EXPERIMENT 2

Histopathological Response of *Solanum nigrum* L. Infected with Root-Knot Nematode *Meloidogyne incognita*

INTRODUCTION

The second stage juveniles of *Meloidogyne incognita* penetrate the young roots, move intercellularly towards the region of vascular differentiation and induce giant cells (Endo and Wergin, 1973; Parveen, 2006; Niyaz and Hisamuddin 2008; Bhat et al., 2009; Azam, 2009). The giant cells are generally formed from undifferentiated vessels elements or from xylem parenchyma (Christie, 1936; Hisamuddin, 2008). They have also been reported arising from protophloem cells (Byrne et al., 1977; Yasmeen 2002), or from provascular strand (Krusberg and Nielsen, 1958; Littrel, 1966).

In addition to giant cell formation, the root-knot nematodes also cause hypertrophy and hyperplasia in the cells adjacent to the giant cells that lead to the formation of the galls. *Meloidogyne* infections accompany cortical and stellar proliferations (Davis and Jenkins, 1960), hypertrophy and hyperplasia in the cortex, pericycle and the stele of the roots (Ibrahim and Massoud, 1974; Azam and Hisamuddin, 2008).

The present experiment was carried out to observe anatomical changes leading to the formation of galls on the roots of *Solanum nigrum*, formation of giant cells, abnormalities in the vascular elements in the galled roots and their relationships with giant cells.

MATERIALS AND METHODS

Collection and Maintenance of *Meloidogyne incognita* Culture:

*Meloidogyne incognita* (Kofoid and White) Chitwood, infected egg-plant roots were collected from vegetable crop fields. The species was identified on the basis of characteristic perineal pattern. The egg masses were picked out from
galled roots and were washed gently with water. A single egg mass was placed it in 0.5% NaOCl solution for five minutes, washed thrice with sterilized distilled water and allowed to hatch at 27°C in an incubator.

Egg-plant seedlings raised in microplots (2 meter x 2 meter) in the Department of Botany, A.M.U., Aligarh, containing autoclaved soil were inoculated with the juveniles thus obtained. In order to maintain sufficient inoculum throughout the course of investigation, new egg-plants were inoculated with at least 15 egg masses obtained from pure culture.

**Raising the Test Plant:**

The seeds of *Solanum nigrum* L. were procured from National Seeds Corporation (I.A.R.I), New Delhi. The seeds were axenized by NaOCl method (Koening and Barker, 1985). About 250 seeds were placed in sterilized beaker containing a mixture of 95% ethanol and 5.25% NaOCl in the ratio of 1:1. The mixture was stirred gently and the seeds were allowed to soak for about 10 minutes. The mixture was drained off and the seeds were rinsed thrice with distilled water.

The axenized seeds were sown in 30 cm diameter clay pots filled with one Kg of steam sterilized soil (7 clay: 3 sand: 1 farmyard manure) and were allowed to germinate. The seedlings were thinned to one seedling per pot, before inoculation.

**Inoculation:**

For, inoculation, the egg masses were handpicked from the egg-plant root galls and allowed to hatch. The second-stage juveniles were collected after the intervals of 48 h in sterilized distilled water, and counted with the help of counting dish.

When the seedlings became one week old, holes of 5-7 cm depth around the plants within the radius of 2 cm of the plant were made. Through these holes second-stage juveniles (2,000 J2 per 10 ml DDW per pot) were introduced with
the help of sterilized pipette. The holes were plugged with sterilized soil. To maintain soil moisture, the pots were regularly watered. Non-inoculated plants served as control.

**Harvesting:**

Five seedlings (one from each pots), were harvested at an interval of 24 h, for 72 h and after 60 days. The roots were washed thoroughly but gently under tap water to remove all soil particles. The galled roots were then cut into one cm long pieces and processed for histopathological studies.

**Processing for Histopathology:**

(i) **Fixation:** One cm long pieces of galled roots and healthy roots were immersed in a fixative of formalin-aceto-alcohol, prepared by mixing 90 ml of 50% ethanol, 5 ml of glacial acetic acid and 5 ml of 37% formaldehyde (Johansen, 1940). Depending upon the thickness the galled tissue was kept in the fixative for a minimum of 24 h to several days.

(ii) **Dehydration:** The galled and healthy roots were dehydrated through tertiary-butyl-alcohol (T.B.A) schedule as given by Johansen (1940) (Table- 6).

(iii) **Infiltration:** After dehydration, paraffin was introduced into the root tissues. After step-8 of table-1, the tissues were transferred to a mixture of 100% paraffin oil and tertiary-butyl-alcohol (T.B.A.) in the ratio of 1:1. These were kept at least for one hour or more in this mixture. Another container was filled 3/4th with melted wax and allowed to solidify. The tissues were placed on wax and then filled with T.B.A. + paraffin oil mixture and placed in an incubator at 65°C for three h. The mixture thereafter was poured off and replaced with pure melted wax. The container was kept at the same temperature for about three hours. This step was repeated twice.

(iv) **Embedding:** For embedding, paper molds in the form of small shallow trays were prepared. The inner surfaces of the molds were first coated
with thin layer of glycerine and afterwards melted paraffin was poured into the bottom of the molds. As the wax started solidifying on the bottom of the mold, the root pieces were kept gently on the solidifying surface with the help of heated forceps. More melted wax was added when the wax around the tissue was solidified into the molds until the tissue was completely immersed in the wax. The molds were immediately transferred to a container filled with chilled water for solidification of wax. After hardening, the whole block was cut into smaller pieces according to the position of the root tissue.

(v) **Sectioning:** The small blocks of wax having root tissues were trimmed to remove extra wax. The wax blocks were mounted on the wooden blocks, and then fixed in rotary microtome. With the help of sharpened knife, 10-12µm thick transverse and longitudinal sections in the form of ribbon were obtained.

(vi) **Ribbon Mounting:** The ribbon was cut into smaller pieces corresponding to the length of slides. The surfaces of the slides were coated with synthetic adhesive. Ribbon was placed gently on the slide and flooded with freshly prepared 3% formalin solution. These slides were then kept in an incubator at 40°C for 6-8 h and then stored in slide boxes.

(vii) **Staining:** The sections were stained with safranin and fast green after removing paraffin wax by the method described by Sass (1951) (Table-7). After staining, Canada balsam was applied on the slide before evaporation of the xylene, cover glass was lowered gradually over the slide. Prepared slides were left at room temperature for at least 24 h and then transferred to incubator at 60°C. The slides were examined under the light microscope, and necessary photographs were taken.

**OBSERVATION:**

**Anatomy of Normal Root:**

Morphologically, the normal root system of *Solanum nigrum* comprised of a primary root and enormous lateral branches arising from it. The secondary and
tertiary lateral branches constituted the root system. Anatomically, the root of *S. nirgum* comprised of a uniseriate epidermis, well differentiated and multilayered parenchymatous cortical cells. The size and the number of vascular bundles are few, the size and the number of vessels are small, many and appeared together within the cortex. One or two calcium oxalate crystals were observed in the cells of cortex (P1, P2, P3 and P4).

**24 h After Inoculation:**

The second-stage juveniles of *Meloidogyne incognita* penetrated the young roots of *Solanum nigrum* within 24 h of inoculation at or near the root tip and then migrated intercellularly to the zone of elongation and vascular differentiation (P-5). Other juveniles selected the zone of cell division for their entry. The juvenile had selected any part of the root for their entry (P-7). The juveniles penetrating at the root tip made a prominent narrow passage to move through it, the same passage were used by other juveniles (P-8, P-9). Epidermal and cortical layers of the root were found damaged during the penetration of the juveniles (P-6, P-8).

The juveniles while migrating towards the zone of elongation caused hypertrophy and hyperplasia. Some large cells with enlarged nuclei were observed near the body of the juvenile (P-10). Some cells, near the nematode head were also enlarged and binucleate (P-11). Around the nematode head, the cells on which nematode start feeding, became enlarged. Three to four became crown shaped and formed rosette (P-12). The cells along the nematode body length were small and compactly arranged.

Hypertrophied and hyperplastied cells were seen near the head of the nematode (P-13). The cells of the undifferentiated phloem around the nematode head were severely hypertrophied (P-14). The developing giant cells were observed in the zone of elongation. In these developing giant cells the nuclei were large that enclosed enlarged nucleoli (P-15, 16). The giant cells were long and wide, and were having dense cytoplasm (P-17, 18). The incipient giant cells
contained granular and deep stained cytoplasm (P-19, 20, 21). The nucleoli were either globular, conical, elongated, constricted, cordated or elbow-shaped (P-22). Both the nucleoli and nuclei were hypertrophied (P-23).

Around the giant cells, a large number of small parenchymatous cells were found. The length of giant cell was about 10 times greater than the neighboring cells (P-23, 24, 25). Vascular differentiation was not observed generally adjacent to the nematodes. In some sections the giant cells were found near the vessel elements (P-27, 28). Hyperchromatic tissue was seen adjacent to the vascular tissue in some of the sections (P-26, P-14). The juveniles that reached the zone of differentiation occupied a place near the phloem differentiating cells (P-29, 30).

**72 h After Inoculation:**

The developing giant cells were observed in the zone of elongation. In these developing giant cells the nuclei were larger that enclosed larger nucleoli. The cytoplasm of the giant cells was dense (P-31). Differentiation of vessel elements was observed near the developing giant cell complex (P-32, 33). The nematode head was now inside the fully differentiated giant cells (P-34, 35, 37). The cytoplasm became more granular and dense, and occupied almost entire space of the giant cells.

The adjacent cells situated at both the ends of the giant cells were separated due to the enlargement of the giant cell size. The separating cells were compressed and were placed outward creating an intercellular space which were later filled by the developing giant cell (P-36, 38).

Smaller nuclei and nucleoli were found near the head of nematode, while very large nuclei that enclosed large nucleoli were found away from the nematode head. The width of the galled root increased, 72 h after inoculation. The width of uninoculated (normal root) was slightly less than the nematode inoculated root. The average size of giant cells was 210 x 60 µm. The largest giant cell measured was 312 x 80 µm.
Near the giant cell, the length of the vessel element decreased in the xylem strands. The diameter of the vessel elements was greater near the giant cells. The vessel elements nearer to the nematode body were wider than the others (P-39, 40). The abnormal vessel element resembled with the shapes of neighboring parenchyma cells (P-40, 41), but their size was larger than the parenchyma cells. A juvenile with its head in phloem cells was found in the infected root. The giant cells were found well connected to the phloem strands (P-42).

60 Days After Inoculation:

The giant cell cytoplasm became extremely dense and granular. The cell wall of the giant cell became thicker. The giant cells measured 385 x 133 µm. The cytoplasm was so dense that it became impossible to count the exact number of nuclei. Some very large and heavily stained nucleoli were quite prominent in the nuclei of the giant cells (P-44, 45). The giant cells attained their largest size after 60 days of inoculation. The mature pyriform female nematodes were found to be settled in such a way that their heads were in the giant cell complex and their posterior part was towards the periphery of the root (P-46, 47, 48, 49, 50).

The increased vacuolation and decreased cytoplasmic contents were found in some of the sections (P-51, 52, 53, 54, 55, 56). There was remarkable reduction in the number of nuclei in the giant cells. The adult females were found associated with their eggs masses (P-57, 58, 59, 60). In many sections, the mature pyriform females were found associated with the egg sacs and also with egg masses (P-61, 62, 63, 64, 65, 66, 67, 68). The width of the gall was measured to 1.5 cm after 60 days of inoculation, in comparison to the normal roots thickness of 0.5 mm.

The abnormal vessel elements were found dispersed with abruptly broken normal vascular strands after 60 days of inoculation (P-69, 70). In some of the sections, the giant cells were found in close association with vessel
elements (P-71, 72, 73, 74). The abnormal vessel elements were of various sizes and of various shapes like elongated to isodiametric (P-73, 75, 76).

After 60 days of inoculation, the anatomy of the infected roots became totally distorted. The orientation of the vascular strands became disturbed by the activity of mature nematodes and by the formation of giant cell complex (P-77, 78, 79, 80). The giant cell complex was found surrounded by phloem elements (P-81, 45). Abnormal phloem elements were observed near the giant cells (P-42, 82).

**DISCUSSION**

From the study, it is evident that the second-stage juveniles of *M. incognita* entered into the roots of *Solanum nigrum* plants, frequently at or just behind the root cap. This happened because *S. nigrum* plant roots were unable to prevent their entry as root exudates or any phytotoxic compound was not secreted at this level. The plants appeared as susceptible hosts for *M. incognita*. Further migration of nematode juveniles was intercellular and no cellular damage was observed. A prominent passage was formed and the cells were compressed along the body length of nematode at the time of their journey. Such penetration and intercellular migration was also observed by Christie, 1936 in tomato, Hisamuddin, 1992 in *Luffa cylindrica*, Parveen in 2006 (*Ocimum sanctum*) and Azam (2009) in Tomato. In the region of cell differentiation and elongation in wheat by *M. nassi* (Siddiqui and Taylor, 1970). The observations on migration of juveniles of *M. incognita* in the roots of *S. nigrum*, supporred the findings Endo and Wergin, (1973); Jones and Pyne, (1978) and Hisamuddin, (1992), that juveniles of root-knot nematodes migrated intercellularly by separating the cells walls along the middle lamella. Immediately after migration the tissue of the roots responded to the nematode and the enlargement of the cells initiated. The cells along with their nuclei became hypertrophied near the head region as well as along the body of juveniles. The effect of stimulation was transmitted to the adjacent parenchyma cells. The effects were expressed in the form of cell enlargement, thickening of the cell wall, greater number of nuclei and
increase in the amount of cytoplasmic contents. The cells became metabolically hyperactive. Nucleolus plays an important role in the formation of ribosomes which are involved in protein synthesis in the cell. The larger nucleoli played an important role in synthesis of a large amount of cytoplasm than small inactive nucleoli (Johnson and Johnson, 1986).

In addition to hypertrophy, the affected cells exhibited nuclear division without subsequent cell wall formation. After 72 h of inoculation, formation of 5-6 giant cells, indicated the host-parasite relationship had been stabilized successfully in such a short interval of time. Six to eight nuclei were formed in the giant cells and each nucleus contained one or more nucleoli. Azam (2009) had observed giant cell formation by *M. incognita* in the roots of tomato, 48 hr after inoculation. The multinucleate condition had arisen due to nuclear division without cytokinesis as was supported by Haung and Maggenti (1969a). Synchronous nuclear divisions within the same giant cells have been reported by several other workers like Krusberg and Nielsen (1958); Birds (1961); Pasha *et al.*, (1987); Hisamuddin (1992) and Robab *et al.*, 2009. The present study also supported this mode of giant cell formation.

Increase in the size of giant cells, increase in the number and size of the nuclei, changes in the shapes of nuclei, and increase in granulation and cytoplasmic density was observed after 72 h of inoculation. The cell walls of the giant cells became thick and hence cell did not burst even after their further enlargement. In the developing giant cells, cell organelles became abundant which indicated that cellular activities had peaked at this stage (Jones and Northcote, 1972; Jones and Dropkin, 1976; Jones and Pyne, 1978).

Abnormalities in the xylem strands were observed clearly in the sections after 72 h of inoculation. The xylem strands were found pushed away from their original position and were found placed in a zig-zag manner, when observed in longitudinal sections. This alteration in orientation was probably due to proliferation in giant cell and also due to the hypertrophy and hyperplasia in the parenchymatous cells. The vessel elements became irregular in shapes,
shortened in size and proliferated and were found in scattered patches. The giant cells were found surrounded by the xylem as was reported by Christie (1936); Krusberg and Nielsen (1958); Parveen (2006). Cells were completely surrounded by xylem, 9 days after inoculation.

To compensate the losses of water and nutrient due to root growth inhibition, probably the plant produced a large number of lateral branches as has been earlier reported by Christie (1936); Krusberg and Nielsen (1958); Davis and Jenkins (1960) and Hisamuddin (1992). It is clear from the present study that the additional lateral branches facilitated the uptake of frequent water and nutrients in large amount. The mineral nutrient absorbed by the lateral roots was not properly translocated upwards to the shoots and this fact was already advocated by earlier workers like Oteifa and Elgindi, (1962). In this respect, infected plants tried to produce large number of xylem elements, to overcome the absorption and translocation.

This kind of adaptation of plant in the formation of abundant abnormal xylem might be protective in nature and provide support and strength to the infected plant. The giant cells were given protection from being ruptured and surrounded by abnormal vessel elements that were transformed from its neighboring parenchyma cells.

Thus, it might be concluded from this experiment, that the orientation of vascular strands was severely distorted because of multiple hypertrophic and hyperplastic reactions that had been taken place in the affected area of the plants.

The phloem region appeared to be the preferential feeding site of the nematodes. Ediz and Dickerson (1976) found that most of the giant cells occurred in phloem region. Primary phloem or adjacent parenchyma cells were selected as the feeding sites by nearly all the nematodes (Byrne et al., 1977; Finley, 1981). The phloem elements were found to be highly affected and
disturbed during the gall formation. The orientation and shapes of sieve tube elements were altered due to enlargement of nematode body.

This study revealed that the giant cells were always found connected with the phloem elements. The phloem elements were found supplying photosynthates to giant cells. The sieve tube elements in the secondary phloem were diverted towards the giant cells to translocate metabolites through the phloem elements, and the supply of these assimilates was unintrupted.

Formation of the giant cells in the cortex has been reported in many plants (Azam, 2009). This observation supported that the giant cells were not derived from cortical parenchyma but from undifferentiated meristematic cells.
EXPERIMENT 3

Anomalies in Root Anatomy, Plant Growth and Photosynthetic Pigment Concentration of *Solanum nigrum* L. at Different Inoculum Levels of *Meloidogyne incognita*.

INTRODUCTION

The root-knot nematode (*Meloidogyne* spp.) is an important pathogen of several Solanaceous crop plant, especially pepper, potato, and tomato (Dropkin and Nelson, 1960; Sasser, 1977). Roots attacked by this parasite exhibit characteristic root galls, and infected plants grow poorly or even die because of vascular dysfunction. As sedentary endoparasites, *Meloidogyne* spp. have complex trophic relationships with their host plant and induce specialized feeding structures known as giant cells, which are essential to obtain nutrition and development of the nematode. These nematodes infect thousands of different herbaceous and woody, monocotyledonous and dicotyledonous plants and cause serious losses to numerous agriculture crops worldwide (Sasser and Freckman, 1987; Eisenback and Triantaphyllou, 1991). Inoculation with the *M. incognita* lead to the formation and establishment of permanent feeding sites comprising of multinucleated giant cells surrounded by deformed xylem elements; hypertrophy of the cortex and hyperplasia of the pericycle; endodermis; and vascular tissues of the host roots. In addition, deformation and blockage of vascular tissues at the feeding sites limit the translocation of water and nutrients, which further suppresses plant growth and crop yield, (Hussey and Williamson 1997). Low or high population densities of these nematodes produce different effects on different plants. Wallace (1971) found an increased rate of plant growth at lower, and decreased at higher population densities. More giant cells with the higher dimensions were formed and more eggs/egg mass were produced on the plants inoculated with increasing inoculum levels. *Meloidogyne*-induced anatomical alterations were investigated in eggplant roots (Pasha *et al*., 1987 and Ekanayake *et al*., 1988) as well as in other plants like
*Luffa cylindrica* (Hisamuddin, 1992), tobacco (Mohamed et al., 1993), papaya (Sabir, 2001) tulsi (Parveen, 2006), tomato (Azam, 2009) and soybean (Robab et al., 2009).

The following study was carried out to determine the effects of different inoculum levels on (i) the plant growth (ii) photosynthetic pigment concentrations, (iii) the number and size of galls (iv) the number of egg masses per plant, (v) the number of eggs per egg mass and more importantly (vi) nematode population, (vii) reproduction factor and (viii) on the vascular and non-vascular tissues in the affected parts of the roots of *Solanum nigrum*.

**MATERIAL AND METHODS**

**Raising and Maintenance of Test Plant:**

The seeds of *Solanum nigrum* L. procured from National Seeds Corporation, New Delhi, were axenized by NaOCl method (Koenning and Barker, 1985). About 100 seeds were placed in sterilized beaker containing a mixture of 95% ethanol and 5.25% NaOCl in the ratio of 1:1. The mixture was stirred gently and the seeds were allowed to soak for about 10 minutes. The mixture was drained off and the seeds were rinsed thrice with distilled water.

The axenized seeds were sown in 30 cm diameter clay pots containing steam sterilized soil (7 clay: 3 sand: 1 farmyard manure) and allowed to germinate. The seedlings were thinned to one seedling per pot, before inoculation.

**Inoculation with Nematode:**

*Meloidogyne incognita* (Kofoid and White) Chitwood was selected as a test pathogen. To perform experiment during the period of research, pure culture of *M. incognita* race 1 was maintained on egg plant (*Solanum melongena* L.) roots in a glass house by using single egg mass. The egg masses from the galled roots of egg plant were picked with the help of sterilized forceps and allowed to hatch. The second-stage juveniles were collected in sterilized distilled
water and counted with the help of counting dish. Three leaf stage seedlings were inoculated by making holes of 5-7 cm depth around the plant within the radius of two centimeters. The second-stage juveniles, at the rate of 5J₂, 50J₂, 500J₂ and 5,000J₂ per 10 ml water, were pipetted into the holes, which were covered with the soil soon after inoculation. Each treatment was replicated five times and the pots were arranged in randomized complete block design. Un-inoculated set of plants served as control.

\[
\begin{align*}
C & : \text{ control} \\
T_1 & : 5J_2/pot \\
T_2 & : 50J_2/pot \\
T_3 & : 500J_2/pot \\
T_4 & : 5,000J_2/pot
\end{align*}
\]

Watering was done regularly. The plants were uprooted after 60 days of inoculation. The data for different parameters were collected and statistically analyzed.

**PARAMETERS**

**Plant Growth:**

After 60 days of inoculation, the mature plants were uprooted with the help of hoe and gently washed with running tap water. The plants were cut at the margin of the root and the shoot. Length of the shoot and the root was measured in centimeter with the help of meter scale. Fresh weight of the shoots and the roots was determined by physical balance. The shoots and the roots were kept, separately, in bamboo paper envelopes and kept in an incubator maintained at 72 °C temperature for 5 days. Dry weight of the root and the shoot was determined. Ten mature leaves from five plants were randomly selected to calculate leaf area, for this purpose an outline of the shape of each leaf was
drawn on rice papers and the area occupied was measured with the help of planimeter.

**Estimation of Chlorophyll:**

For estimation of chlorophyll, one g fresh leaves of *S. nigrum* from different treatments were crushed in mortar and pestle containing 50 ml of 80% acetone and filtered through Whatman’s (No.1) filter paper. The filtrate was transferred to 100 ml volumetric flask and the volume was made upto the mark with 80% acetone. The transmittance was read at 645 and 663 nm on spectrophotometer. The amount of chlorophyll a and b was determined as mg/g fresh leaf according to the formula given by MacLachlan and Zalik (1963).

**Number and Size of Galls:**

The number of the gall was counted visually. And the size of gall was obtained by measuring maximum length and width (in mm$^2$) on meter scale.

**Number of Egg Masses:**

The number of egg masses per root system on infected roots was counted after staining with phloxin B, prepared by dissolving 0.12 g phloxin B per liter of water. The galled roots were placed in this solution for 15-20 minutes. The roots were gently rinsed in tap water. The egg masses were stained red and counted directly.

**Number of Eggs per Egg Mass:**

Ten mature egg masses randomly selected from galled roots of each treatment were treated with 20 ml of NaOCl (2%) solution and stirred vigorously for one minute. The egg masses were stained with acid Fuchsin (Byrd *et al.*, 1972) and then counted under stereoscopic microscope.

**Nematode Population (Root and Soil):**
Root nematode population was determined by macerating 5g of infected root in a waring blender. The suspension was passed through 100 to 400 mesh sieves and the juvenile catch on the 400-mesh sieve was collected in a beaker. Soil of each pot was thoroughly mixed with and juveniles were extracted by Cobb’s sieving and decanting and Baerman funnel methods. The number of nematodes per root system and per kilogram soil was counted using counting dish.

**Reproduction Factor:**

Reproduction factor (R_f) was calculated by the formula:

$$R_f = P_f/P_i$$

Where P_f is the final population and P_i is the initial population.

**Size of Female Nematode:**

The length and width of female nematode body were measured using camera lucida.

**Root - Knot Index (RKI):**

The gall indices were rated on Taylor and Sasser (1978) scale.

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<tr>
<th>Scale</th>
<th>Root-knot index</th>
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<tr>
<td>0</td>
<td>0</td>
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<tr>
<td>1</td>
<td>1-2</td>
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<td>2</td>
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<td>4</td>
<td>31-100</td>
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<td>5</td>
<td>≥ 100</td>
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**Histopathology:**
The roots were cut into one cm long pieces and processed for histological studies. The galled roots were fixed in F.A.A and dehydrated by passing through Tertiary-Butyl-Alcohol (TBA) paraffin oil and paraffin mixture, and finally paraffin wax. The wax embedded tissues were trimmed into small blocks (Johansen, 1940). Sections were cut with the help of rotary microtome of 10-12μm thickness. The sections were stained with safranin and fast green (Sass, 1951), and necessary photographs were taken. The details of the method have been described in experiment-2.

OBSERVATIONS

Plant Lengths (Shoot and Root):

The length of the shoot and the root of *Solanum nigrum*, inoculated with different inoculum levels of second-stage juveniles of *Meloidogyne incognita* decreased, when compared with uninoculated (control) plants (Table-8). The data revealed that at lower inoculum level (T₁), the length of the shoot and root were decreased non-significantly (p=0.05) over the control, while a significant (p=0.01) reduction in the plant inoculated with 50 J₂ per pot was observed. Significant (p=0.01) reductions were observed in T₃ and T₄ plant, over the control. The reduction was maximum in T₄ plants grown at 5,000 J₂ per pot and minimum in T₁ (5 J₂ per pot) Table-8.

Fresh and Dry Weight of Shoots and Roots:

The fresh weight of the shoot and the root decreased in all the treatment when compared with control plants (Table-8). The non-significant (p=0.05) reductions were noticed in T₁ plants while significant (p=0.01) reductions were observed in rest of the treatments over the control. On the other hand the dry weight of the shoots of T₁ plant decreased non-significantly (p=0.05) while that of T₂, T₃ and T₄ plants were decreased significantly (p=0.01), when compare with the control plants. The maximum reductions were found in the T₄ plants which were inoculated with the highest level of inoculum (5,000 J₂ per pot) (Table-8).

Chlorophyll Contents:
There were significant reductions (p=0.01) in Chl-a contents in all the plants inoculated with the nematode. The Chl-b decreased non-significantly (p=0.01) at the initial inoculum levels of 5 j$_2$ and 50 j$_2$ per pot (T$_1$ and T$_2$). In comparison to control the reductions were significant (P=0.01) in the plants inoculated with higher inoculum levels (T$_3$ and T$_4$) (Table-8).

**Leaf Area:**

The leaf area of the plant inoculated with the root-knot nematode, when compared with control, decreased non-significantly (p=0.05) at lowest inoculum level (5 j$_2$ per pot) and significantly at higher levels (50 j$_2$, 500 j$_2$ and 5,000 j$_2$). Highest and significant (p=0.01) reduction in leaf area was noticed at the inoculum level of 5,000 j$_2$ per pot, over the control (Table-8).

**Number of Galls:**

Galls were observed on all the nematode inoculated plants. The galls were smaller and almost unnoticeable on the roots of T$_1$ plants that were grown at the lowest initial inoculum level of 5 j$_2$ per pot. The number of galls increased with the increase in inoculum level. The number of galls on the root of T$_2$ plants was higher but non-significant (p=0.01), when compared with the T$_1$ Plant. Significant increase in the gall number on T$_3$ and T$_4$ plants, over the control was observed. The highest number of gall were present in plant which were inoculated with the maximum number of second-stage juveniles (5,000 j$_2$) and the gall number was found significantly increased when compared with the plants inoculated with lowest initial inoculum level (Table-9).

**Size of Galls:**

The size of the galls was found to be increased at higher inoculum levels when compared with the lowest inoculum levels (Table-9). Non-significant (p=0.05) increase in the size of galls was found on T$_2$ plants, but significant (p=0.01), increase was noticed on T$_3$ and T$_4$ Plants, when compared with the T$_1$
plants. On $T_1$ plants the size of the galls was smallest (3.97 mm$^2$) and largest on $T_4$ plants (22.57 mm$^2$) (Table–9; P-83, 84, 85, 86).

**Number of Egg Masses:**

An increase in the number of juveniles per plant increased the number of egg masses per plant. An average number of egg mass recovered from the plant inoculated with 5 j$_2$ per pot was very low (2.56 per plant). Non-significant (p=0.01) increase in the number of egg masses per plant was observed on $T_2$ plant, when compared with $T_1$ plant. The number of egg masses significantly increased as the initial inoculum level increased. Maximum number of egg masses were noticed on the $T_4$ plants, the inoculated with highest number of juveniles (5000 j$_2$) (Table -9).

**Number of Eggs per Egg Mass:**

The number of eggs per egg mass decreased with an increase in initial inoculum level. Non-significant (p=0.05) reduction was observed in $T_2$ plant. Maximum number of eggs per egg mass was noticed in $T_1$ plant that was inoculated with lowest inoculum level. The reduction were significant (p=0.01) in rest of the plants ($T_3$ and $T_4$) when compared with $T_1$ plants (Table – 9).

**Nematode Population, Reproduction Factor (Rf) and RKI:**

Final population (root population + soil population) of the nematode was minimum at the initial inoculum level of 5 j$_2$ per pot, and maximum in $T_4$ plants that were inoculated with highest number of juveniles. Reproduction factor (Rf) decreased with an increase in initial level of inoculum, maximum being at lowest and minimum at highest inoculum levels. Whereas the root–knot index followed reverse pattern. The RKI was minimum at $T_1$ and maximum at $T_4$ plants that were inoculated with the highest initial inoculum level (Table – 10).

**Size of Mature Female:**
Average size of the mature female was not affected very much with an increase in initial inoculum level. The reduction in the size of the female in T₃ and T₄ plants were non-significant (p=0.05), when compared with T₁ and T₂ plants.

**Histopathological Studies:**

*Solanum nigrum* infected with the root-knot nematode (*Meloidogyne incognita*) exhibited severe nature of infection when observed through longitudinal and transverse sections of the infected roots and examined under compound microscope as compared to the healthy roots. The juveniles that entered the roots established a successful host-parasite relationship by introducing giant cells at the site of feeding. The juveniles were seen clearly in the infected portion of the roots (P-87 and P-88) under microscope. After successful penetration and migration, the nematode settled and led to the introduction of 6-8 multinucleate giant cells (P-89, P-90). The number of giant cells and the number of mature females were increased as the initial inoculum level increased from 50 j₂ to 500 j₂ per pot (P-91, P-92, P-93, P-94). The giant cell complex and the number of pear-shaped mature females were found maximum in the roots of plants that were inoculated with the highest number of initial inoculum level (5,000 J₂ per pot) in T₄ plants (P-95, 96, 97, 98, 99, 100).

There were great variations in the size of giant cells (P-101, 102). At the lower inoculum levels, the nematodes as well as the giant cells were found at one or two places (P-103, 104), while at higher inoculum levels, entire portion of the root was found associated with the mature female (P-105, 106).

At lower inoculum level the (5 j₂ and 50 j₂), the giant cell cytoplasm was dense and more granular, whereas at higher inoculum levels (500 j₂ and 5,000 j₂) it was less dense. The giant cell had larger vacuolation at higher inoculum levels, and the cytoplasm was less in the larger giant cell. At lower inoculum levels, the vacuolation was less and cytoplasm was more dense.

The number of nuclei was higher at lower inoculum levels and lower at higher inoculum levels. The nuclei stained deep at lower inoculum level and
became very prominent (P-107). The number of nuclei was higher and the nucleoli were clearly visible in the plants inoculated with less number of juveniles (5 j$_2$ and 50 j$_2$).

In the vicinity of giant cell complex there were several vessel elements of abnormal shape and size that comprised abnormal xylem (P-108, 109, 110, 111). Distortion of Xylem strands occurred as a result of nematode infection. The amount of abnormal xylem elements was more at higher inoculum levels that at lower inoculum levels. Similarly the orientation of phloem elements was broken due to nematode infection. The abnormal phloem elements were observed in the vicinity of the giant cells (P-112, 113). Abnormal xylem was formed in large amount as it is evident from plate-P-114, 115, 116, 117, 118, 119. The mature female after completing their life cycle laid eggs encapsulated in egg sac (P-121, 122).

Internal structure of the roots of *S. nigrum* plant was distorted heavily and specially at higher inoculum levels as it is evident from the plate-123, 129, 125, 126).

**DISCUSSION**

Inoculation of *Solanum nigrum* with second–stage juveniles of *Meloidogyne incognita* caused reduction in plant length as is evident from table–8. An increase in number of juveniles at different inoculum levels decreased the length of plant. The relationship between the nematode population and plant length was inversely proportional. Increase in inoculum level from 5 j$_2$ per pot to 5,000 j$_2$ per pot brought about a gradual decrease in length, fresh weight and dry weight of plant (Table -8).

Reduction in plant length and weight of the plant with increase in initial inoculum levels of *Meloidogyne* spp. have been reported by several workers like Jonathan and Rajendran, 2000; Nehra and Trivedi, 2002; Hisamuddin *et al.*, 2005; Azam and Hisamuddin, 2008; Azam *et al.*, 2008; Robab *et al*; 2009. The damage caused by nematode on growth of susceptible plants probably involved
several mechanisms. Nematode removed plant nutrients, altered nutrient flow pattern, and retarded root growth, all of these contributed in suppressing the plant yield (Hussey, 1985). There are several reports narrating that the root-know nematode influenced the transport mechanism from root to shoot and translocation of metabolites from leaves to other organs, and reduced the amount of photosynthetic pigment concentrations (Loveys and Bird, 1973; Wallace, 1974 and Melakberhan et al., 1985).

In the presence of higher number of nematodes the chances of infection are greatly increased. The growing roots as well as newly emerged roots are frequently attacked by the juveniles causing severe infection in roots leading to heavier damages to the plants. The nutrient materials were prevented, up to certain extent to reach the top of the plant which resulted in the reduction of leaf area. Jonathan and Rajendran (2000) also reported significant reduction in leaf area of Musa sp. at 1,000 and 10,000 juveniles per Kg of soil.

The observations from the present experiment revealed that the number and size of galls increased with an increase in initial inoculum levels. Smaller galls indicated low level and large gall indicated higher level of inoculum. Maximum numbers of galls were observed at 5,000 J$_2$ per pot. This trend might be due to the fact that at higher inoculum levels more feeding sites were explored by large number of juveniles which resulted in increased number of galling on the infected roots. The findings are in accordance with the earlier reports, (Yasmeen, 2002; Parveen 2006; Robab, 2006; Niyaz and Hisamuddin, 2008; and Azam, 2008).

With an increase in inoculum levels numbers of egg masses per plant were increased which is obvious as lower the inoculum level, lower will be number of mature females and consequently fewer be the egg masses. Number of eggs per egg mass significantly decreased at the highest inoculum levels, however at lower inoculum levels the difference in the number of eggs per egg mass were non-significant. Limited food and space, probably, produced detrimental effects on the maximum development of the nematode and
consequently on egg production. The insufficient nutrition might be the cause of less number of eggs.

Reproduction factor (Rf) was found decreased with the increase in initial inoculum level which was highest at lowest inoculum level and vice-versa. The decrease in the rate of nematode population was perhaps due to destruction of the root system with high population of nematode and due to competition for nutrients among the developing nematodes within a given root system as was reported by Chitwood (1951); Pathak et al., 2000, Khan and Ashraf (2005).

There were major devastating anatomical changes in the infected roots when observed in the transverse and the longitudinal sections. The development of elaborate feeding sites, called giant cells, were the main among the other charges like hypertrophy, hyperplasia, formation of abnormal xylem and distortion in phloem. When the giant cells and the nematode developed in the protophloem, they exerted considerable pressure on the surrounding tissue resulting in abnormality in the neighboring cells and tissue. At higher inoculum level average size of giant cell was reduced. It might be because at higher inoculum level all the parenchyma rays were occupied by the giant cells and the nematode and usually more than one nematode were observed feeding at the same site.

At lower inoculum level, dense cytoplasm indicated that metabolites were sufficiently supplied to the giant cells by the plant. One of the discernible events in the giant cells, in addition to granular cytoplasm, was the enlargement of the endomitotically divided nuclei. Occurrence of larger nuclei and larger nucleoli is essential for the accumulation of dense and granular cytoplasm in larger amount (Hisamuddin, 1992).

Formation of abnormal xylem as a result of root-know nematode infection has been reported in almost all the histopathological studies. Abnormal phloem has been scantily mentioned. Increased amount of phloem elements was
probably due to higher number of giant cells that were associated with abnormal phloem elements.

From the present experiment, it might be concluded that, with increase in initial inoculum level, length and weight of the plant decreased, number and size of galls, number of egg masses per plant increased, size of the giant cell and number of eggs per egg mass were decreased.

Stimulation induced by *M. incognita*, on the roots of *Solanum nigrum*, resulted in anatomical changes in the roots. At higher inoculum levels, the strength of inoculum was high consequently more abnormalities in the affected roots were encountered. The abnormalities ranged from subcellular (nucleus and nucleolus), to cellular (giant cell, abnormal vessels element, abnormal phloem elements) to tissue level (distortion in vascular strand).