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Histopathological study of nanocomposite treated okra roots infected with *Meloidogyne incognita*

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Abstract
Histopathological study of nanocomposite treated okra roots showed few and poorly developed giant cells with substantial necrotic layer formation around the cells which may be due to hypersensitivity reactions (HR) in the tissue thereby inhibiting the further development of giant cells. Whereas, roots infected with *Meloidogyne incognita* shows induction of galls and specialized feeding sites called giant cells, disrupting the histological profile of root tissues. *Meloidogyne incognita* was able to enter the host tissue, feed and reproduce normally showing prominent giant cell formed around the infecting nematodes and causes significant cellular alteration, like multinucleate giant cells, hypertrophy and hyperplasia cells, dense cytoplasm and displacement of xylem and phloem parenchyma and other elements from their normal position.

Keywords: Nanocomposite, *Meloidogyne incognita*, okra, histopathology

Introduction
Okra, *Abelmoschus esculentus* (L.) Moench, a commercial vegetable crop cultivated throughout India. In native language, it is called 'Bhindi' is a flowering plant and it is prized for its palatable green seed pods. It belongs to Malvaceae family. It is extensively distributed in tropical, subtropical, and warm temperate regions of the world. Okra is low in calories and high in minerals like vitamin B6 and C, fibre, K, Mg, Na, Ca and Fe (Santos *et al.*, 2013) [20]. It also contain vit A and B (Gemede *et al.*, 2014) [8]. *Abelmoschus esculentus* is grown for its fibrous fruits or pods that contain spherical, white seeds it is essential to human nutrition and a good source of total minerals, vitamins, enzymes, and other nutrients that are frequently lacking in impoverished nations' diets. Okra plants that have suffered root-knot nematode disease are typically stunted and appear unhealthy, with both large and small galls on the roots. The significant factors which leads to decline in okra productivity throughout the globe is due to numerous abiotic and biotic constraints, such as lack of improved varieties, drought, diseases from number of insect pests and pathogens such as fungus, viruses, mycoplasmas and plant parasitic nematodes has been identified (Ahmad *et al.*, 2012; Srivastava *et al*., 2012, Khan *et al.*, 2017; Kayani *et al.*, 2017, 2018; Mukhtar *et al.*, 2017a, b; Mukhtar, 2018; Tariq-Khan *et al.*, 2017) [1, 24, 10, 11, 17, 18, 25]. Nanotechnology, can be used to manage soil borne pathogens including nematodes due to their ultra sub microscopic size, nanoparticles have gain the high degree of reactivity and sensitivity and thus have potential to prove very useful in controlling root-knot nematode and other plant parasitic nematodes (Cromwell *et al.*, 2014) [6]. Thus, the goal of the current study was to evaluate how the root-knot nematode changes the histopathology of okra roots and on nanocomposite treated okra roots.

Materials and Methods
The histopathological studies of nanocomposite treated okra roots (AgNP @ 50 ppm + ZnONP @ 50 ppm) and nematode inoculated roots showed significant differences in the development of females as well as giant cells and egg production.
Okra roots infested with nematode and nanocomposite treated roots (AgNP @ 50 ppm + ZnONP @ 50 ppm) were uprooted and cut into bits of 1-2 cm length and fixed in Formalin Acetic Alcohol (F.A.A.) and processed for histological studies of nematode infected roots by methods given by Sass (1964). Dehydration process was carried out in a serial concentration of ethanol (50, 60, 70, 80, 90 and 100) percent for 2-3 hours in each solution. Then it was cleared in a mix of absolute ethanol and xylol (3:1) for three hours and subsequently passed through

2:2 and 1:3 ethanol and xylol. The dehydrated root tissues were infiltrated with paraffin wax (60 ºC) to replace alcohol and then embedded in clear paraffin and made into blocks which were turned hard after hardening. Sections of 10-15 µm thickness were cut with a rotary microtome on a clean glass slide smeared with 'Mayer's albumin'. The ribbon sections were placed on 2-3 rows on clean glass slide. A few drops of water were added and the ribbon was stretched by passing the slide on the flame of a spirit lamp. The slide was dried overnight in an incubator at 40 ºC.

Processing of slide

The wax of the ribbon in the slide was dissolved by dipping in xylol for 2-5 minutes. The slide was then passed through absolute ethanol (95, 70, 50, 30) percent and distilled water for 2-5 minutes in each solution. The slide was stained for 1-2 hrs in 1 percent saffranin solution. The excess of stain was washed in distilled water until the slide was then passed through (30, 50 and 95) percent ethanol for five minutes in each solution. It was then counterstained with fast green for 5-30 seconds. Then the slide was passed through absolute ethanol and was cleared in clove oil for 5-10 minutes and then the slide was mounted in DPX mounting. DPX mounting is a chemical used to observed a nematode under the microscope.

Results and Discussion

Histopathology of okra roots infected with *Meloidogyne incognita*

Infective juveniles (J_2) cause the initial infection in the roots by penetrating the epidermis of the roots by puncturing the roots with the stylet. The transverse sections of the infected roots of okra exhibited severe infestation by the nematodes, the second stage juveniles after penetration were seen migrating towards the cortical zone. Female nematodes after feeding from the cell content acquired a swollen shape with egg mass inside gelatinous matrix where seen in the cortex region of the infected root section (Fig 2). In general, the treatment with inoculated control (Nematode only) exhibited well-formed multinucleated giant cells, when adult female feed voraciously in the giant cells, nearby cells, cortical and vascular tissues in those cells began to disintegrate, resulting in cell injury and exhibit extensive necrotic region around the cells (Fig 3). Giant cells being highly sensitive to nematode infection, were almost void of protoplasmic contents (Fig 4). Giant cells are multinucleated with large vacuoles that serve as a metabolic sink for nutrients that are supplied to the developing female throughout its parasitism. After giant cell development, the nematode infected roots showed disruption of xylem and phloem parenchyma from their normal position and abnormal cells due to the hypertrophied and hyperplastic cells (Fig 1). All types of tissues are affected by nematode development, formation of giant cells, hyperplasia and hypertrophy of cells with dense cytoplasm making it difficult to find endodermis, pericycle and structures of normal xylem and phloem (Fig 1-4). The giant cell complex is produced around the nematode head, while hypertrophied and hyperplastic cells were noticed at a little distance away from the nematode's body. The giant cells' shape and size was not uniform, some were ovoid, elongated or irregular shapes with multinucleated condition (Fig. 3 & 4).

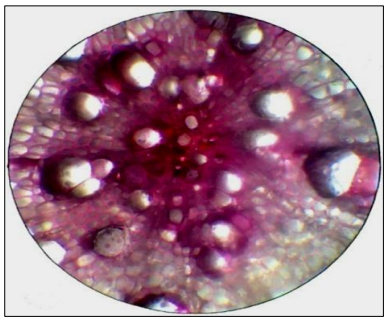

Fig 1: Transverse section of okra root showing abnormal cells formed by feeding of root-knot nematode, *Meloidogyne incognita*

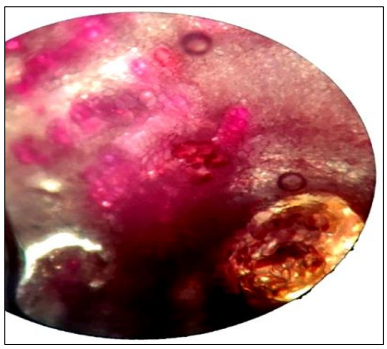

Fig 2: Transverse section of infected okra roots showing giant cells (Gc) with female nematode (N) and egg mass (EM) inside the gelatinous matrix

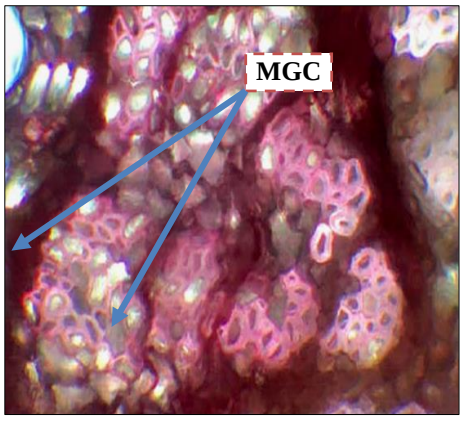

Fig 3: Transverse section of infected okra roots showing multinucleated giant cells (MGC)

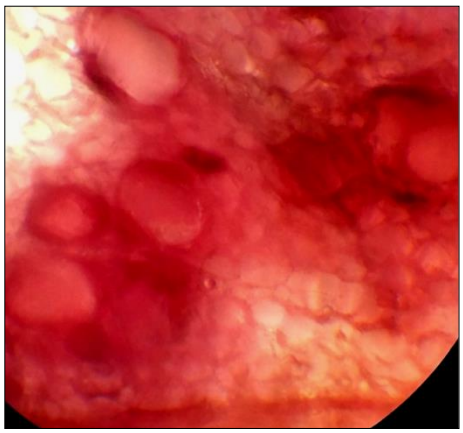

Fig 4: Transverse section of infected okra roots showing female nematode (N) and giant cell complex (Gcc) void of protoplasmic contents

These giant cells are the feeding sites for the nematode. After completing the life cycle, the adult female moved away from the roots in search of a new host, leaving behind egg masses in gelatinous matrix embedded within the cells laid by adult female (Fig 2).

Histopathology of nanocomposite treated okra roots (AgNP @ 50 ppm + ZnONP @ 50 ppm) infected with *Meloidogyne incognita*

The transverse section of nanocomposite treated okra roots (AgNP @ 50 ppm + ZnONP @ 50 ppm) were uniseriate showing parenchymatic cortex, a vascular cambium with xylem and phloem and lacked pith (Fig. 5). Though the adult female nematodes were seen in the nanocomposite treated okra roots, but it was less developed and no giant cell formation was observed (Fig. 6). Some forms of defence response could be distinguished through histological examination. As initial defence response HR like response (Hypersensitive reaction) was discernible in nanocomposite treated okra roots, showing significant necrosis and necrotic region around the cells with few egg masses (Fig 7 & 8) and also not much modification in pericycle area was noticed (5 & 6). Although the giant cell was seen in the treated okra roots, but it was fewer and less developed than root-knot nematode infected roots (Fig. 7).

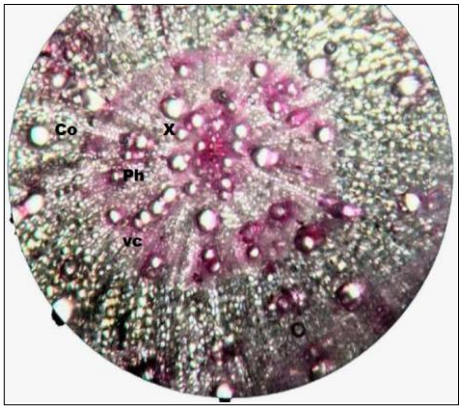

Fig 5: Transverse section of Nanocomposite treated okra roots showing xylem (X), phloem (Ph), vascular cambium (VC) and cortical parenchyma (Co)

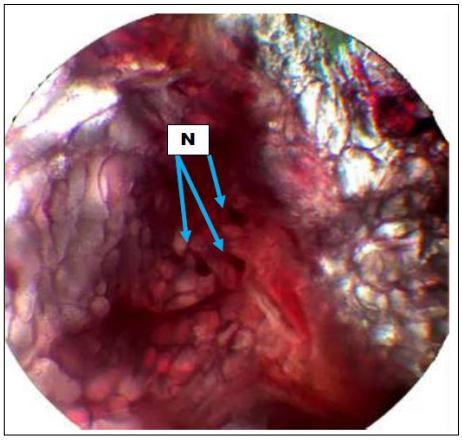

Fig 6: Transverse section of Nanocomposite treated okra roots showing less developed female nematodes (N) with no giant cell formation

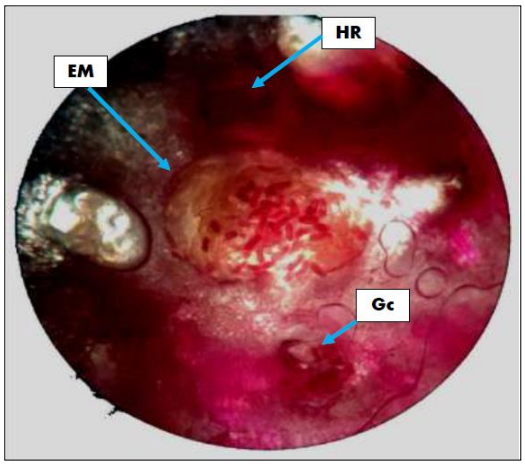

Fig 7: Transverse section of Nanocomposite treated okra roots showing poorly developed giant cells (Gc) with hypersensitive reaction (HR) and eggmass (EM)

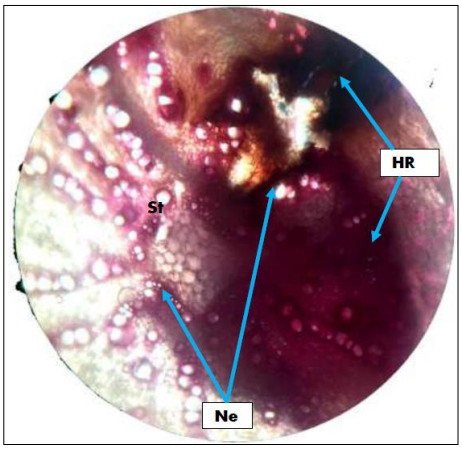

Fig 8: Transverse section of Nanocomposite treated okra roots showing hypersensitive reaction (HR) and necrotic tissue (Ne) at the periphery of the stele region (St)

The root-knot nematode infected roots and nanocomposite treated root sections of okra were observed for histopathological changes under 40 x magnifications. The root-knot nematode induces a root thickening galls and giant cells on the roots of okra. Gall formation is the most well-known sign of *Meloidogyne incognita* infection. According to Mitkowski and Abawi (2003) [16], galls are present throughout the root system and have an elongated shape and swollen appearance. However, the roots disorganised anatomical structure had no impact on the growth and development of okra. While observing transverse section of nanocomposite treated roots the penetration and development of *M. incognita* is very less and hence there was formation of necrotic layer around the cells, that may have resulted from the hypersensitivity reactions (HR) thereby inhibiting the further development of giant cells. This may be due to the secretion of phenolic compounds and certain enzymatic activities which are increased during the nematode plant incompatible responses (Plate 7 & 8). This findings is similar with Seo *et al*. (2014) [22] where they observed modified cells, along with substantial necrotic layers formation around the infecting tiny and degenerative nematodes and across middle lamella in the resistant carrot. Sreekavya *et al.,* (2019) [23] he studied the mechanisms of resistance against *M. enterolobii* in guava by

taking microtome sections of infected root tissue of *P. cattleianum* and *P. gujava.* The results revealed that in *P. gujava* giant cells were thick walled, multinucleated with rich cytoplasm. The giant cells in *P. cattleianum* appeared to be disrupted and with less dense cytoplasm. No complex galls or group feeding of nematodes was observed in *P. cattleianum.* Development of nematodes was hindered, smaller females were observed in resistant species when compared to *P. gujava* species. Due to the penetration of J_2 of *M. incognita*, the roots shows some hypersensitive like reaction thereby inhibiting the further development of giant cells. The formation of necrotic layers around the modified cells may be an incompatible response to the nematode infection and reflect the expression of resistant genes against the infecting nematode (Canto-Sáenz, 1985; Kim *et al.*, 1987) [5, 15]. The root-knot nematode causes large number of galls on the infected roots of okra. These galls are caused by cell hypertrophy of the cortex or of adjacent cells surrounding the nematode, as well as tissue hyperplasia mainly of the pericycle, this findings is in conformity with Vilela *et al.* 2021 [26] who reported that there is formation of galls and giant cell in the root-knot nematode infection site and this giant cell served as nematode feeding sites in the cortical region. The transverse section of infected okra showed the penetration and migration of 2nd stage juveniles resulting in the production of multinucleate giant cell, hypertrophy and hyperplasia with extensive necrotic region around the affected regions (Fig. 3) same finding was reported by Assumi *et al.* (2017) [4] they reported that 2nd stage juveniles penetrated into root cortex and moved along the cortical layer of cells and started feeding. During feeding process, they produced some hypertrophied cells in vascular cylinder and hyperplasia in pericycle regions. The hypertrophied cells were metabolically highly active, multinucleate and contained dense cytoplasm. As a result, locating endodermis, pericycle and structure of xylem and phloem become challenging. Giant cells typically have dense cytoplasm, multinucleate condition and thick cell walls (Fig. 21) same findings was reported by Akhtar and Hisamuddin (2015) [19]. Adjacent to the adult females head was enveloped by giant cells with thick walls. Giant cell are multinucleated with more than 10 nuclei per cell, the nuclei are enlarged and hence very prominent and extremely hypertrophied cell in the vascular cylinder and cortical cells (Fig. 3). These findings are supported by Khan *et al.* (2010) [12] on apple roots, Robab *et al.* (2010) [19] on soybean and Hamidi and Hajihassani (2020) [9] where they revealed the presence of multinucleated giant cells in root-knot nematode infected cultivars of oilseed radish and oats with multiple giant cells and severe hypertrophy in the vascular cylinder and cortical cells of the root tissues. The formation of necrosis in the roots was typically caused by fully developed female with egg masses that were surrounded by necrotic tissue. The hatched out juveniles moves around the necrotic rings and hypertropied and hyperlastic cells, and leaves the necrotic ring in pursuit of healthy tissue. Dipali *et al.* (2014) [7] also observed giant cells with very few normal cells and the fully developed female produce the eggs and were usually surrounded by necrotic tissue. Khan *et al.* (2017) they also observed that the cortex was completely destroyed, cells showing hypertrophy and hyperplasia were common, adult female deposited eggs mass into a gelatinous matrix. But in nanocomposite treated okra roots (AgNP @ 50 ppm + ZnONP @ 50 ppm) the pericycle and cortex area were visible due to the non and few invasion of 2nd stage juveniles of *M. incognita* (Fig. 5). Due to the penetration of J_2 of *M. incognita*, the nanocomposite treated roots produces some hypersensitive like reaction thereby preventing the further establishment of giant cells. This may be due to the secretion of certain phenolic compounds and enzymatic activities which is stimulated during the plant-nematode interactions. The findings are in conformity with Khan *et al.* (2010) [12] on apple roots, Robab *et al.* (2010) [19] on soybean and Vilela *et al.* (2021) [26] in okra.

Conclusion

Histopathological study of okra roots infected with *M. incognita*, induces galls and specialized feeding sites called giant cells on the roots of okra, disrupting the histological profile of root tissues. Second stage juveniles of *M. incognita* was able to enter the host tissue, feed and reproduce normally showing prominent giant cell formed around the infecting nematodes, looking intact and well grown and causing significant cellular alteration, with multinucleate giant cells, hypertrophy and hyperplasia cells condition and displacement of xylem and phloem parenchyma from their normal position with abnormal cell formation due to the feeding of nematode. Which results in noticeable damage to roots and consequently may have an impact on okra yield. Whereas, poorly developed giant cell were noticed in nanocomposite treated okra roots (AgNP @ 50 ppm + ZnONP @ 50 ppm). As initial defence response hypersensitive reaction was visible with significant necrosis and necrotic region around the cells with few egg mass thereby inhibiting the further development of giant cells. The slow growth and development of nematode may be related to the inhibition of giant cell formation and development.

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