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# Studies on scanning electron microscopy and fungal association with root knot nematode in major mulberry growing areas of Southern Karnataka

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**Abstract**
The present investigation was carried out in view of destructive nature of root knot nematode (*Meloidogyne incognita*) incidence in mulberry and to identify the associated root rot causing fungal pathogens. A roving survey was carried out in five traditional mulberry growing districts of southern Karnataka in two consecutive years (2016 & 2017). The results indicated that incidence of root knot nematode was found in all the five districts of southern Karnataka. The highest RKN incidence was noticed in Ramanagara district (50%) whereas; the lowest was recorded in Mysore district (20%). Remaining three districts *viz.*, Mandya, Chamarajnagar and Hassan showed 30, 40 and 35.5 percent incidence, respectively. The scanning electron microscopy (SEM) examinations indicated the penetration of infective second stage juveniles (J2) results in degeneration of epidermis and showed the presence of nematode egg mass and juveniles in outer periphery of root epidermis. On the basis of isolation and morphological studies, four different fungal pathogens were associated with root knot nematode and identified as *Fusarium solani* (Mart.) Sacc., *F. oxysporum* Schlecht., *Macrophomina phaseolina* and *Pythium* spp. In addition to that bio-control agents *Trichoderma harzianum* Rifai. and *T. viride* Pers and other saprophytic fungal organisms were found in the root zone of mulberry.

**Keywords:** Mulberry, root knot nematode, *Meloidogyne incognita*, SEM

## Introduction
Mulberry is a perennial crop with deep rooted, fast growing tree species and is widely adoptable to different environmental climatic conditions and only food plant for silkworm (*Bombyx mori* Linn.) [29]. In mulberry cultivation, soil-borne diseases are the major constraints in leaf production. Since, mulberry leaf is the major economic component and the quality of the leaf produced has a direct bearing on cocoon harvest. Due to repeated harvesting of leaves, the soil nutrients get depleted and make the plant vulnerable to soil-borne pathogens like root rot and root knot [7, 18].

The root knot nematode (RKN) is one of the major limiting factors in crop production throughout the world. Since, its first report on mulberry by Bessey in 1911, the RKN has been recorded in many mulberry growing countries of the world [7, 18]. RKN in mulberry is caused by *Meloidogyne incognita* (Kofoid & White) Chitwood in India [14] and is widespread and more prevalent in red sandy soils followed by red loamy soils. The severity of the RKN increases with the age of the garden and the estimated leaf yield loss is 12-25%, besides affecting leaf quality [11, 24]. The RKNs control is very difficult, because of wide host range and its ability to survive in soil for several years. Four races of *M. incognita* have been identified and all of them are found in India and other countries [9] and Race-2 has been reported to infect mulberry in India [7]. The severely infected mulberry plants show stunted growth with marginal chlorosis and necrosis of leaves. Symptoms include the formation of knots/galls on the roots which are spherical and vary in size; young galls are too small and yellowish, while old galls are big and blackish brown in colour [6, 23]. Infected plants become weak and predisposed to other diseases while severely infected plants ultimately die. The roots damaged by the disease lose their efficiency to absorb the available moisture and nutrients from the soil resulting in reduced metabolic function leading to the deterioration in leaf quality and yield [17].

*M. incognita* is an endo-parasite inhabiting mulberry roots and is a non-segmented worm belonging to the family Heteroderoidea, order Tylenchida of class Secernentea under phylum Nematoda [7]. The males are small and worm like while the females are pear shaped. Each female lays 400-500 eggs in a mucilaginous egg sac attached to the root surface. The young ones hatch out as second stage larvae after undergoing first moult within the egg. They are highly infective and enter the roots to induce the galls [27]. Soil moisture is essential for the movement of young larvae and hence the RKN is prevalent under irrigated conditions.

*M. incognita* has a wide range of host plants and attacks more than 2000 species of plants, including almost all agricultural, horticultural, oil based, ornamental, plantation and other crops [30]. *M. incognita* is more hazardous to mulberry not only because of causing direct damage to the crop but also due to its role in predisposing the plants to the attack of other pathogens. It is, therefore, vital to play appropriate control measures at early stage to avoid the loss of mulberry leaf production [18].

In consideration of all the facts and figures of mulberry root knot nematode in mulberry cultivating lands, a field survey was conducted to know the incidence of root knot nematode and association of fungal pathogens in major mulberry growing areas of southern Karnataka *viz*., Ramanagara, Mandya, Chamarajanagar, Mysore and Hassan districts. The root knot nematode infected samples were also subjected to scanning electron microscopic studies.

## 2. Materials and Methods

**2.1 Mulberry root knot nematode incidence:** A roving survey was conducted to know the root knot nematode severity in major sericulture practicing districts of southern Karnataka *viz*., Ramanagara, Mandya, Chamarajanagar, Mysore and Hassan during the year 2016 & 2017. The information on mulberry variety, area of the garden, age of the plantation, soil type, source of irrigation, plant spacing and nematode incidence was recorded. Infected root samples collected randomly from infected plants in sterile polythene bags. Further, the samples brought to laboratory for the isolation of associated micro-organisms (Plate 1). The laboratory experiments were carried out in the Molecular Biology Laboratory-I, Central Sericulture Research and Training Institute, Mysore, Karnataka.

The root knot nematode incidence was assessed by recording the number of plants showing symptoms and the total number of plants examined. In each districts, 40 gardens were selected and examined randomly and scored for nematode incidence.

**2.2 Isolation and identification of fungi associated with the mulberry root knot:** The fungi were isolated from mulberry showing typical symptoms of the disease. Roots with such symptoms were collected for the isolation purpose and followed 'root bit method' [1, 18]. The infected region from the root samples were cut into small pieces, surface sterilized with 0.1% Mercuric Chloride ($HgCl_2$) and washed with sterile distilled water for about 2-3 times; these root bits were blot dried with Whattman No. 1 filter paper discs and kept on Petri dishes containing sterilized solidified potato dextrose agar medium (PDA) in aseptic condition. The plates were incubated for 5-7 days at room temperature (27±2 °C). The pure culture of the fungus was obtained after eight days of inoculation. Such pure culture obtained was again sub cultured in potato dextrose agar slants and kept in the refrigerator at 5 °C for further studies.

Unknown fungi can be identified on the basis of their morphological and cultural characters. Morphological characters of vegetative and reproductive structures, which include the shape, size color and arrangement or attachment of spores on the sporophores or the fruiting bodies were considered for fungal species identification. These characters can be examined under the microscope directly after preparation of mounted slides using cotton blue - lactophenol stain. For studying cultural characters of a fungus, it is cultured on different synthetic/natural media in Petri plates and for the color of vegetative and aerial mycelium, appearance of colonies (margin, elevation and forms), formation of soluble pigment and color of reverse of colonies. These characters are of great significant for identification of fungi. The characters observed are compared with those of recognized groups of fungi which have already been published in various monographs/books/journals, *etc* [4, 16, 5].

**2.3 Scanning Electron Microscopy:** The transverse sections of mulberry root infected with root knot nematode and without nematode (Control) were fixed in 2.5% glutaraldehyde prepared in 0.2 M cacodylate buffer (pH 7.2) for 2 hr, washed in cacodylate buffer followed by double distilled water dehydrated in ethanol series. The dehydrated samples were critically dried in Critical Point Dryer (EMS-850) and coated by gold (20 nm thickness) in Sputter Coater (EMS-550), mounted onto copper stubs using double side sticky tape and scanned under JEOL 100 CX ASID-4D scanning electron microscope (JEOL Ltd., Tokyo Japan) at 20 kV [26].

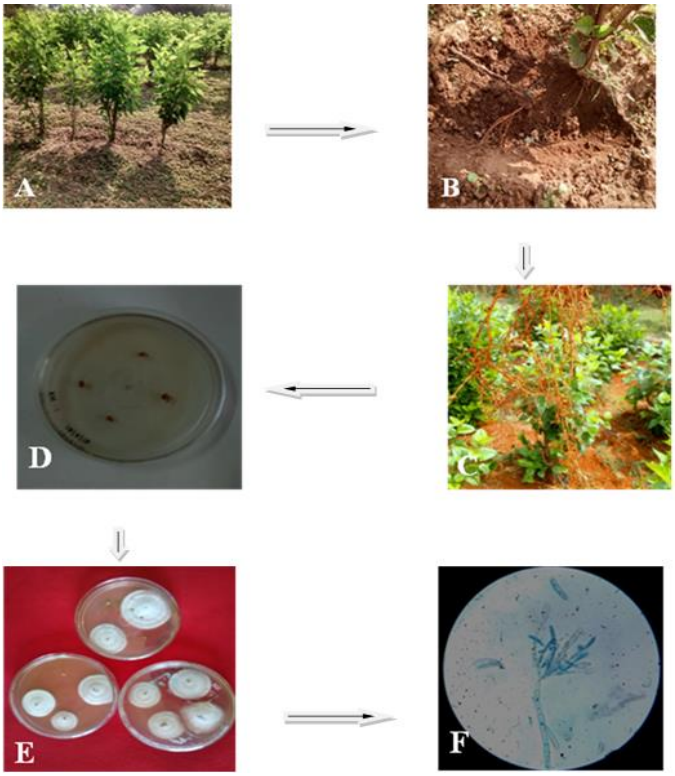



**Plate 1:** Pictorial representation of isolation of root knot nematode associated fungal organisms in mulberry; A) RKN infected mulberry gardens, B) Mulbery rhizoshpere, C) Root bit isolation method, D) Mulberry root knots, E) Fungal growth in Petri dishes, F) Microscopic examination for identification.

## 3. Results and Discussion

**3.1 Root knot nematode incidence:** RKN incidence was noticed in all the surveyed districts during February and March of 2016 & 2017. The RKN incidence ranged from 20 to 50 percent in the districts surveyed. Highest RKN incidence (50%) was found in Ramanagara district whereas, least (20%) RKN incidence was recorded at Mysore district. Remaining three districts *viz.,* Mandya, Chamarajnagar and Hassan showed 30, 40 and 35.5 percent incidence, respectively (Table 1 & Fig 1). It was in agreement with earlier reports [18] and these results are also in contradiction to earlier report and they showed that there was no incidence of RKN at Chamarajanagar district [30]. This may be due to age of the garden, since they were conducted survey during 2007-2009 and also due to the edaphic factors for reproduction of RKN [12, 13, 23, 32]. Apart from the RKN incidence, the information on type of soil, varieties, growing condition, age of the gardens and spacing were recorded in each surveyed districts and given in the Table 1.

**3.2 Type of Soil:** The RKN incidence was recorded more in red sandy loam, sandy loam soil and majority of the surveyed mulberry gardens were grown in red sandy loam soil. In Mysore and Ramanagara districts 80 percent surveyed gardens grown in red sandy loam soil, fifteen and five percent were grown in sandy loam and black soil, respectively. Whereas in Mandya, Chamarajnagar and Hassan districts 60, 40 and 37 percent surveyed gardens grown in black soil, respectively and remaining mulberry gardens grown in red sandy loam and sandy loam soil. The RKN incidence was highest in Ramanagara district this may be because of majority of the gardens grown in red sandy loam soil under irrigated condition and also age of the gardens were found more than 10 years. These results are in agreement with earlier reports, revealed that the RKN incidence and intensity were found to be very high in red sandy soils followed by red loamy soils while in black cotton soils, the incidence was found very less [11, 24]. Among the different forming systems, the incidence and intensity found to be very high under irrigated condition, poor under rainfed condition [8, 19, 22, 12, 13, 15]. The disease spread primarily through contaminated soil, farm implements and run-off irrigation water from infected plantation to healthy plantation. Plantation of infected saplings, cultivation of other susceptible crops along with mulberry and growth of some susceptible weeds in and around the mulberry gardens acts as the secondary sources of infection [6, 23].

**3.3 Variety:** Victory-1 was the major variety grown in all the surveyed districts of southern Karnataka. V-1 variety showed moderate RKN incidence as compare to other varieties like K-2 and S-36 which were showed severe incidence of root knot nematode in farmer's field. Since, K-2 and S-36 were too old varieties and become susceptible to RKN [24, 25]. In Mysore, Hassan and Ramanagara districts 85 percent of the gardens grown with V-1 variety and remaining gardens with other old varieties. Whereas, in Chamarajnagar and Mandya districts 65 and 75 percent gardens were grown with V-1 variety, respectively and remaining area with other varieties. As the V-1 variety is high yielding and more popular in major sericulture growing states of southern India. The present results are in agreement with earlier findings [11, 12, 13].

**3.4 Age of garden:** The RKN incidence was found higher in case of gardens attained >10 years of age. Whereas, low to medium level of incidence was observed in case of <10 years aged garden and it was also found true with root rot disease incidence studied by several researchers [23, 12, 13]. Old mulberry gardens may be attributed to the ability of the fungal pathogen to establish in younger root tissues [20, 24].

**3.5 Growing condition:** The surveyed districts showed majority of the mulberry gardens grown in irrigated condition in which higher RKN incidence was recorded. Soil moisture is essential for the movement of young larvae and hence, the RKN is prevalent under irrigated conditions [3, 32]. In Mysore, Hassan and Ramanagara districts eighty five percent gardens grown in irrigated condition. Whereas, in Chamarajnagar and Mandya district 65 and 75 percent gardens were grown in irrigated condition, respectively and remaining gardens were grown in restrictive irrigation and rainfed condition and results were found on par with the past studies [12, 13].

**3.6 Spacing:** Majority of the surveyed districts adopted 90 cm x 90 cm spacing in mulberry gardens. In Mysore, Hassan and Mandya districts seventy percent of gardens adapted 90 cm x 90 cm spacing and remaining gardens observed with (150 + 90) cm x 60 cm spacing. Whereas; in Chamarajnagar fifty percent gardens with 90 cm x 90 cm spacing and remaining fifty percent with (150 + 90) cm x 60 cm and also in Ramanagara, sixty percent gardens were grown with 90 cm x 90 cm spacing and forty percent with (150 + 90) cm x 60 cm spacing. There was no much difference in the incidence of RKN in different spacing and it was in agreement with earlier studies [12, 13, 24].

**3.7 Isolation and identification of fungi associated with mulberry root knot nematode**

**3.7.1 Pathogens:** A total of four plant pathogenic fungal organisms were isolated from root samples collected from the root knot infected mulberry gardens. The dry root rots causing *Fusarium solani* (Mart.) Sacc. and *F. oxysporum* Schlecht. were found to be more associated with *M. incognita* in all the surveyed districts and other root rot causing *Macrophomina phaseolina* (Tassi.) Goid. [syn. *Rhizoctonia bataticola* (Taub.) Butler] and *Phythium* spp. were found only in Ramanagara district (Table 2 & Plate 2). These results are on par with recent reports [7, 17, 18].

**3.7.2 Bio-control agents:** Two fungal bio-control agents were isolated and identified as *Trichoderma harzianum* was associated in root samples of Ramanagara and Hassan districts and *T. viride* was associated in root samples Ramanagara and Mysore districts (Table 2). The similar results were found in recent past studies [13, 18].

**3.7.3 Saprophytes:** The common contaminant saprophytic fungal cultures isolated and identified as *Aspergillus* spp. and found associated with all the root samples of surveyed districts whereas, *Penicillium* spp. was found associated with the root samples of Ramanagara, Hassan and Mysore districts (Table 2). These findings are in concurrent with recently reported studies [13].

The results of associated fungal pathogens obtained are in confirmation with the earlier reports [12, 13, 18] where they have studied the disease complex of root rot and root knot in major mulberry cultivating areas of southern Karnataka and majority of the root samples were isolated with *Fusarium* spp. In the

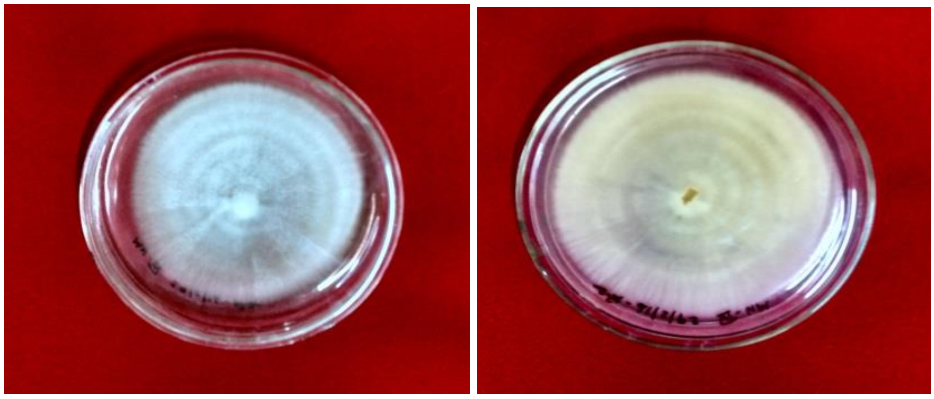
past, several studies showed that *F. solani* and *M. incognita* causes root disease complex in mulberry. It was noticed that V-1 variety grown in red sandy loam soil under irrigated condition was severely infected by *Fusarium* spp. similarly the present results are on par with the earlier survey results [19, 22, 13].

Interestingly, in the present study revealed that *Phythium* spp. was associated with root knot nematode. In India, interactions of root knot nematodes with other pathogens have been studied and in majority of the cases the association of nematode is as a predisposer of the soil-borne pathogens. Root knot nematodes interact synergistically with large numbers of root infecting fungi [10]. Among the soil-borne fungal pathogens, *Fusarium*, *Rhizoctonia*, *Sclerotium*, *Pythium*, *Phytophthora*, *Macrophomina* etc., most frequently interact in the rhizosphere of different crops like vegetables, pulses, tobacco, potato, ginger, cardamom, betelvine, jute, cotton, carnation, banana etc. Most of the interactions of fungi with *Meloidogyne* spp. result in root rotting or wilt complex of crops. *M. incognita* is one of the important limiting factors in production and productivity of black pepper in various districts of Karnataka. Further, it is involved in creating disease complexes along with fungi apart from inflicting the disease on its own. In major black pepper growing districts of Karnataka revealed the heavy incidence of root-knot nematode (RKI). The maximum mean RKI (3.52) was observed in Udupi district followed by Shimoga (3.58) and least mean RKI was observed in Kodagu district (2.73). Further, in all the districts, fungal nematode associations were observed leading to slow wilt complex [21].

### 3.8 Scanning Electron Microscope study

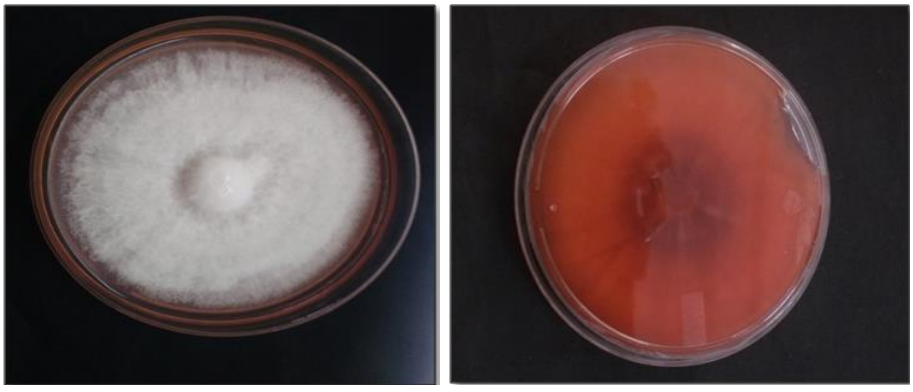
The root knot nematode infestation can be identified based on the formation of galls in root system. However, the developmental stages of the root knot nematode can be observed after penetration into root tissues (Plate 3). The SEM examinations indicated the penetration of infective second stage juveniles (J2) results in degeneration of epidermis (Fig 2 & 3). The infestation by RKN in mulberry takes place in two ways, primary infestation by J2 present in soil and secondary infestation by developed J2 within the root tissue [27]. The infected roots exhibit radial swelling due to hypertrophy and hyperplasia which is accompanied by proliferation of root hairs on the surface (Fig 4). The similar observations were made by earlier SEM study on tomato root knot nematode [28]. The egg mass of root knot nematode was observed in outer periphery of epidermis (Fig 5 & 6), within the eggs juvenile stage-I was observed under high magnification and also it was found that freshly hatched juveniles (J2) of RKN near the egg mass (Fig 7). The growing female remains initially embedded in the tissue, but when matures it induces the development of a distinct cavity around it into which eggs are deposited. The J2 hatched within the cavity migrate to other parts of root through cortex, thus completing the life cycle within the root and the adult females positioned towards root periphery develops cavity which opens to outside to release eggs inside a gelatinous matrix [27].

**1. *Fusarium solani***



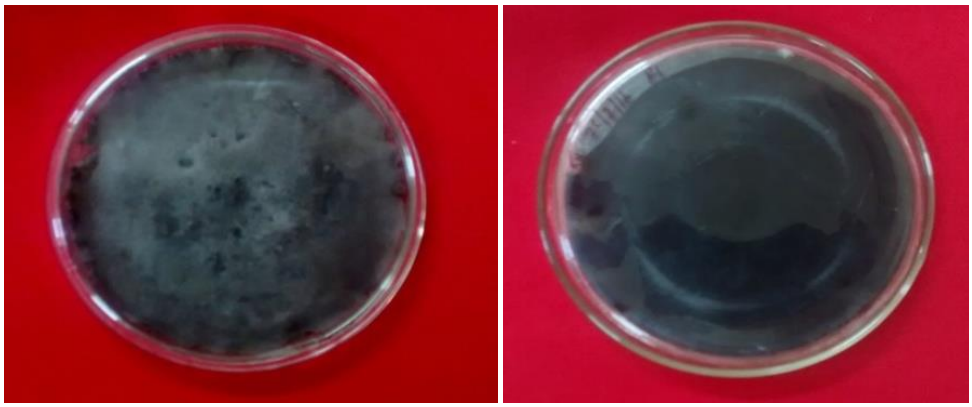
Front view Back view

**2. *Fusarium oxysporum***



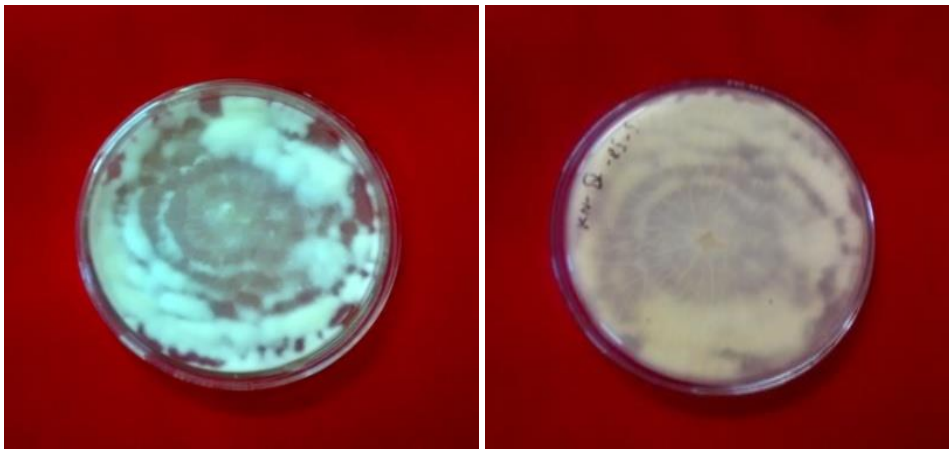
Front view Back view

**3. *Macrophomina phaseolina***



Front view Back view

**4. *Pythium* spp**



Front view Back view

**Plate 2**: Root rot causing fungal organisms isolated from root knot nematode infected mulberry root samples

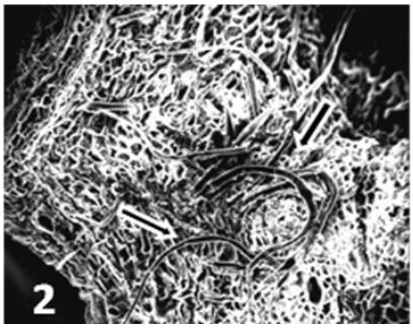



**Fig 2:** Second stage juveniles (J2) penetrating roots of mulberry (Arrows) x 330

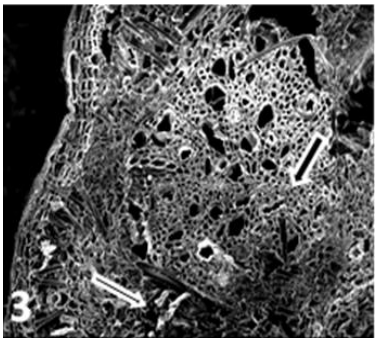



**Fig 3:** Degenerated epidermis showing the differentiated xylem tissue (Arrows) x 500

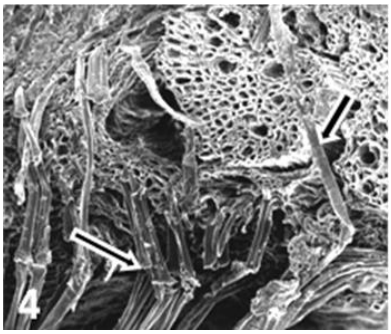



**Fig 4:** Fractured sections of epidermis layer with finger like projections (Arrows) x 500

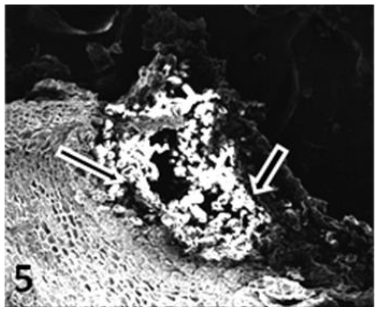



**Fig 5:** Egg mass laid by female adult nematode in outer periphery of epidermis (Arrows) x 330

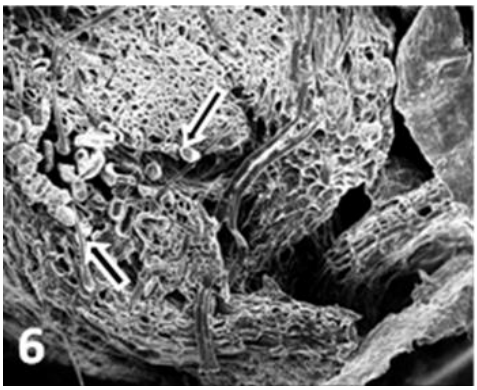


**Fig 6:** Egg mass of RKN present in the outer layer of epidermis (Arrows) x 500

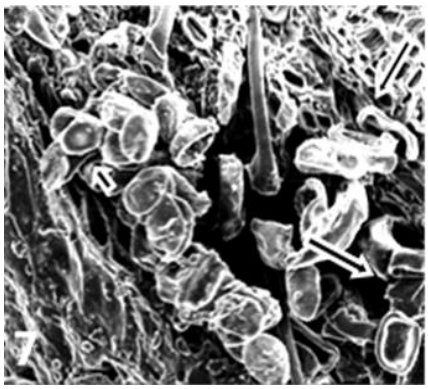


**Fig 7:** Magnified view of eggs and freshly hatched juveniles (J2) of RKN (Arrows) x 1330

**Plate 3:** Scanning electron microphotographs of developmental stages of root knot nematode in mulberry

**Table 1:** Root knot nematode incidence in traditional sericulture areas of southern Karnataka

| Name of the Districts | Variety | Area (%) | Soil type | Area (%) | Spacing (cm) | Area (%) | Growing condition | Area (%) | RKN Incidence (%) |
|---|---|---|---|---|---|---|---|---|---|
| Mysore | V-1 | 85.00 | Red sandy loam | 80.00 | 90 × 90 | 70.00 | Irrigation | 85.00 | 20.00 |
| | Others | 15.00 | Sandy loam | 15.00 | (150+90) × 90 | 30.00 | Restricted Irrigation | 10.00 | |
| | | | Black | 5.00 | | | Rainfed | 5.00 | |
| Hassan | V-1 | 85.00 | Red sandy loam | 15.00 | 90 × 90 | 70.00 | Irrigation | 85.00 | 35.50 |
| | Others | 15.00 | Sandy loam | 25.00 | (150+90) × 90 | 30.00 | Restricted Irrigation | 8.00 | |
| | | | Black | 60.00 | | | Rainfed | 7.00 | |
| Mandya | V-1 | 75.00 | Red sandy loam | 20.00 | 90 × 90 | 70.00 | Irrigation | 75.00 | 30.00 |
| | Others | 25.00 | Sandy loam | 40.00 | (150+90) × 90 | 30.00 | Restricted Irrigation | 20.00 | |
| | | | Black | 40.00 | | | Rainfed | 5.00 | |
| Chamarajanagar | V-1 | 65.00 | Red sandy loam | 23.00 | 90 × 90 | 50.00 | Irrigation | 65.00 | 40.00 |
| | Others | 35.00 | Sandy loam | 40.00 | (150+90) × 90 | 50.00 | Restricted Irrigation | 15.00 | |
| | | | Black | 37.00 | | | Rainfed | 20.00 | |
| Ramanagara | V-1 | 85.00 | Red sandy loam | 80.00 | 90 × 90 | 60.00 | Irrigation | 85.00 | 50.00 |
| | Others | 15.00 | Sandy loam | 15.00 | (150+90) × 90 | 40.00 | Restricted Irrigation | 13.00 | |
| | | | Black | 5.00 | | | Rainfed | 2.00 | |

**Table 2:** Fungal spp. associated with root knot nematode in mulberry

| Fungal Organisms | Districts Surveyed | | | | |
|---|---|---|---|---|---|
| | Ramanagara | Mandya | Chamarajanagara | Hassan | Mysore |
| I Pathogens | | | | | |
| *a. Fusarium solani* | + | + | + | + | + |
| *b. F. oxysporum* | + | + | + | + | + |
| *c. Macrophomina phaseolina* | + | - | - | - | - |
| d. *Phythium* spp. | + | - | - | - | - |
| II Bio-control agents | | | | | |
| *a. Trichoderma harzianum* | + | - | - | + | - |
| *b. T. viride* | + | - | - | - | + |
| III Saprophytes | | | | | |
| *a. Aspergillus spp.* | + | + | + | + | + |
| *b. Penicillium spp.* | + | - | - | + | + |

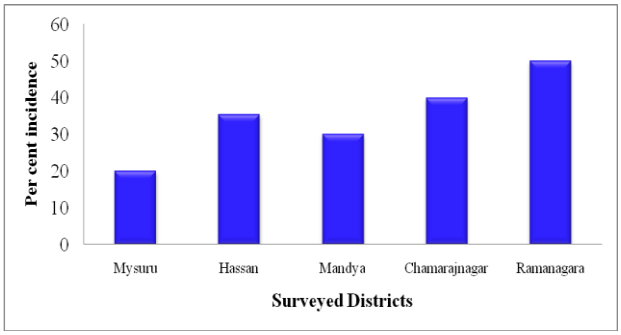



**Fig 1:** Root Knot Nematode incidence in five southern Karnataka districts

## 4. Conclusion

The present study concluded that incidence of root knot nematode in mulberry was found in five districts of southern Karnataka. The highest RKN incidence was noticed in Ramanagara district whereas; the lowest was recorded in Mysore district. A total of four root rot causing fungal pathogens were isolated from RKN infected samples. Since, the RKN is more prevalent in traditional sericulture area which further leads to the disease complex situation with root rot causing fungal pathogens. From the present study, it is understood that RKN problem in mulberry growing areas is increasing year by year and hence, resistant mulberry variety is a need of the hour to address the farmers problem along

with best management practices including broad spectrum bio-control agent (consortium of microbes) which can control both RKN and associated root rot causing fungal pathogens to manage existing mulberry gardens. Thus, RKN can be minimized at greater extent.

**5. Acknowledgment**
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