Biological Forum – An International Journal **14(1): 108-114(2022)**

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

Biopolymer Chitosan for the Management of Root Knot Nematode, *Meloidogyne incognita* and Root Pathogenic Fungus, *Fusarium solani* Infecting Tomato

R. Mouniga[1*], B. Anita[2], A. Shanthi[3], A. Lakshmanan[4] and G. Karthikeyan[5]
[1]Ph.D. Scholar, Department of Nematology,
Tamil Nadu Agricultural University, Coimbatore, (Tamil Nadu), India.
[2]Professor, Directorate of Open Distance and Learning,
Tamil Nadu Agricultural University, Coimbatore, (Tamil Nadu), India.
[3]Professor and Head, Department of Nematology,
Tamil Nadu Agricultural University, Coimbatore, (Tamil Nadu), India.
[4]Professor and Head, Department of Nano Science and Technology,
Tamil Nadu Agricultural University, Coimbatore, (Tamil Nadu), India.
[5]Professor and Head, Department of Plant Pathology,
Tamil Nadu Agricultural University, Coimbatore, (Tamil Nadu), India.

(Corresponding author: R. Mouniga)*
(Received 12 October 2021, Accepted 06 December, 2021)
(Published by Research Trend, Website: www.researchtrend.net)

ABSTRACT: The endoparasitic root knot nematode, *Meloidogyne incognita* and wilt pathogen, *Fusarium solani* cause severe yield loss in tomato. At present more emphasis is being given to develop environmentally safe pest and disease management technologies for sustainable crop production. Experiments were conducted to access the efficacy of chitosan at different concentrations against root knot nematode, *M. incognita* and root pathogenic fungus, *Fusarium solani*. Under lab conditions nematode egg hatching and juvenile mortality was recorded. In pot culture experiments, nematode population in roots, gall index and plant growth were recorded. The effect of chitosan on fungal growth was also assessed using poison food technique. Exposure of *M. incognita* egg mass to 5000 ppm and 10,000 ppm chitosan decreased the hatchability of eggs by 96.19% to 100% within 24 hrs time interval , compared to control. Similarly chitosan at 5000 ppm and 10,000 ppm concentration caused 100% infective juvenile mortality. Chitosan was also found to inhibit the growth of *F. solani.* Under pot culture condition, application of chitosan at 10,000 ppm concentration decreased the number nematode population and enhanced plant growth. There was 51.72% reduction in the number of *M. incognita* adult females in roots of treated plants and 61.84% reduction in eggs over untreated control.

Keywords: Chitosan, egg masses, *Fusarium solani*, infective juveniles, *M. incognita* and tomato.

INTRODUCTION

Agriculture is fundamental source of national prosperity. It plays the most significant role of India's economy (Pathak, 2009). Almost 43% of geographical area is occupied by the agriculture sector (Arjun, 2013). The endoparasitic root knot nematode, *Meloidogyne incognita* has been reported to causes severe yield loss in all vegetables including tomato (Khanna and Hema, 2018). Ramyabharathi *et al.,* (2012) reported tomato *Fusarium* wilt caused drastic yield loss up to 45 % in India Since the availability of nematicides is limited, the use of alternative nematode control methods is warranted. Chitosan is the second most abundant bio-polymer in the earth and is extracted from the crustaceous shell, shrimps (Mirzadeh *et al.,* 2002) and fungi (Pochanavanich and Suntornsuk, 2002). Crab exoskeleton is considered as the major source of chitosan (15–30%) and it is composed of minerals (30–50%) and 15–50% protein (Joesph *et al.,* 2021). It possess anti-bacterial (Fei *et al.,* 2001*)*, anti-viral, anti-fungal (Rabea *et al.,* 2003) and nematicidal properties (Asif *et al.,* 2017). It also enhances plant growth parameters *viz.,* root length, shoot length and shoot weight (Singh *et al.,* 2015). The use of chitosan in plant protection against pests and pathogens is more advantageous compared with the chemical nematicides, as it is physically and biologically functional,

biodegradable and biocompatible with tissues and cells (Rabea *et al.,* 2003; Chung *et al.,* 2004). The use of chitosan can be integrated with various nematode management programs and a safe method for human health and environment. Chitosan being a biopolymer and considering its biodegradable nature, this study was conducted with the objective to assess the efficacy of chitosan against root knot nematode, *M. incognita* and root pathogenic fungus *F. solani* in tomato.

MATERIALS AND METHODS

Root knot nematode culture. Pure culture of root knot nematode, *M. incognita* was maintained in a glasshouse at the Department of Nematology, TNAU, Coimbatore. Fifteen days old tomato seedlings (variety-Shivam) were transplanted in to 5kg pot containing pot mixture (1 part organic manure: 2 part red earth: 1 part sand). Egg masses were collected from the root knot nematode infected galled roots. Freshly hatched juveniles (5000 J2) were inoculated to tomato seedlings for experiments. For laboratory studies egg masses were collected from *M. incognita* infected galled roots of tomato and sterilized with 0.5% sodium hypochlorite solution for 10 seconds. Infective juveniles were obtained from fully matured egg masses of *M. incognita*.

Root pathogenic fungus culture: Roots samples were collected from tomato plants at Thondamuthur, Narasipuram region of Coimbatore district with the latitude and longitude of 10.991 and 76.77 respectively. Roots of wilted plants were cut longitudinally (1cm diameter) and surface sterilized with 70 % ethanol and sterile water and placed in half plate Potato Dextrose Agar media (PDA). The fungus from the root was further sub-cultured in Potato Dextrose Agar medium.

Characterization of fungus:

Morphological characterization. The fungal culture was observed under a compound microscope for micro conidia, macro conidia and chlamydaspores (Rao *et al.,* 2019).

Molecular characterization: Extraction of DNA from the fungus was done by CTAB method (Lee *et al.,* 1988).

The isolated DNA was used for Polymerase Chain Reaction (PCR) and two universal (ITS1 5'-TCCGTAGGTGAACCTGCGG-3' and ITS4-5' TCCTCCGCTTATTGATATGC-3') primers were used for identification of species. Reaction were carried out in a 25 µl reaction mixture (Master mix-12.5 µL, ITS1-2.5 µL, Reverse primer- 2.5 µL, Distilled water -5 µL and DNA 2.5 µL). DNA amplification were performed in a PCR machine (Ependrof master cycle Germany) using initial denaturation 94°C, 5 minutes, followed by 35 amplification cycle (denaturation 94°C for 1 minutes, primer annealing at 55°C for 30 sec and extension 72°C for 1 minutes) and a final extension at 72°C for 10 minutes. Amplified PCR products were casted in agarose gel electrophoresis for 60 minutes and documented.

Preparation of different concentration of chitosan. Medium molecular weight chitosan Sigma Aldrich was used in this study. Different concentrations of chitosan *viz.,* 100 ppm, 500 ppm, 1000 ppm, 5000 ppm and 10,000 ppm were prepared from 3% chitosan dissolved in acetic acid.

Bio-efficacy of chitosan under *in-vitro* conditions:

Hatching of root knot nematode eggs. Different concentrations (100 ppm, 500 ppm, 1000 ppm, 5000 ppm and 10,000 ppm) of chitosan were tested on sterilized egg masses of tomato root knot nematode, *M. incognita* under *in-vitro* conditions (Hallmann *et al.,* 1999). From each concentration, 2ml was taken and tested against one nematode egg masses per replicate. Totally six treatments and four replications were maintained in a Completely Randomized Block Design. Number of hatched juveniles from the egg mass was observed at 24 hour interval for four days.

Root knot nematode juvenile mortality. One ml suspension of water containing 100 infective juveniles was added to different concentrations of chitosan (100 ppm, 500 ppm, 1000 ppm, 5000 ppm and 10,000 ppm) in Petri-plates. Totally six treatments and four replications were maintained in a Completely Randomized Block Design. Number of dead juveniles was observed at different 24 hour intervals for four days.

Bio-efficacy of chitosan on root knot nematode, *M. incognita* under pot culture condition. Based on the laboratory studies three best concentrations of chitosan were evaluated for its effect on, *M. incognita* under pot culture condition. Fifteen days old tomato seedlings were transplanted in to sterilized pot mixture. Five hundred numbers of infective juveniles were inoculated on tomato plants. After one week of nematode inoculation, soil drenching was done with 20ml of 3% chitosan from each concentration (1000 ppm, 5000 ppm and 10,000 ppm). One treated check, carbofuran was also maintained. After 35^{th} days of nematode inoculation, plants were uprooted and observed for plant growth parameters and nematode population. Totally five treatments and four replications were maintained under Completely Randomized Block Design. Number of egg masses/ plant, gall index (Heald *et al*., 1989) and plant growth parameters were observed after 35th day of inoculation.

Bio-efficacy of chitosan against root pathogenic fungus. The bioefficacy of chitosan against root pathogenic fungus was evaluated using poison food technique (Gupta and Tripathi, 2011). Various concentrations *viz.,* 300 ppm, 500 ppm, 1000 ppm and 2000 ppm were prepared from 3% chitosan and added to Potato Dextrose Agar (PDA) media in Petri plates. After solidification of PDA media, one loop of fungus was placed in the centre of each Petri-plate. The radial

growth of the fungus in Petri plates was recorded. Five treatments and three replications were maintained under Completely Randomized Block Design.

Statistical analysis. The data obtained from the various experiments were analyzed statistically using ANOVA and Duncan's Multiple Range Test (DMRT) (Panse and Sukhatme, 1954).

RESULTS AND DISCUSSION

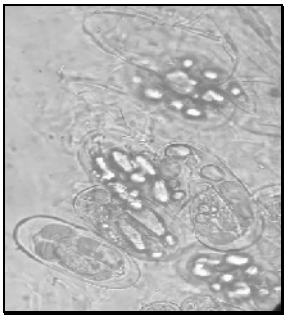
Bio-efficacy of chitosan against root knot nematode, *M. incognita* under in vitro condition:

Among the six different concentrations of chitosan (3%) evaluated, exposure of root knot nematode egg mass to 5000 ppm and 10,000 ppm concentration decreased the egg hatching of *M. incognita* significantly by 96.19 and100 % within 24 hours of exposure compared to control (Table 1 and Fig. 1). Chitosan was found to degrade the gelatinous matrix of egg masses and eggs were vacuolated with deformed juveniles. Mortality of *M. incognita* infective juveniles was significantly high *in -vitro* at all concentrations of chitosan compared to control. *In- vitro* results revealed that exposure of juveniles of *M. incognita* to chitosan at 5000 ppm and 10,000 ppm concentration caused 100 per cent mortality within 24 to 72 hrs time interval period (Table 2). In this present study it was also observed that the chitosan treated juveniles exhibited severe distortion of internal contents.

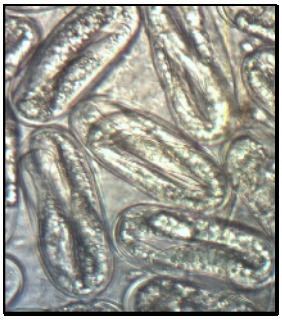
Table 1: Effect of Chitosan on hatching of *Meloidogyne incognit*a eggs under *in-vitro* conditions.

Treatments	Number of hatched juveniles (Mean of four replications)			
	24 hrs	48 hrs	72hrs	96 hrs
Chitosan- 100 ppm	74.00^{d} (8.60)	83.00^{c} (9.10)	96.00^{c} (9.81)	96.00^{c} (9.81)
Chitosan – 500 ppm	59^{c} (10.18)	72.75^{c} (8.53)	84.75^{c} (9.22)	93.75^{c} (9.70)
Chitosan – 1000 ppm	25^{b} (5.02)	26.25^{b} (5.14)	28.25^{b} (5.35)	30.25^{b} (5.52)
Chitosan – 5000 ppm	3.00^{a} (1.41)	3.00^{a} (1.39)	3.00^{a} (1.31)	3.00^{a} (1.40)
Chitosan - 10,000 ppm	0.00^{a} (0.87)	0.00^{a} (0.92)	0.00^{a} (0.92	0.00^{a} (0.92)
Control - tap water	78.75^{d} (8.86)	87.75^{c} (9.52)	103.75^{c} (10.11)	111.75^{c} (10.52)
SEd	0.389	0.463	0.462	0.407
CD (=0.01%)	1.121	1.334	1.331	1.172

*Figures in parentheses are square root transformed value. In a column, means followed by common alphabet are significantly different from each other at 1% level by DMRT.



(a) Treated nematode eggs



(b) Untreated nematode eggs

Fig. 1. Effect of chitosan on *M. incognita* eggs.

Table 2: Effect of Chitosan on mortality of *Meloidogyne incognit*a juveniles under *in- vitro* conditions.

Treatments	Mortality of infective juveniles (Mean of four replications)			
	24 hrs	48 hrs	72 hrs	96 hrs
Chitosan – 100 ppm	4.00^{c} (2.11)	4.00^{c} (2.11)	4.00^{c} (2.16)	4.00^{c} (2.11)
Chitosan – 500 ppm	66.75^{b} (8.19)	88.0^{b} (9.40)	88.0^{b} (9.40)	89.25^{b} (9.47)
Chitosan – 1000 ppm	92.25^{a} (9.61)	100.00^{a} (10.02)	100.00^{a} (10.02)	100.00^{a} (10.02)
Chitosan – 5000 ppm	100.00^{a} (9.95)	100.00^{a} (10.02)	100.00^{a} (10.02)	100.00^{a} (10.02)
Chitosan-10,000 ppm	100.00^{a} (10.02)	100.00^{a} 10.02)	100.0^{a} (10.02)	100.00^{a} (10.02)
Control -tap water	0.00^{d} (0.7)	0.00^{d} (0.7)	0.00^{d} (10.02)	1.25^{d} (1.16)
SEd	0.221	0.067	0.051	0.067
CD (=0.01%)	0.635	0.194	0.147	0.195

*Figures in parentheses are square root transformed value. In a column, means followed by common alphabet are significantly different from each other at 1% level by DMRT.

Bio-efficacy of chitosan against root knot nematode, *M. incognita* under pot culture conditions. *In-vivo* results revealed that chitosan reduced nematode populations in roots. Application of chitosan at 10,000 ppm concentration decreased galls by 87.41%, number of females by 51.72 % and number of egg mass in root by 61.84% compared to control (Table 3 and Fig. 2). Chitosan treated plants produced dense lateral roots. The treated plants had less number of galls in lateral roots. The plant height increased by 41.73% and root length by 42.47%. Chitosan has been reported to act as a plant growth promoter, stimulating responses associated with both primary and secondary metabolism, including: carbon and nitrogen metabolism, primary photochemistry and photosynthesis, the tricarboxylic acid cycle, and terpenoid and phenolic compounds biosynthesis (Mansilla *et al.*, 2013).

Table 3: Efficacy of chitosan against *M. incognita* infecting tomato under pot culture conditions.

Treatments	Plant growth parameters[1]		Root knot nematode population in roots[2]			
	Plant height (cm)	Root length (cm)	No. of galls/plant	Gall index/ plant	No. of females/plant root	No. of egg masses/plant root
T1-Chitosan 1000 ppm	40.75 (6.38)	16.75^{a} (4.14)	21.6^{b} (4.60)	3	17.50^{b} (4.23)	28.75^{b} (5.35)
T2-Chitosan 5000 ppm	49.00 (6.96)	21.20^{a} (4.56)	19^{ab} (4.28)	3	16.00^{b} (3.89)	23.25^{b} (4.79)
T3-Chitosan 10000 ppm	40.75 (6.36)	22.75^{a} (4.75)	8.75^{a} (2.40)	2	14.00^{b} (3.70)	16.50^{a} (3.78)
T4-Carbofuran	36.25 (5.99)	22.05^{a} (4.60)	8.00^{a} (2.80)	2	12.5^{a} (1.77)	13.5^{a} (3.65)
T5- Control	28.75 (5.23)	7.00^{b} (2.58)	69.5^{c} (8.28)	4	29.00^{c} (5.37)	43.25^{c} (6.48)
SEd	0.52	0.43	0.80		0.29	0.32
CD (0.05%)	1.12 NS	0.91	1.89		0.62	0.69

*Figures in parentheses are square root transformed value. In a column, means followed by common alphabet are significantly different from each other at 1% level by DMRT.
NS- Non Significant

(a) Chitosan treated plant root (b) Chitosan untreated plant root

Fig. 2. Efficacy of chitosan on *M. incognita* under pot culture conditions

Various reports have indicated the nematicidal activity of the biopolymer chitosan. Spiegal *et al.*, (1985), reported that chitosan from crustacean chitin at the rate of 0.05%-0.55% concentration reduced the root knot nematode, *Meloidogyne javanica* population in bean and tomato. Chitosan with irrigation water enhanced the colonization of *Pochonia chlamydasporia* and reduced the *M. javanica* infection and nematode population by 54.23% compared to control (Khalil and Badway, 2012). Different molecular weight chitosan was effective against *M. javanica* infecting tomato (El-Sayed and Mahdy, 2015). Under *in-vitro* conditions, high molecular weight chitosan decreased the egg hatching of root knot nematode, *M. javanica*. Studies of Mota and Santos, (2016), indicated that the application of chitosan in soil and as leaf spray reduced root knot nematode, *M. javanica* population in roots by 44.63% and 63.88% respectively under pot culture studies. They also reported the chitosan application in soil and promoted root growth. (Asif *et al*., 2017), concluded chitosan along with fenugreek leaf extract increased egg plant length and decreased nematode population in root. (Hu *et al.*, 2020) revealed that the accumulation of secondary metabolites like chitosanase from *B. cereus* at feeding sites paralyses the nematode or reduces the populations of next generation by induction of Induced Systemic Resistance (ISR).

Bio-efficacy of chitosan against against *Fusarium* spp: Based on the morphological and molecular characterization, the root pathogenic fungus isolated from tomato roots was identified as *Fusarium solani* (Accession number SUB7093058 seq MT138582). Chitosan @ 1000 ppm and 2000 ppm was found to completely inhibit the growth of the fungus (Table 4, Fig. 3 and 4). Chitosan has been reported to possess broad spectrum fungicidal activity. It inhibits *in- vitro*

fungal growth of many pathogenic fungi, for example, *Botrytis cinera, Alternaria alterata, Colletotrichum gleosporoides and Rhizopus solonifer*. The inhibition was observed at different pathogen development stages such as mycelia growth, sporulation, spore viability and germination on the production of fungal virulence factors (Badawy and Rabea, 2011). The anti-fungal activity was also demonstrated *in-vivo* in many different plant pathogen systems, such as in pear against *Alternariam kikuchiana* and *Physalophora piricola* (Meng *et al.,* 2010) in grapevine and in strawberry against *B. cinerea* (Feliziani *et al.,* 2015) and in dragon fruit against *C. gleosporoides* (Zahid *et al.,* 2015). Chitosan applied at 4.0 g/L of acetic acid-distilled water solution significantly decreased the mycelial growth of *Fusarium oxysporum, Fusarium sambucinum* and *Fusarium graminearum* by 88.4%, 89.0% and 89.8%, respectively (Mejdoub *et al.,* 2020*)*. The reason for the inhibition of the growth of the fungus is attributed to the ability of chitosan to bind with the cell wall or cell membrane of fungi and disrupt the stability of cell membrane layer as reported by Ke *et al.,* (2021).

To conclude, the findings of current study revealed that chitosan effectively reduced egg hatchability and mortality of infective juveniles of root knot nematode under *in-vitro* conditions. Preliminary studies indicated that chitosan inhibits growth of *F. solani*. Under pot culture conditions, chitosan decreased root knot nematode population in roots and enhanced plant growth in tomato.

Table 4: Bioefficacy of chitosan on root pathogenic fungus, *Fusarium solani*.

Treatments	Colony growth diameter (in cm) on 14th day after inoculation Mean of three replications
T1- Chitosan 300 ppm	1.43[b] (1.37)
T2- Chitosan 500ppm	1.10[b] (1.26)
T3- Chitosan 1000ppm	0.00[a] (0.70)
T4- Chitosan 2000ppm	0.00[a] (0.70)
T5- Control	4.50[c] (2.23)
SEd	0.61
CD(.01%)	0.1933

*Figures in parentheses are square root transformed value. In a column, means followed by common alphabet are significantly different from each other at 1% level by DMRT.

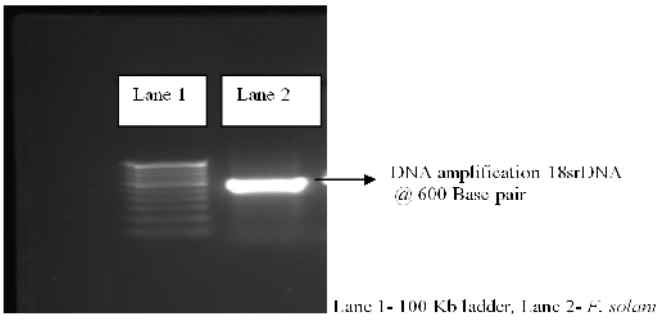
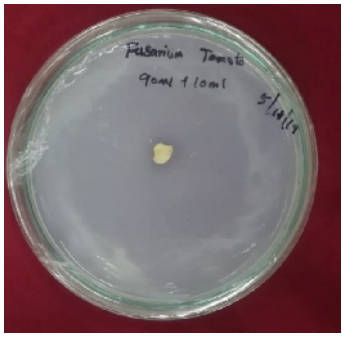
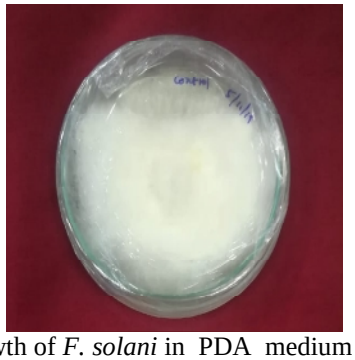


Fig. 3. Molecular characterization of *Fusarium* spp.



(a) Growth of *F. solani* in PDA medium with Chitosan 2000 ppm



(b) Growth of *F. solani* in PDA medium without Chitosan

Fig. 4. Effect of chitosan on *Fusarium solani* (Poison food technique).

Acknowledgement. The authors express gratitude to the Department of Nematology, TNAU, Coimbatore for providing laboratory facilities to conduct this research work.
Conflict of Interest. None.

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How to cite this article: Mouniga, R.; Anita, B.; Shanthi, A.; Lakshmanan, A. and Karthikeyan, G. (2022). Biopolymer Chitosan for the Management of Root Knot Nematode, *Meloidogyne incognita* and Root Pathogenic Fungus, *Fusarium solani* Infecting Tomato. *Biological Forum – An International Journal*, *14*(1): 108-114.