecht, the rate of nematode reproduction was double in lycorine treated plants as compared to untreated plants. Conversely, susceptible cultivars irrigated with water solution of ascorbic acid reacted similarly to resistant cultivars. The amount of ascorbic acid in susceptible plants was unaltered but in resistant cultivars ascorbic acid synthesis was always stimulated by nematode attack. Un-infected okra plants, the higher occurrence of ascorbic acid indicated a limited resistant tresponse and simultaneously the inherent capacity of the plant to defend itself against the pathogen.

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