Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
www.entomoljournal.com
JEZS 2021; 9(2): 587-592

Received: 07-01-2021
Accepted: 09-02-2021

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Biocontrol potential & rhizosphere competence of *Trichoderma harzianum* against *Meloidogyne incognita* infecting tomato cv. Pusa ruby

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Abstract
One indigenous isolate of *Trichoderma harzianum* (ITCC accession number 6888) which was effective in causing Juvenile mortality in previous lab studies was tested for its bioefficacy against *Meloidogyne incognita*. Cell Free Filtrate of isolating in petriplate bioassay on pluronic gel exhibited significantly reduced J2 movement towards tomato root at 6h compared to water as a control. Soil application of *T. harzianum* ITCC 6888 @3% w/w could significantly reduce penetration of root-knot J2. The effectiveness of ITCC 6888 was seen in sterilised and unsterilized soil on tomato cv Pusa Ruby, as the isolate exhibited rhizosphere competence with a significant reduction in root galls, egg mass production, eggs/eggmass and reproduction factor in both sterilised and unsterilized soil. The effects of ITCC 6888 @ 3% w/w talc based formulation were at par with those of carbofuran (1kg a.i/ha) in reducing eggmasses/plant, and the effect of carbofuran was significant in Sterilized Soil but exhibited lowered effects in Un Sterilized Soil. ITCC 6888 was compatible with carbofuran application in soil, but no significant difference was observed in bioefficacy of the combination treatment. The number of *M. incognita* J2s recovered from soil 3 months after inoculation was significantly low in pots treated with ITCC 6888 alone or in combination with carbofuran, compared to untreated control. Thus this isolate holds the potential to develop into a biopesticide for field application as it reduced the nematode population significantly.

Keywords: root-knot nematode, *Trichoderma harzianum*, carbofuran, management

Introduction
Tomato (*Solanum lycopersicum*), a Solanaceae family crop. It is an important vegetable crop worldwide because of its high consumption, year-round availability and high content of health-related components such as minerals, vitamins and antioxidants. Among different pests causing yield loss in tomato root-knot nematode is a major constraint for the successful cultivation of tomato. In India, tomato is infected with *Meloidogyne incognita* and *M. javanica*, which are distributed in all the agro-ecological zones of the country. Nematode infection in tomato causes characteristic galling in the roots, stunting of plants and yellowing of leaves. In India, the annual yield loss in tomato due to this nematode is estimated to be around 23% which amounts to a monetory loss of Rs. 6035.20 million [1]. Managing the *Meloidogyne* species (comprising more than 95 species) is a challenging problem because of their wide host range, short life cycle, high reproduction rates & endoparasitic nature. The use of chemical nematicides has been restricted due to ecological and human health hazard concerns as well as development of resistance in target organism. One of the alternatives of chemicals for controlling the nematodes is the use of antagonistic microorganisms. Among them fungi are unique candidates for use in biocontrol and are known to regulate nematode densities in soil. Biological control of plant-parasitic nematodes is receiving an increasing attention as it is safe to the user and does not disturb the beneficial flora and fauna in the soil, thus, maintaining the soil biodiversity and health.
Among different fungal biocontrol agents *Trichoderma* is also known to suppress plant-parasitic nematodes through mechanisms like predation, parasitism and antibiosis [2]. *Trichoderma* is a genus of asexually reproducing filamentious fungi that belong to the order Hypocreales. Many species of *Trichoderma* have shown efficacy in controlling root-knot [3, 4]. In India, the bio-control potential of *T. harzianum* is reported by many people[5]. However, very little information is available on nematicidal effects of indigenous isolates of *T. harzianum* maintained by Indian type culture collection (ITCC), Division of Plant Pathology, ICAR-IARI [6]

Trichoderma harzianum ITCC 6888 was effective in causing Juvenile mortality and parasitism on root-knot nematode in our previous lab studies [7]. Thus the aim of the following study is to understand bioefficacy and rhizosphere competence of potential nematicidal isolate *i.e, Trichoderma harzianum* ITCC 6888 in pots on tomato cv. Pusa Ruby in sterilized soil as well as in unsterilized soil.

Materials and methods

Maintenance of pure culture of *M. incognita:* The soil was collected from the experimental field near Glass house of Division of Nematology, ICAR-IARI, New Delhi. The sieved soil was autoclaved at 121 °C at 15 lbs for 3 h. The sterilized soil was mixed with sand in the ratio of 2:1 in earthen pots for raising the culture of root-knot nematode, *Meloidogyne incognita.* Egg masses of
Root-knot nematode was collected from infected tomato roots and the population was multiplied on susceptible tomato variety (Pusa Ruby) grown in pots containing sterilized soil.

Preparation of *Trichoderma harzianum* culture: Fungal culture was grown in 150 mL of Potato dextrose broth (PDB) (Source:Hi-Media chemicals) in 250 mL Erlenmeyer flasks. Each flask was plugged with the cotton plug and wrapped with aluminum foil and sterilized at 121°C at 15 lbs/in pressure for 20 min. The flasks were inoculated with a 5 mm disc of *Trichoderma* cultures under aseptic conditions in a laminar air flow cabinet. The flasks were kept in a shaker at 130 rpm and constant temperature of 25 °C were maintained for 7 days. This culture containing 4.3×10^6 spores/cc mixed with talc to prepare @ 3% w/w formulation. Cell free filtrate (CFF) of a fungal isolate is prepared by centrifuging 7 day old culture broth at 10000 rpm for 10 minutes and filtering the supernatant through the bacterial filter of 0.20µm size.

Nematode attraction Study in Pluronic gel: Two ml of 23% pluronic gel (Sigma Aldrich chemicals) was prepared and poured in a 4.0cm calibrated Petri dish. Young tomato seedlings of 6day old 0.5cm cm root length were dipped in CFF of the *T. harzianum* isolate and placed at one end of the plate. *M. incognita* J2s were released at the other end @ 100 J_2 per replication. The average number of J2s within 1.0- 1.5, 1.5-2.0, 2.0-2.5, 3.0-3.5 and 3.5-3-4.0cm from the point of inoculation was recorded at two, four and six hours. Other treatments included were Sterile distilled water (SDW) and media (PDB) treated root tips (Plate 1).

Treatments and experimental layout for Bioefficacy of *Trichoderma harzianum* ITCC 6888 in pots: The experimental trial was conducted in 8 inch earthen pots, filled with sterilized and unsterilized soil (2 different sets) for 6 replications.Talc based formulation as well as Carbofuran were uniformly mixed with soil. Different treatments *viz.*, T1:*T.harzianum* ITCC 6888 3%w/w +2 J2/cc;T2:*T.harzianum* ITCC 6888 3% w/w+ Carbofuran @ 0.5 kg *a.i* /ha+2 J2/cc; T3:Carbofuran @ 1 kg a.i /ha+2J2/cc T4:J2 alone 2 J2/cc;T5:*T.harzianum* ITCC 6888 3% w/w; T6: Media alone (talc); T7:Uninoculated Control. 20 day old seedlings of Tomato cultivar Pusa Ruby were sown @ single plant per replication & J2 were inoculated after 3days. Observations such as number of J2s per cc of soil, number of egg masses/plant, number of eggs/egg mass, gall index and plant growth parameters like plant height, fresh weight, root length, root weight, Dry shoot weight and Dry root weight were recorded after 90 days of transplanting in earthen pots. Regarding the penetration experiment where 15 day old Tomato seedlings were grown in 100cc cups in sterilized soil *Meloidogyne incognita* J2 were inoculated @2/cc. Different treatments include T1:*T.harzianum* ITCC 6888 3%w/w;T2:*T.harzianum* ITCC 6888 3% w/w+ Carbofuran @ 0.5 kg *a.i* /ha; T3:Carbofuran @ 1 kg a.i /ha T4:J2 alone. Observations were taken on a number of juveniles penetrated the root tips after 7days by using Byrds staining procedure and the number of J2s penetrated inside the root were counted [8].

Statistical analysis: Data was analyzed using analysis of variance (ANOVA) by using SAS version 9.0 as per the standard method of CRD. The CD values were calculated at 5 per cent probability to determine the significance of treatment and time differences

Results and Discussion

Effect of *T. harzianum* ITCC 6888 Cell Free Filtrate (CFF) treated tomato roots on movement of *M.incognita* in pluronic gel: 6 day old tomato seedlings (cv. Pusa Ruby) dipped in CFF of *T.harzianum* ITCC 6888 resulted in significant reduction in movement of J2s in pluronic gel, compared to untreated control (Plate 1). The effect was most evident at 2 and 6h (Table 1). The average number of J2s that reached a distance of 1.5-2.0 cm (4.0), 2.0-2.5cm (1.67), 2.0-2.5cm (0.0) from the point of inoculation was significantly low in CFF-treated root treatments than in treatments where the root was dipped in SDW at 2 h. This effect became more apparent at 6h, when an average of 23.3 at 0.0-0.5cm, 15.33 at 0.5-1.0cm and 2.67 at 1.5-2.0cm and 0.67 J2s at 2.0-2.5cm were observed in CFF treated plates, compared to an average of 8.0 at 0.0-0.5cm, 4.00 at 0.5-1.0cm and 32 at 1.5-2.0cm, 12 at 2.0-2.5cm and 9.0 at 2.5-3cm, close to the roots in SDW treated roots. These observations exhibited the repellent effect of the filtrate on the J2s. No inhibitory effect on J2 movement was also observed in the media (PDB) treated roots compared to SDW. Root-knot nematode (*Meloidogyne* spp.) second-stage juveniles can move easily through the pluronic gel and we can observe attraction toward roots of hosts like a tomato. Pluronic gel (F-127) is a copolymer of propylene oxide and ethylene oxide. It is utilized as a medium to study bacteria, fungi, plant tissues and nematodes due to its nontoxic nature. A 23% solution is a liquid at temperatures below 15 ºC and a semisolid gel at room temperature. As nematodes can move freely through the transparent gel, it offers an excellent tool to study host attraction studies in a three dimensional assay. Besides, the nematodes can also be recovered easily by keeping the gel at a low temperature [9]. In the present work, the pluronic gel bioassay further supported the repellant effect of the fungal toxin on juvenile mobility towards the host root, which was treated with CFF and compared with juvenile mobility towards the host root, which was treated with water or media (PDB) as control. The repellent activity of *Trichoderma* could be due to the presence of some nematicidal metabolites such as gliotoxin, trichodermin [10] and peptide cyclosporin A [11].

Effect of *T. harzianum* ITCC 6888 on Penetration of root-knot J2 in tomato roots: The pot trial in sterilised soil, maintained for 7 days, revealed a significantly low number of J2s penetrated in *Trichoderma* treated pots than in untreated control as indicated by Byrd's acid fuchsin method. An

average of 2.0 J2/plant was observed in T1 (*Trichoderma* 3% W/W), compared to 2.33 in T2 (*Trichoderma* + half dose of Carbofuran), 0.67 in T3 (Carbofuran 1 kg a.i/ha) and 21.8 in T4 (Nematode control) (Fig 1).

Effect of talc based formulation of *T. harzianum* ITCC 6888 on multiplication of *M.incognita* infecting tomato cv. Pusa Ruby: A preliminary lab trial was conducted to test the compatibility of carbofuran (3G) with *T. harzianum* ITCC 6888. *T. harzianum* did not show any growth inhibition on PDA mixed with carbofuran, tested at 2 concentrations, 2000µg/mL and 1000µg/mL, till 7 days of incubation, although insignificant growth inhibition was observed on day 3.

The bioefficacy of nematicidal isolate ITCC 6888 in the soil as a talc based formulation @3%w/w with an average of 4.3×10^6 spores per cc and inoculum level of 2J2/cc *M. incognita* per cc soil, supported the nematicidal potential of the isolate in both sterilized (SS) and unsterilized soil (USS), indicating its rhizosphere competence.

The application of *T. harzianum* ITCC 6888 alone (T1SS and T1USS) or in combination with half of the recommended dose of carbofuran i.e, 0.5kg a.i / ha (T2SS and T2USS) resulted in a significant reduction in root galls ranging from 78.2 to 84.0 per cent, compared to untreated control. The effect was evident in both SS and USS (Table 2; Plate 2). observations on reduced galling by different isolates of *T. harzianum* have been reported by several other workers in sterilized soil [6, 12]. The significant reduction in galling was a consequence of reduced juvenile penetration (Fig 1).

The effect of carbofuran on root galling in unsterilsed soil was significantly less with an average of 138.8 galls/plant resulting in 65.75 per cent reduction in galling compared to control. On comparing this effect with *Trichoderma* application (T1USS) in unsterilized soil, ITCC 6888 alone resulted in 81.66 per cent reduction which increased to 84.00 per cent in combination with carbofuran (Table 2). The application of carbofuran in combination with *T harzianum* 6888 was compatible as indicated by the observations (Table 2) although no synegisic effect on the bioefficacy of the isolate for any of the factors was observed. The isolate *T harzianum* 6888 could be able to survive well & control nematode population in the natural soil, thus observed to be a good rhizosphere competitor in USS. The effect of carbofuran in USS was significantly reduced compared to that in SS.The lowered effect of Carbofuran in unsterilized soil was possibly due to microbial degradation of the pesticide. An enhancement in fungal and bacterial population densities on carbofuran application has been reported in soil [13].

The per cent decrease in egg masses ranged from 87.05 to 92.36 per cent, both in T1 and T2, at par with carbofuran treatment in T3 USS and T3 SS which resulted in per cent decrease of 92.36 to 91.96 (Table 2) indicating the suppressive effect of the ITCC 6888 on nematode reproduction which was at par with carbofuran treatment. The significant reduction in egg masses was due to reduced penetration and development of the nematode in the treated plants. However, the effect of the fungal isolate on developmental changes of the nematode needs to be observed to confirm the observation.

The average number of eggs per egg mass were significantly low in presence of *Trichoderma* alone (257.33 in T1SS and 232.00 in T1USS or combination with carbofuran (282.00 in T2SS and 323.00 in T2USS), compared to untreated control (503.00 and 541.3), (CD (0.05P)=77.64), although the treatments T1, T2 and T3 did not show any significant difference among one another. The better performance in combination treatments (T2SS and T2USS) could be attributed to the compatibility of Carbofuran with *Trichoderma.* Similarly the compatability of *Trichoderma* species with nematicides have been reported by several other workers [14].

The J2s/cc soil were the lowest in T1SS (2.95) but was at par with T1 USS,(4.09), T2SS (5.32), T2USS (4.47), and T3SS (3.28), but was significantly lower than that in T3USS (8.28) [CD (0.05) =3.5]. Although all the above four treatments exhibited low J2 densities in soil compared to controls T4SS and T4 USS. This indicated that carbofuran effects varied in SS and USS.The reproduction factor was low in all treatments with *T. harzianum* 6888 alone (1.48 in SS, 2.04 in USS) or in combination with carbofuran (2.66 in SS, 2.23 in USS), at par with carbofuran (1.64) in SS. Though the present isolate is proved to be potential biocontrol agent the establishment and survival of the isolate in soil/rhizosphere need to be further investigated. This can be better understood using GFP transformed *Trichoderma* which can help us to distinguish the active fungal biomass from the inactive propagules like conidia or chlamydospores that are enumerated by plate counts. It is also important to investigate if the isolate penetrates the root system and acts like an endophyte as reported for some *Trichoderma* species. This is also achievable after GFP transformation that will elucidate the mechanism of nematode suppression in the plant by the antagonist.

Most of the plant growth parameters (shoot and root length, shoot and root weight), however did not exhibit any significant differences among the treatments in our pot trial (Tab 3). Albeit the dry root weight was significantly more in T1SS *i.e. Trichoderma* along with nematode, over other treatments, but this effect was not observed in T5 treatments, indicating that the effect was not due to *Trichoderma* application (Table 3). But the improvement in plant growth parameters due to the release of growth promoting substances or by producing toxic metabolites which inhibit nematodes has been reported for some *Trichoderma* species/isolates [15].

Conclusions

Thus the following isolate (*Trichoderma harzianum* ITCC 6888) could be developed into a biopesticide for field application as it reduced the nematode pest population significantly.There is a need to develop the isolate ITCC 6888 into a bioformulation, evaluate its shelf life and carry out multilocation trials to confirm its bioefficacy on different crops and against different economically important nematode pests. Further attempts can be made to identify the genes responsible for its nematicidal activity and also to develop a GFP construct to understand the mechanism of action of the isolate on the nematode as well as on the plant.

Acknowledgement

The authors are thankful to Division of Nematology, ICAR-IARI for providing facilities to conduct the research.

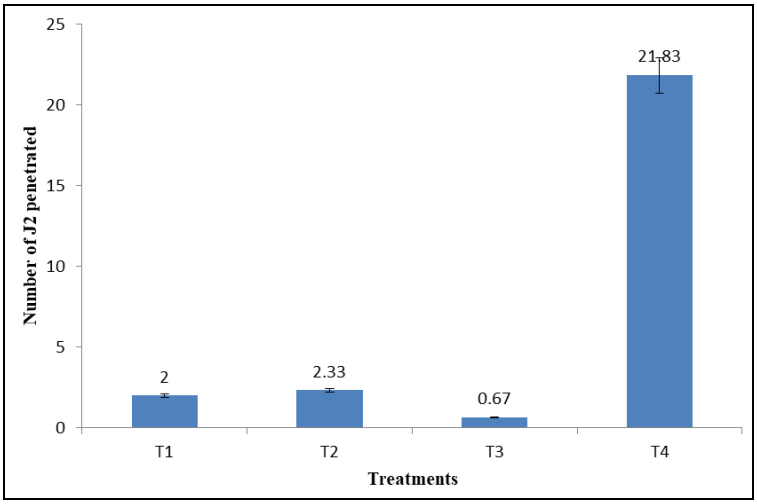

Fig 1: Effect of *Trichoderma harzianum* 6888 CFF on *M. incognita* penetration in tomato cv Pusa Ruby in sterilized soil

T 1: *T. harzianum* ITCC 6888 3%w/v +2 J2/cc,
T 2: *T. harzianum* ITCC 6888 3%w/v+ carbofuran @ 0.5 kg a.i /ha+2 J2/cc,
T 3: Carbofuran @ 1 kg a.i /ha+2J2/cc
T 4: J2 alone 2 J2/cc

Number of replications =6
Inoculum level =2 J2 /cc

Table 1: Effect of *Trichoderma harzianum* ITCC 6888 CFF on *M. incognita* J2s movement towards tomato cv Pusa Ruby seedlings on pluronic gel at 2, 4 and 6h.

	Average number of J2s at 2h at different distance from the root tip						
Treatments/cm	0-0.5	0.5-1.0	1-1.5	1.5-2.0	2-2.5	2.5-3.0	Mean
CFF	41.67	17.00	4.00	1.67	0.0	0.00	10.72
SDW(control)	20.00	13.67	10.67	4.67	1.67	0.00	8.44
Media	26.7	15.7	7.7	2.7	0.0	0.00	8.80
CD(0.05 P)	14.53	NS	3.19	2.82	1.76	0.0	

	Average number of J2s at 4h at different distance from the root tip						
Treatments/cm	0-0.5	0.5-1.0	1-1.5	1.5-2.0	2-2.5	2.5-3.0	Mean
CFF	18.67	15.00	8.00	3.0	0.0	0.0	7.45
SDW(control)	21.00	16.33	11.33	12.00	4.67	3.0	11.38
Media	21.33	19.33	9.33	10.33	7.0	1.0	11.39
CD(0.05 P)	NS	NS	NS	NS	6.95	NS	

	Average number of J2s at 6h at different distance from the root tip						
Treatments/cm	**0-0.5**	**0.5-1.0**	**1-1.5**	**1.5-2.0**	**2-2.5**	**2.5-3.0**	**Mean**
CFF	23.33	15.33	5.33	2.67	0.67	0.00	7.88
SDW(control)	8.00	4.00	7.00	32.00	12.00	9.00	12.00
Media	10.00	8.00	15.00	23.00	10.00	6.33	12.05
CD(0.05 P)	8.10	5.90	NS	11.95	7.41	3.80	

Number of replications=6
Average number of J2 per replication=100

Table 2: Bioefficacy & rhizosphere competence of *Trichoderma harzianum* ITCC 6888 on *Meloidogyne incognita* multiplication, gall reduction, egg mass and egg production in Tomato cv Pusa Ruby in sterilized soil (SS) and unsterilized (USS) soil Number of replications= 6 Inoculum level=2 J2/cc of soil

Treatment	**No of galls/plant**	**%Decrease over control**	**Egg masses/plant**	**%Decrease over control**	**Eggs /egg mass**	**%Decrease over control**	**J2/cc of soil**	**RF (pi/pf)**	**%Decrease over control**
T1SS	52.67	83.60	9.33	92.22	257.33	48.87	2.95	1.48	84.32
T1USS	74.33	81.66	14.50	87.05	232.00	57.14	4.09	2.04	75.99
T2SS	70.00	78.20	12.30	89.72	282.00	43.97	5.32	2.66	71.72
T2USS	64.83	84.00	13.50	87.95	323.30	40.27	4.47	2.23	73.76
T3SS	43.67	86.41	9.17	92.36	384.66	23.57	3.28	1.64	82.59
T3USS	138.80	65.75	9.00	91.96	463.30	14.41	8.28	4.14	51.36
T4SS	321.20	0.0	120.00	0.0	503.30	0.0	18.80	9.40	0.0
T4USS	405.30	0.0	112.00	0.0	541.30	0.0	17.00	8.51	0.0
T5SS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

T5USS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T6SS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T6USS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T7SS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
T7USS	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
CD(0.05P)	58.22		23.38		77.64		3.51		

T1: *T. harzianum* 6888 @ 3% w/v+2 J2/cc, T2: *T. harzianum* 6888 @ 3% w/v + Carbofuran @ 0.5 kg a.i /ha+2 J2/cc, T3: Carbofuran @ 1 kg a.i /ha+2 J2/cc
T4:J2 alone 2 J2/cc, T5: *T. harzianum* 6888 @ 3% w/v Alone, T6: Media alone (talc), T7:Uninoculated Control
(SS: Sterilized soil, USS: Unsterilized soil)

Table 3: Bioefficacy & Rhizosphere competence of *Trichoderma harzianum* ITCC 6888 on *Meloidogyne incognita* & effect on plant growth parameters in Tomato cv Pusa Ruby in sterilized soil (SS) and unsterilized soil (USS) Number of replications= 6 Inoculum level=2 J2/cc of soil

Treatment	Shoot Length (cm)	Root Length (cm)	Fresh Shoot Weight (g)	Fresh Root Weight (g)	Dry Shoot Weight (g)	Dry Root Weight(g)	Fruit Yield (g)
T1SS	38.23	23.88	17.73	6.46	4.68	3.10	48.18
T1USS	43.17	26.00	20.50	4.68	4.78	1.80	56.72
T2SS	72.25	25.67	46.08	9.25	10.70	0.90	33.63
T2USS	59.83	29.33	31.08	5.24	10.70	1.80	35.72
T3SS	95.33	23.08	90.10	10.10	17.00	1.71	77.23
T3USS	63.60	28.20	33.78	5.28	6.03	1.78	45.82
T4SS	62.03	20.63	16.02	7.17	3.48	0.90	28.97
T4USS	51.67	23.92	42.40	5.20	3.25	1.37	36.50
T5SS	73.67	26.67	33.20	3.84	7.82	0.91	55.93
T5USS	57.67	25.00	34.40	7.85	12.00	2.30	43.17
T6SS	61.83	23.50	30.93	3.07	6.87	0.81	23.67
T6USS	61.67	25.88	41.70	7.80	10.10	1.67	40.17
T7SS	66.90	28.00	78.68	7.22	14.40	1.86	62.80
T7USS	58.50	20.67	69.40	8.43	6.88	1.73	30.33
CD(0.05P)	25.33	N/S	43.60	3.61	7.76	1.09	N/S

T1: *T. harzianum* 6888 @ 3% w/v+2 J2/cc, T2: *T. harzianum* 6888 @ 3% w/v + Carbofuran @ 0.5 kg a.i /ha+2 J2/cc, T3: Carbofuran @ 1 kg a.i /ha+2 J2/cc
T4:J2 alone 2 J2/cc, T5: *T. harzianum* 6888 @ 3% w/v Alone, T6: Media alone (talc), T7:Uninoculated Control
(SS: Sterilized soil, USS: Unsterilized soil)

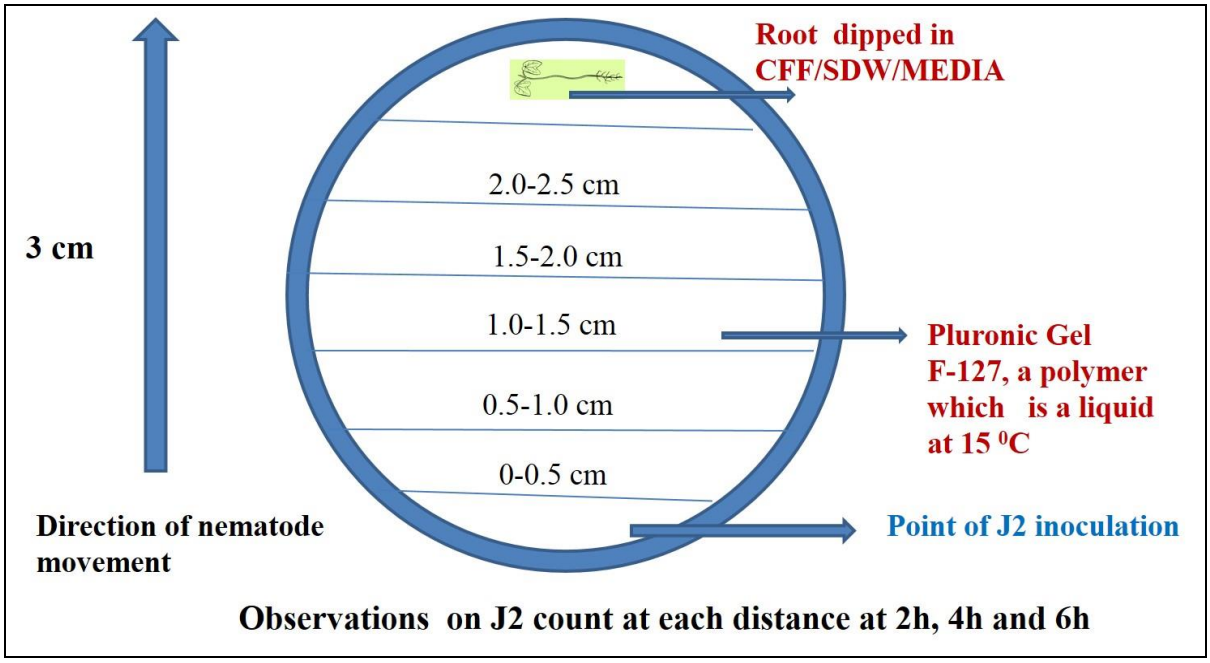

Plate 1: *Meloidogyne incognita* J2s attracted to tomato cv Pusa Ruby root dipped in CFF, Media and SDW on pluronic gel.

Root galling in tomato cv Pusa Ruby sterilized soil

Root galling in tomato cv Pusa Ruby unsterilized soil

Plate 2: Effect of *Trichoderma harzianum* ITCC 6888 and carbofuran alone and in combination on root galling in tomato cv Pusa Ruby in sterilized and unsterilized soil.

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