Biological Forum – An International Journal **14(2a): 398-408(2022)**

ISSN No. (Print): 0975-1130
ISSN No. (Online): 2249-3239

In vitro Studies on the Nematophagous fungus *Clonostachys rosea* (TNAU CR 01) against *Meloidogyne incognita*, a Root-knot Nematode

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(Received 01 May 2022, Accepted 18 June, 2022)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: *Meloidogyne incognita* larval mortality and egg hatching were investigated using *Clonostachys rosea* culture filtrate. Higher concentrations of the fungal culture filtrate infested *M. incognita* juveniles and impaired egg hatching. The hatching rate of *M. incognita* eggs decreased as concentrations increased. Due to the lytic effect the morphological alteration of embryo and eggshell with hyphal penetration and internal egg colonization were observed. When compared to control, culture filtrate of *C. rosea* was found effective against the nematode *M. incognita* in terms of J_2 mortality (100 percent), egg hatching inhibition (94.12 percent), and parasitization of *M. incognita* eggs (76.94 percent) after 96 hours of exposure time, indicating high biocontrol capability against the root knot nematode, *M. incognita.* Similarly different dilutions of culture filtrate significantly inhibited hatching *of M. incognita* eggs and larval emergence was inversely proportional to filtrate concentrations.

Keywords: *Clonostachys rosea*, culture filtrate, *in vitro*; *Meloidogyne incognita*, parasitism, bio-control agent, nematophagous fungus.

INTRODUCTION

Nematodes belong to the phylum Nematoda (Kingdom Animalia), which has over 25,000 species with a wide range of feeding habits, including animal-parasitic, plant-parasitic, bacterivorous, fungivorous, omnivorous, and predatory (Blaxter, 2003). Plant-parasitic nematodes are a major global hazard in field crops, vegetables, and fruit crops (Singh and Kumar, 2015). When compared to other plant parasitic nematodes that are polyphagous, endoparasites, and sedentary vascular root feeders, root knot nematodes (*Meloidogyne* spp.) are considered cryptic adversaries (Sharon *et al*., 2001).

Meloidogyne arenaria, *M. incognita*, *M. hapla*, and *M. javanica*, four common species of this genus have been identified as dangerous (Dong *et al*., 2014). *M. incognita* is the most dangerous of these species due to its wide host range, rapid reproduction rate, potential to cause complex diseases in collaboration with other pathogens, and quick generation time (Vos *et al*., 2013). Pandey and Nayak (2018) discovered that *M. incognita* alters host metabolic processes, causing alterations in the infected host at the cellular, physiological, and biochemical levels which are difficult to manage.

Nematicides based chemicals have been used to control plant-parasitic nematodes, which is now restricted due to environmental concerns, nematode resistance development (Abad *et al*., 2008), high costs, toxicity to plants, livestock, and biodiversity (Beketov *et al*., 2013). The need for more ecofriendly alternatives, such as the use of microorganisms such as fungi, bacteria, protozoa, viruses, nematodes, and mites and their derivatives from various sources, is urgent because most synthetic nematicides will likely be withdrawn from the market soon (Sarrocco & Vannaci 2018).

In the soil, nematophagous fungi are commonly associated with nematodes. These fungi either actively parasitize nematodes with sticky branches, networks, knobs, constricting and non-constricting rings, or indirectly assault them by releasing harmful chemicals and enzymes (Zouhar *et al*., 2013).

Clonostachys is a mycoparasite and opponent of pathogenic fungi, insects, and parasitic nematodes that is well known (Goh *et al*., 2020). *Clonostachys rosea* (syn. *Gliocladium roseum*) fungi are microscopic fungus that has been used as a biological agent to control plant parasitic nematodes (Trainer *et al*., 2014). *C. rosea* has showed potential lethal effects against *M. incognita* under *in vitro* condition (Wang *et al*., 2011).

In this study, *in vitro* investigations such as egg hatching inhibition, juvenile mortality, egg parasitization, and parasitization in bittergourd against root knot nematode have been assessed to study the biocontrol potential of the nematophagous fungus *C. rosea*.

MATERIALS AND METHODS

A. Pure culture of root knot nematode

Root knot nematodes were grown and maintained as pure culture in tomato plants under glasshouse conditions at the Department of Nematology, TNAU, Coimbatore. After confirming the species of root knot nematodes, egg masses were obtained from diseased roots, placed in a beaker containing sterile distilled water and allowed to develop for 4 days at room temperature. The clay pots were filled with a sterile pot mixture at the ratio of red soil, sand and FYM as 2:1:1. Root knot nematode juveniles were introduced at the root area of 25-day-old tomato seedlings. The pure culture egg masses were collected for future studies.

Morphological Identification of *M. incognita* through the Posterior Cuticular Pattern

Posterior Cuticular Pattern (PCP) was used to identify the species of root knot nematode. The root portions were stained with acid-fuchsin lactophenol, which was followed by a 24-hour period of plain lactophenol to remove the stain from the root tissue. The stained female posterior cuticular pattern, which includes a high dorsal arch of smooth to wavy striae but no obvious lateral incisures, identified it as *M. incognita* (Eisenback *et al.*, 1981).

Pure culture of fungus

Clonostachys rosea (TNAU CR 01) was isolated from Ooty and provided by the Department of Nematology, TNAU, Coimbatore. Potato Dextrose Agar (PDA) medium was used to develop and maintain the fungus.

Fungalculture filtrate preparation

C. rosea culture filtrate was produced by culturing the fungus in potato dextrose broth. The PDB medium (100 mL in 250 mL flasks) was autoclaved for 30 minutes at 15 psi. Each flask was then infected with four (5mm diameter) scoops of the fungus from an actively developing culture on Potato Dextrose Agar under sterile conditions and incubated at 25°C for 10 days. The cultures were passed through Whatmann filter paper No. 1 at the conclusion of the incubation period to remove the mycelial mats. The resulting filtrate was assigned a concentration of 100 percent. By adding the required amount of sterilized distilled water, the concentration was prepared as 25, 50, 75, and 100 percent.

Incubation test. Three ml of culture filtrate from each concentration was pipetted to 4.5cm diameter petri plates to study the effect of different concentrations of *C. rosea* culture filtrate on egg hatching of *M. incognita*. Three egg masses of *M. incognita* of uniform size were hand-picked and placed in each dish. As a control, egg masses were put in distilled water. Each treatment was carried out four times. The temperature of each petri dish was maintained at 25 °C. After 24, 48, 72, and 96 hours, data on egg hatching was collected. To compare the impact of culture filtrate with that of the medium, the same method was done using potato dextrose broth.

$$\text{Egg hatching inhibition (\%)} = \frac{\text{Total number of unhatched eggs}}{\text{Total number of eggs}} \times 100$$

Test for mortality. Three ml of culture filtrate from each concentration was placed into each petri dish, and roughly 100 freshly hatched second stage juveniles of *M. incognita* in 0.2 ml distilled water were transferred to each petri dish to evaluate the effect of varying concentrations of the culture filtrate on juvenile mortality. As a control, juveniles were placed in distilled water. Each treatment was replicated four times. To compare the effect of culture filtrate and plain medium, the number of dead (unmoved) larvae in each petri dish was counted after 24, 48, 72, and 96 hours, and their percentage were calculated.

$$\text{Mortality (\%)} = \frac{\text{Number of dead juveniles in treatment}}{\text{Number of juveniles inoculated}} \times 100$$

Parasitization of eggs. Each egg mass was placed in a petri plate with fungal culture before being incubated at 25±2°C. At 24, 48, 72, and 96 hours, observations were made. There were four replicates and the control eggmass in distilled water. Counting parasitized and non-parasitized eggs under a microscope were done to determine the % egg parasitism. Eggs that were infected by direct hyphal penetration or disintegration of their contents were considered as infected, but eggs that contained living juveniles or eggs from which juveniles had hatched were counted as viable (Khan, Williams, and Nevalainen 2006).

$$\text{Egg parasitism (\%)} = \frac{\text{Total parasitized eggs}}{\text{Total number of eggs}} \times 100$$

Penetration study. The study was to observe the effect of *C. rosea* on root knot nematode penetration in bittergourd. Fungal culture filtrate (5.4×10^7 spores/ml) was used to inoculate fifteen days old bittergourd seedlings. 100 second-stage juveniles were injected after 7 days. Plants without fungus served as a control. The observations were taken every 24 hours. Uprooted roots were dyed with acid fuchsin – lactophenol and then destaining was done with ordinary lactophenol.

Data Analysis. The AGRES programme was used to analyze the data from the above-mentioned studies, and Duncan's Multiple Range Test was used to interpret the significant means (Fisher, 1935). p=0.01 was considered as the level of significance. When the "F" test was determined to be significant, critical difference (CD) values were generated for p=0.01.

RESULTS AND DISCUSSION

The analysis of variance revealed a significant interaction between filtrates and concentrations (F × C), filtrates and time interval (F × T), concentrations and time (C × T), and among filtrates, their concentrations, and time (F × C × T).

Effect of culture filtrates on *Meloidogyne incognita* egg hatching. The results revealed that there were substantial variations (p=0.01) in the concentrations of different culture filtrates. In addition, the hatching rates of *M. incognita* eggs were inversely linked to the concentrations of filtrates, indicating that the hatching rate reduced as the concentrations increased. After four days, control eggs had the highest hatch rate (96.54%) while eggs treated with 100 percent concentration of *C. rosea* culture filtrate had the lowest hatch rate (5.87%) (Table 1).

The hatching of *M. incognita* eggs was considerably suppressed by various dilutions of the culture filtrate. Percentage of hatching was inhibited the most at 100 percent (94.12) and 75 percent (87.37) concentrations of culture filtrate, followed by 50 percent (85.5). According to Singh and Mathur (2010), eggs that contained juveniles and seemed normal as well as eggs from which juveniles had hatched out were counted as healthy. Eggs that were contaminated by direct hyphal penetration or by the dissolution of their contents were counted as infested. Egg hatching of *M. incognita* in varied concentrations of the medium filtrate was statistically equal in distilled water, it was inferred that the medium lacked ovistatic or ovicidal properties. *Pochonia chlamydosporia* showed highest egg parasitism, egg hatch inhibition and juvenile mortality of *M. incognita* at 25, 50, 75 and 100 percent concentrations (Annapurna *et al*., 2018).

Effect of culture filtrate on *Meloidogyne incognita* juvenile mortality. The concentration of fungal filtrates and the length of exposure were shown to be directly linked to juvenile mortality. At 100 percent concentration of *C. rosea*, greatest death rate of J2 (63.75 percent) occurred after 24 hours of exposure.

The mortality of juveniles increased as the concentrations of the filtrates progressively increased. After 96 hours of exposure at 100% concentration of *C. rosea* filtrate, the maximum mortality (100%) occurred. In *C. rosea* filtrate at 25% concentration, the lowest mortality was 62.75 percent (Table 2). The findings also revealed that *M. incognita* second-stage juveniles were more susceptible to these fungus secondary metabolites. The percentage of highest levels of juvenile mortality were seen in the culture filtrate concentrations of 100 percent (100.00) and 75 percent (86.25), followed by 50 percent (76.75). Nematodes that were rigid and elongated with head and tail sometimes slightly bent were considered as immobilized and if they did not react when probed with a fine needle were considered as paralyzed

The most promising fungus for controlling *M. incognita* was *C. rosea*, and its nematicidal impact was proven (Wang *et al*., 2011). This fungus may generate a huge number of conidia in a short period of time, which adhere to nematodes and germinate in the body cavity. Proteases, collagenase, and chitinase are hydrolytic enzymes produced by the fungus, and they may be implicated in nematode cuticle penetration and host cell disintegration. Hussain (2017) found that incubation in *C. rosea* resulted in 70.2 percent inactivation of *M. hapla* J_2 after 48 hours and an 86 percent inactivation after 72 hours.

***M. incognita* egg parasitization.** In the study of egg parasitization, *C. rosea* had the highest rate (63.97%) (Table 3). The egg shells of the fungi-infested eggs disintegrated and shrank abnormally. Immature eggs that had been parasitized by fungus had destroyed embryos, protruding interior contents, and spores filled the eggs.The eggs, either infected by direct hyphal penetration or disintegration of their contents, were counted as parasitized (Khan, Williams, and Nevalainen 2006), while eggs that contained live juveniles and eggs from which juveniles had hatched were counted as unparasitized. Due to lytic effect the morphological alteration of embryo and eggshell, with hyphal penetration and internal colonization were observed. Filtrates from cultures of *Fusarium* spp., *Paecilomyces lilacinus*, and *Pochonia chlamydosporia* were toxic to *M. incognita* second stage juveniles, inhibited hatching and/or suppressed egg or J_2 populations on plants (Meyer *et al.*, 2004).

***C. rosea* effect on juvenile penetration.** Under glass house conditions, second stage juvenile penetration on bittergourd roots were observed up to 7 days after inoculation (DAI) on 3, 4, 5, and 7 DAI. The number of nematode penetration in *C. rosea* treated seedlings was reduced considerably by 78.82 percent, 80.43 percent, 81.20 percent, 75.56 percent and 76.94 percent respectively, compared to control (Table 4).

Regardless of DAI, *C. rosea* prevented juvenile penetration in roots, according to the outcomes. *C. rosea* showed the greatest decrease (76.94 percent) after 7 days when compared to the control. In order to promote colonization and provide resistance against harmful interactions, fungal partners release bioactive molecules such enzymes, short peptide effectors, and secondary metabolites (Zeilinger *et al.*, 2015). Antibiosis, direct parasitism, and enzyme secretion are the techniques used by *C. rosea* to kill nematodes (Iqbal *et al*., 2018). Its hyphae can parasitize *M. incognita* eggs and adults. Conidia adhere to the nematode's cuticle at first, then germinate and pierce the nematode's cuticle, killing it (Zhang *et al*., 2008). The production of nematotoxic secondary metabolites has also been described, however the precise site of action has yet to be determined (Song *et al*., 2016).

Table 1: Effect of different concentrations of fungal culture filtrate on *M. incognita* egg hatching.

Treatments	Number of egg hatched *															
	25%				50%				75%				100%			
	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
T_1 - *Clonostachys rosea*	49.50^{ac} (7.01)	66.00^{ac} (8.10)	79.50^{ac} (8.89)	99.00^{ac} (9.92)	37.50^{ac} (6.10)	60.7 ^{ac} (7.77)	73.50^{ac} (8.55)	87.00^{ac} (9.30)	25.25^{a} (5.01)	53.25^{a} (7.27)	67.50^{a} (8.19)	75.75^{a} (8.68)	2.25^{a} (1.50)	28.00^{a} (5.27)	28.50^{a} (5.32)	35.25^{a} (5.92)
T_2 -Broth	72.75^{bc} (8.50)	111.25^{bc} (10.51)	288.00^{bc} (16.92)	393.75^{bc} (19.78)	71.00^{bc} (8.40)	106.25^{bc} (10.28)	238.25^{bc} (15.39)	340.00^{bc} (18.38)	98.25^{b} (9.88)	106.00^{b} (10.26)	194.00^{b} (13.88)	241.75^{b} (15.50)	62.7^{b} (7.90)	88.00^{b} (9.35)	186.25^{b} (13.60)	291.25^{b} (17.01)
T_3 -Control	86.00^{bc} (9.24)	148.25^{c} (12.14)	377.75^{c} (19.37)	562.25^{c} (23.64)	88.50^{c} (9.38)	140.25^{c} (11.81)	385.25^{c} (19.57)	568.50^{c} (23.77)	88.00^{c} (9.35)	147.50^{c} (12.11)	384.00^{c} (19.53)	564.50^{c} (23.68)	89.50^{c} (9.43)	137.50^{c} (11.69)	393.75^{c} (19.78)	579.25^{c} (23.99)
SEd	0.5403	0.6755	1.0221	1.2161	0.5255	0.6563	0.9884	1.1813	0.5445	0.6557	0.9512	1.1119	0.4654	0.5961	0.9236	1.1268
CD (p=0.01)	1.7559	2.1953	3.3218	3.9522	1.7078	2.1329	3.2124	3.8392	1.7696	2.1311	3.0915	3.6137	1.5124	1.9373	3.0016	3.6620

*Mean of four replications; square root transformed results are in parenthesis. Duncan's Multiple Range Test (DMRT) shows that means in a column following a different alphabet are significantly different at the 1% level of significance.

Table 2: Effect of different concentrations of fungal culture filtrate on *M. incognita* juvenile mortality.

Treatments	Number of juveniles dead*															
	25%				50%				75%				100%			
	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
T_1 - *Clonostachys rosea*	26.50^{ac} (5.13)	39.75^{ac} (6.91)	53.00^{ac} (7.26)	62.75^{ac} (7.90)	38.75^{a} (6.21)	56.00^{a} (7.46)	67.75^{a} (8.21)	76.75^{a} (8.73)	52.75^{a} (7.24)	73.25^{a} (8.53)	85.50^{a} (9.22)	86.25^{a} (9.27)	63.75^{a} (7.96)	97.75^{a} (9.89)	100.00^{a} (10.00)	100.00^{a} (10.00)
T_2 -Broth	0.00^{bc} (0.71)	6.75^{bc} (2.59)	12.50^{bc} (3.52)	17.75^{bc} (4.20)	0.00^{b} (0.71)	7.25^{b} (2.68)	12.00^{b} (3.45)	12.75^{b} (3.56)	0.00^{b} (0.71)	8.75^{b} (2.95)	13.25^{b} (3.63)	16.00^{b} (3.99)	0.00^{b} (0.71)	7.75^{b} (2.78)	11.25^{b} (3.34)	20.00^{b} (4.46)
T_3 -Control	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)	0.00^{c} (0.71)
SEd	0.1927	0.2553	0.3030	0.3359	0.2331	0.2978	0.3343	0.3542	0.2719	0.3390	0.3335	0.3282	0.2989	0.1172	0.1256	0.1674
CD (0.01)	0.6264	0.8297	0.9848	1.0917	0.7574	0.9677	1.0866	1.1511	0.8837	1.1018	1.0838	1.0668	0.9715	0.3810	0.4081	0.5442

* Mean of four replications; square root transformed results are in parenthesis. Duncan's Multiple Range Test (DMRT) shows that means in a column following a different alphabet are significantly different at the 1% level of significance.

Table 3: Effect of fungus on *M. incognita* egg parasitization.

Treatments	Number of eggs in the egg mass *	Number of eggs infected with fungus*	Percentage of eggs parasitized (Percent)
T_1- *Clonostachys rosea*	182.1^{a} (13.45)	116.5^{a} (10.76)	63.97
T_2- Control	243.2^{b} (15.59)	0.0^{b} (0.71)	0.0
SEd	0.62	0.49	
CD (p=0.01)	2.30	1.83	

* Values in parenthesis are square root transformed values; values in parentheses are mean of ten replications. Duncan's Multiple Range Test (DMRT) shows that means in a column following a different alphabet are significantly different at the 1% level of significance.

Approximately 38 metabolites from *C. rosea* have been described so far, with a considerable structural variation (Abdel-Wahab *et al*., 2019; Supratman *et al*., 2019). Gliocladin C and 5-n-heneicosyl resorcinol displayed nematotoxic action against zooparasitic nematodes (Song *et al*., 2016). *C. rosea* mode of action against nematodes, according to Iqbal *et al*. (2018), is antibiosis rather than parasitism. Chitinase and proteases are enzymes that hydrolyze the nematode cell wall's-(1-4) glycosidic linkages (Yang *et al*., 2010).

Nematicidal action of culture filtrates of *V. chlamydosporium* against *M. javanica* may be attributed to the production of certain enzymes (Segers *et al*., 1994) and toxins like Verticillin A, B and C which help in weakening and dissolving the barriers of its hosts. Species of *Aspergillus*, *Penicillium*, *Talaromyces*, *Curvularia* and *Aternaria* are known to produce toxins and antibiotics like aflatoxin, penicillin, vermiculin, vermicillin, talaron, vermistatin, viridin, fusaricacid, rhizopin, lilacinin, leucinostatin, P-168 and phytoalternarin.

The findings showed that the *Clonostachys rosea* fungus parasitizes nematodes by excreting chemicals that can paralyze or kill J_2 of the root-knot nematode, *M. incognita*. A number of nematophagous fungi are known to have proteolytic and chitinolytic activities which cause alteration in eggs' cuticular structure, changes in egg shell permeability or cause perforations in the cuticle which allows seepage of toxic metabolites into the eggs and cause physiological disorders (Tariq Mukhtar and Ijaz Pervaz 2003).

CONCLUSION

To summarise, the current study found that *Clonostachys rosea* efficiently decreased egg hatchability and mortality of infective juveniles of root knot nematode and parasitized *M. incognita* eggs under *in vitro* studies.

Table 4: Influence of fungus on *M. incognita* penetration (J_2).

Treatments	Number of J_2 penetrated into roots (Days after inoculation (DAI))*							
	3DAI	Percent decrease over control (%)	4DAI	Percent decrease over control (%)	5DAI	Percent decrease over control (%)	7DAI	Percent decrease over control (%)
T_1 - *C. rosea* + Nematodes	4.5[a] (2.09)	78.82	6.75[a] (2.56)	80.43	12.5[a] (3.48)	81.20	17.00[a] (4.06)	76.94
T_2 –Nematodes alone	21.25[b] (4.54)	-	34.5[b] (5.79)	-	66.5[b] (8.08)	-	73.75[b] (8.46)	-
SEd	0.28	-	0.36	-	0.50	-	0.54	-
CD (p=0.01)	0.82	-	1.04	-	1.45	-	1.55	-

* Values in parenthesis are square root transformed values; values in parentheses are mean of four replications. Duncan's Multiple Range Test (DMRT) shows that means in a column following a different alphabet are significantly different at the 1% level of significance.

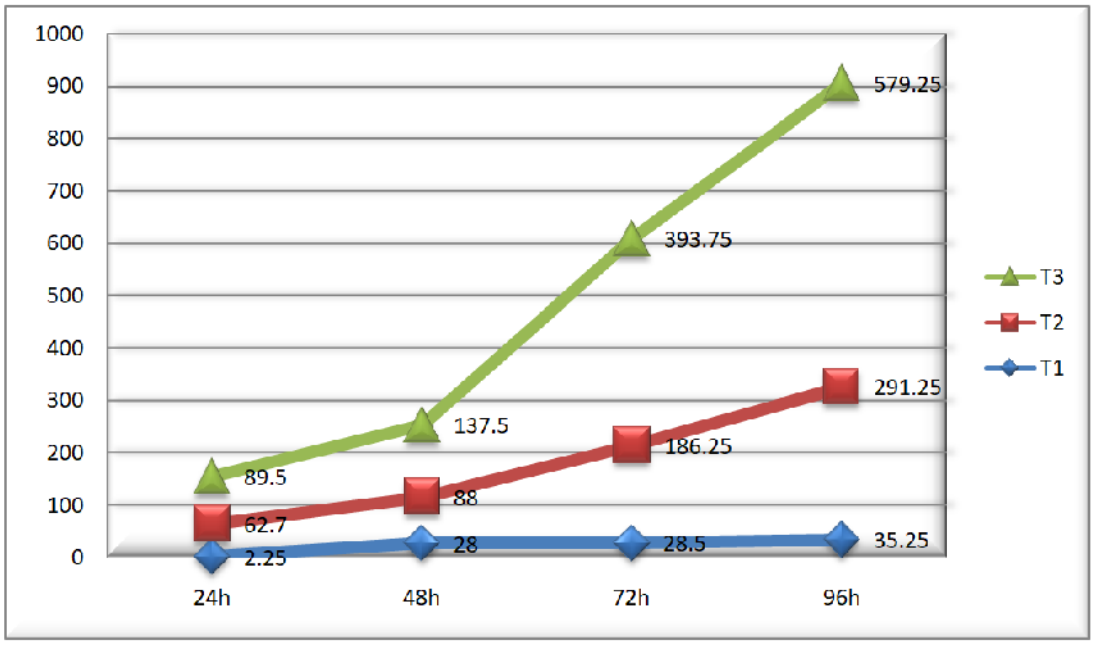


Fig. 1. Effect of fungal culture filtrate on *M. incognita* egg hatching inhibition.

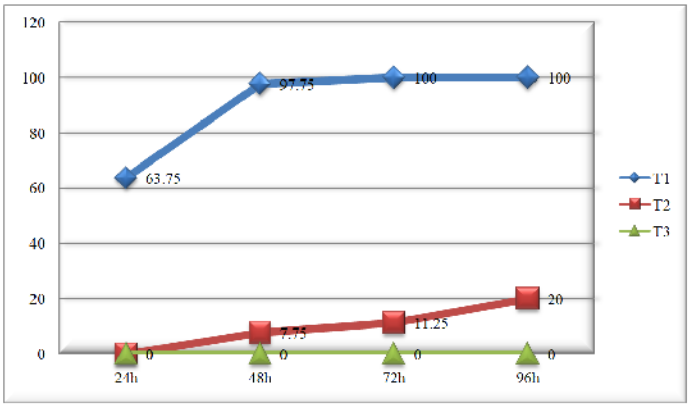


Fig. 2. Effect of fungal culture filtrate on *M. incognita* juvenile mortality.

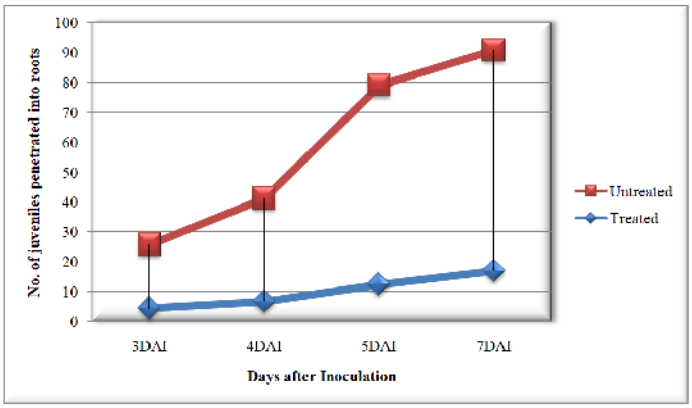
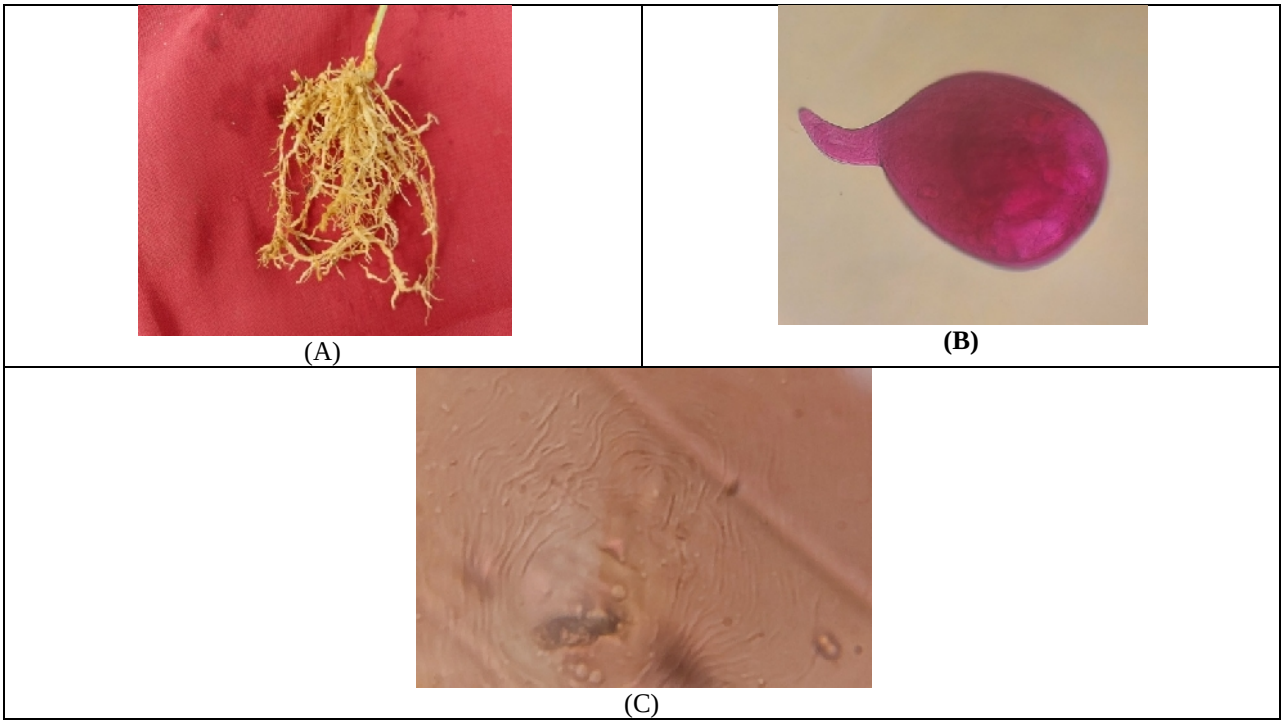


Fig. 3. Influence of fungus on *M. incognita* penetration (J_2).



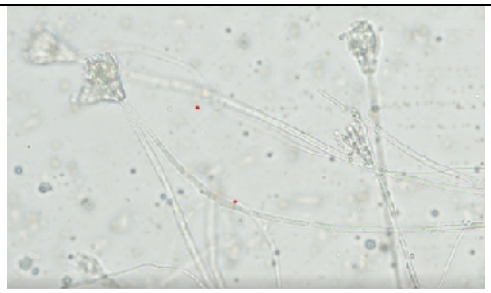
A. Infected root sample B. Adult female (10X) C. Posterior cuticular pattern of *M. incognita* (40X)

Fig. 4. Morphological identification of root knot nematode, *Meloidogyne incognita.*

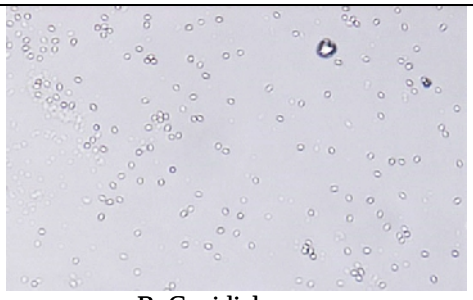
A. Creamy white mycelia

B. Light creamy yellow in reverse

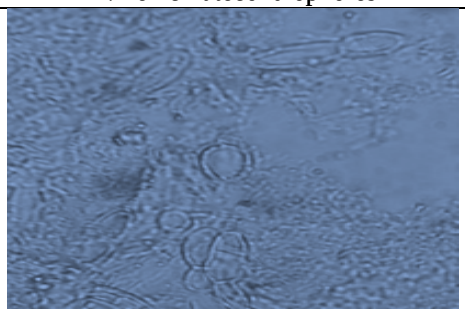
Fig. 5. Colony growth on PDA plates after 25 days.



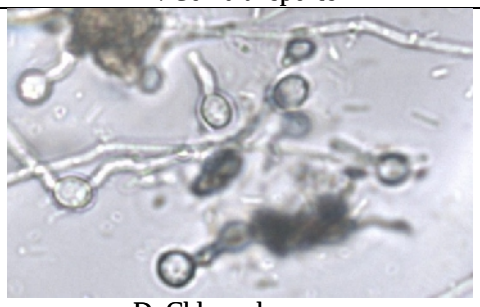
A. Penicillatecondiophores



B. Conidial spores

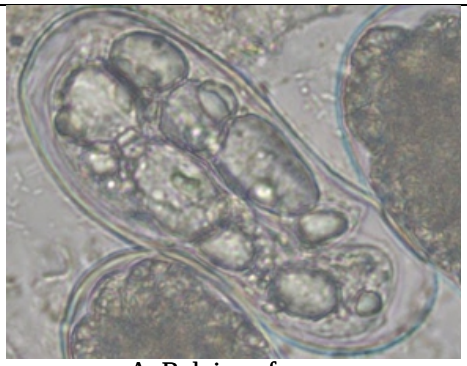


C. Hyphal coil

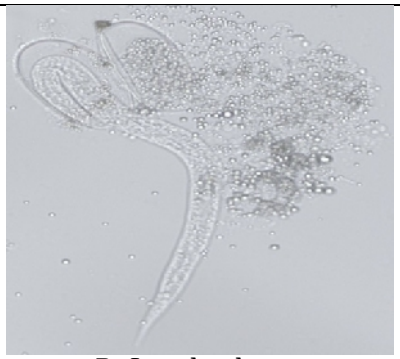


D. Chlamydospores

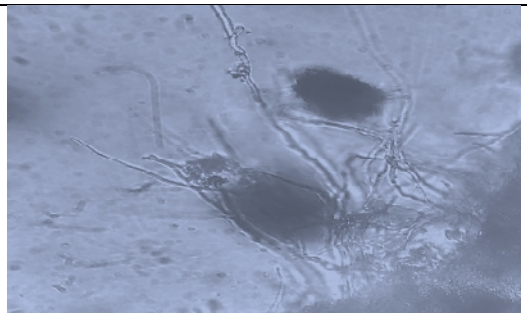
Fig. 6. Morphological description of *C. rosea.*



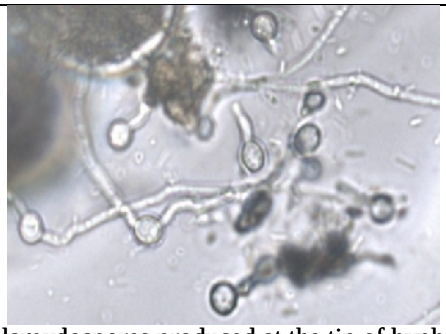
A. Bulging of eggs



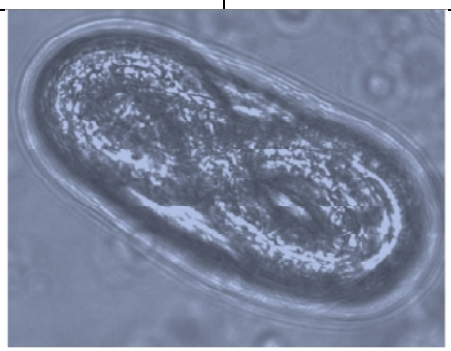
B. Sporulated eggs



C. Hyphal growth on eggs

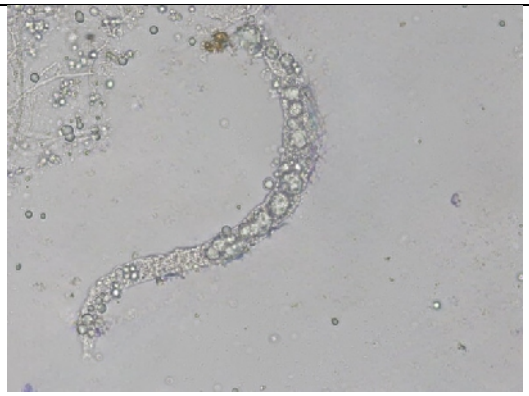


D. Chlamydospores produced at the tip of hyphae on eggs

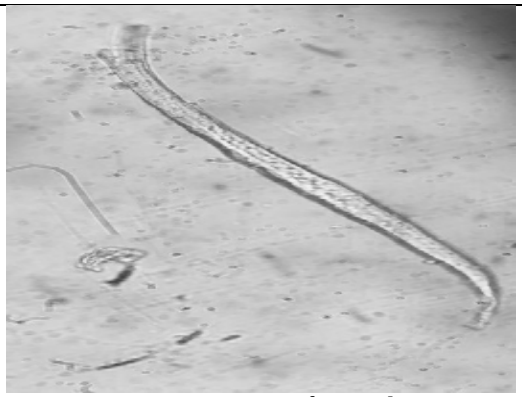


E. Juvenile inside the egg

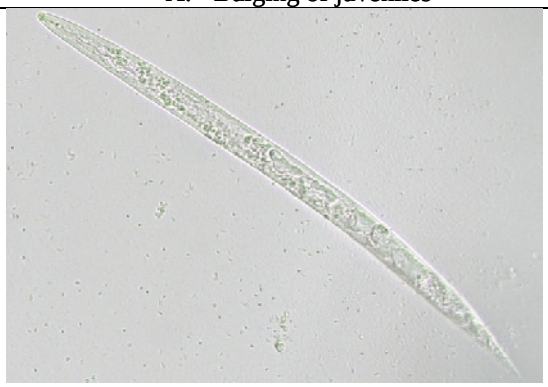
Fig 7. *In vitro* study of *C. rosea* against *M. incognita.*



A. Bulging of juveniles



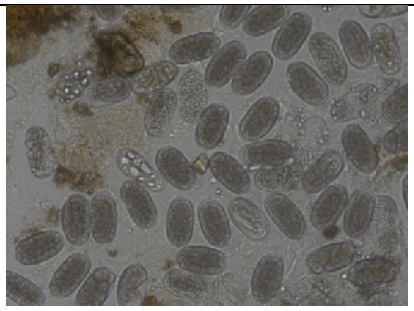
B. Disintegration of juveniles



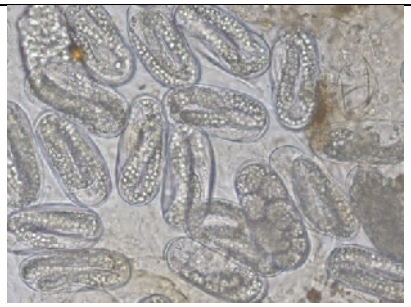
C. Dead juveniles

D. Healthy juveniles

Fig. 8. *In vitro* study of *C. rosea* against second stage juveniles of *M. incognita.*

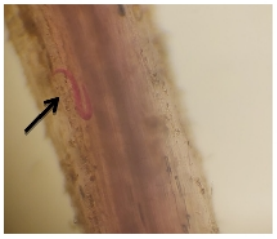


A. Parasitized eggs of *C. rosea*

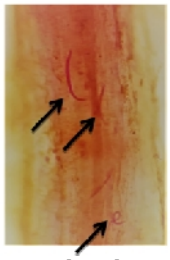


B. Healthy eggs

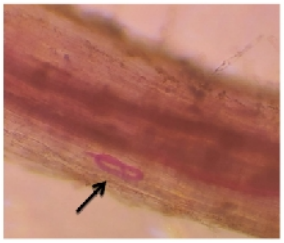
Fig. 9. Egg parasitization of *C. rosea.*



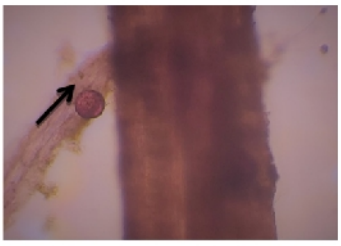
A. J_2 inside the root cortex



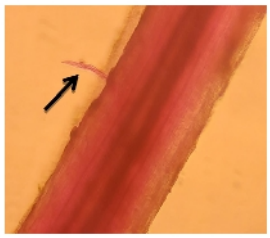
B. J_2 within the root



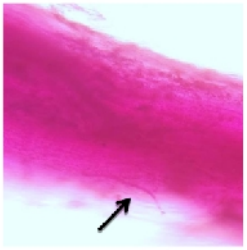
C. In search of feeding site



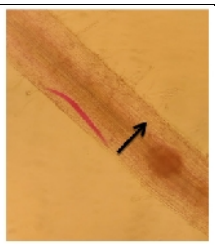
D. Adult female



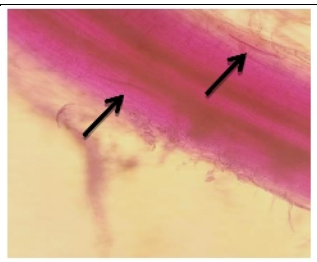
E. J_2 Penetration



F. J_2 within the root



G. In search of feeding site



H. Remain as J_2

A, B, C, D –Untreated: E, F, G and H –Treated roots

Fig. 10. Root penetration study.

Acknowledgement. I acknowledged my sincere gratitude to the Department of Nematology at Tamil Nadu Agricultural University, Coimbatore, for providing the culture of *C. rosea* as well as the laboratory facilities needed to carry out the experiment successfully.

Conflict of interest: None.

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How to cite this article: Shaliha B., G. Jothi, N. Swarnakumari and L. Rajendran (2022). *In vitro* Studies on the Nematophagous fungus *Clonostachys rosea* (TNAU CR 01) against *Meloidogyne incognita*, a Root-knot Nematode. *Biological Forum – An International Journal*, *14*(2a): 398-408.