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Identification of virulent isolate of *Fusarium oxysporum* infesting tomato and exploring its disease severity in the presence of root-knot nematode, *Meloidogyne incognita*

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Abstract

The present study focused on identifying the native virulent *Fusarium* sp. associated with tomato (*Solanum lycopersicon* Mill) cv. ArkaVikas and to investigate its synergistic interaction with root-knot nematode (*Meloidogyne incognita*) in inducing wilt complex in tomato under pot culture conditions. The virulent isolate was identified as *F. oxysporum* f.sp. *lycopersici* through molecular characterization. TF-4 isolate was the most virulent strain among five tested isolates, exhibiting highest wilt incidence (85%) in tomato plants. Sequential inoculation of *M. incognita* followed by *F. oxysporum* exacerbated plant damage, with maximum wilt incidence recorded at 72.50%. It's imperative to study the role of nematodes as a predisposing factor in increasing *Fusarium* wilt severity in tomato.

Key words: *Disease complex, Fusarium oxysporum* f.sp. *lycopersici, Meloidogyne incognita,* tomato, *Solanum lycopersici.*

Introduction

Tomato (*Solanum lycopersicum*), belonging to the Solanaceae family, is one of the widely grown vegetable crops worldwide. It is a rich source of antioxidants, phytochemicals and many essential nutrients such as vitamins, proteins and amino acids (valine, arginine, threonine, leucine). Lycopene, the major dietary carotenoid present in the crop, protects against cancer, osteoporosis, and cardiovascular diseases (Ali *et al.,* 2021). Besides, it is an industrially important commodity since 80% of all commercially cultivated tomatoes are consumed in the processed forms of juice, soup, and ketchup (Collins *et al.,* 2022). It is susceptible to more than 200 diseases caused by fungi, nematodes, bacteria, and viruses (Singh *et al.,* 2017). Among them, infestation of *Fusarium* wilt and root-knot nematode is one of the most economically relevant complexes affecting the crop yield drastically (Wanjohi *et al.,* 2018). Infestation of both *Fusarium oxysporum* f.sp. *lycopersici* and *Meloidogyne incognita* reduce the yield of the crop by 11- 45%, respectively in India (Ramyabharathi *et al.,* 2012; Chandan *et al.*, 2022).

F. oxysporum f.sp. *lycopersici* (FOL) poses a great threat to the tomatoes grown in the fields as well as in greenhouses. An estimated yield loss of 30-40% was observed due to the fungus, which rises as high as 80% under favorable weather conditions (Nirmaladevi *et al.,* 2016). This fungus, being a soilborne pathogen, can persist in the soil for years and can readily infect healthy plants (Kumar *et al.,* 2017) by penetrating and colonizing the vascular bundles of the plant, leading to the characteristic browning and wilting symptoms (Srinivas *et al.,* 2019).

Infestation of root-knot nematodes in tomatoes, besides causing direct damage such as yellowing and stunting, paves the way for the infection of secondary fungal pathogens (Janati *et al.,* 2018). The interaction effect of the nematode with the fungi enhances wilt symptoms and negatively influences the growth and yield of the crop (Zhang *et al.,* 2020). The synergistic interactions wherein nematode aggravated *Fusarium* wilt was documented in Yam (Montalvo and Melendez, 1986), Cotton (Shepherd and Huck, 1989), Pigeon pea (Marley and Hillock, 1996), Common bean (Carneiro *et al.,* 2010), Gerbera (Meena *et al.,* 2015), Carnation (Meena *et al.,* 2016a), Tuberose (Meena *et al.,* 2016b), Cucumber (Patil *et al.,* 2018) and Okra (Yaseen *et al.,* 2024). Entry of nematodes into the plants prior to fungus can lead to various anatomical and physiological changes in the plants, which assist the easy entry and development of fungal pathogens into the plant (Regmi *et al.,* 2022). Infestation of *M. incognita* even breaks down the resistance of the plants towards fungal infection (Ambayeba, 2018). The present study was engrossed to understand the predisposal mechanism of nematode in aggravating the wilt disease through concomitant or sequential inoculation of nematode (*Meloidogyne incognita*) and fungi (FOL) in tomato cv. Arka Vikas under pot culture condition.

Materials and methods

Source and maintenance of nematode inoculum: Pure culture of root-knot nematode, *Meloidogyne incognita* population was maintained on tomato cv. Arka Vikas using single egg mass technique, wherein second-stage juveniles hatched out from a single egg mass of the nematode were inoculated to the roots of 15-day-old healthy tomato seedlings grown in steam-sterilized soil in the glass house of the Crop Protection division of ICAR-Indian Institute of Oilseeds Research, Hyderabad. The plants were watered regularly, and 30 days post-inoculation, egg masses were collected from the plant roots and allowed to hatch. Freshly hatched second-stage juveniles were used for the pathogenicity studies.

Perineal pattern studies of root-knot nematode: Root-knot nematode females collected from the tomato galls were placed on the microscopic slide. A gentle cut was made at the neck region of the female body and the inner body contents were pushed out. The female body was placed in 45% lactic acid solution to clear out the tissue, and further trimming was done to retain the vulva region. This trimmed tissue was placed in a drop of glycerol solution and the perineal pattern photomicrographs were captured to confirm the nematode species in the present interaction (Meena *et al.,* 2015).

Source of fungal isolates: *Fusarium*-infected tomato stem and root portions collected from tomato growing areas of the Yadadri-Bhuvanagiri district of Telangana and Annamayya district of Andhra Pradesh were washed carefully to remove adhering soil particles. The stem and root portions were cut into small bits of 0.5-1cm length and surface sterilized with 1% sodium hypochlorite and 70% ethanol sequentially. Finally, the stem and root bits were passed through three successive changes of sterile distilled water and dried on Whatmann no. 1 filter paper and the dried tissues were placed on the solidified potato dextrose agar (PDA) plates and incubated for seven days at 25◦C. About five *Fusarium* isolates, namely (TF-1, TF-2, TF-3, TF-4, and TF-5) obtained were sub-cultured on PDA and subjected to pathogenicity tests in tomato cv. Arka Vikas.

Pathogenicity tests of the fungal isolates: The 7-day-old cultures of five different *Fusarium* isolates multiplied on sterilized sorghum grains were incubated at 25±1°C for 15 days. Later, the fungus-infected sorghum grains were inoculated into the sterilized potting mixture (red soil: FYM: sand @ 2:1:1) at the rate of 5 % of soil weight (Jaiswal *et al.,* 2023). Each isolate was replicated four times in CRD and the healthy potted plants without inoculum served as control. The pots were covered with a sterile polythene cover and incubated for a week to allow uniform distribution of pathogens in the soil. About ten tomato seeds were sown in each pot, and the percentage of germination and wilt incidence was recorded 45 days after sowing using the following formulae. The re-isolated pathogen was identified as *Fusarium* sp. based on the morphological characters (pigmentation and sporulation).

Germination % = Seeds germinated / Total no. of seeds x 100

Wilt incidence = No. of wilted plants / Total no. of plants x 100

Molecular characterization of fungal isolate: The most virulent *Fusarium* isolate (TF-4) obtained from the pathogenicity study was characterized at the molecular level based on the amplification of the conserved internal transcribed spacer (ITS) region. The fragment of ITS region was amplified by using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The reaction mixture was set with 2.5 µL of 10X buffer (10 mM Tris–HCl, pH 8.8), 2.5 mM MgCl2, 25 pmol ml^{-1} primer (ITS-1 and ITS-4), 2 mM each of dNTP, 1U of Taq DNA Polymerase, and 60–100 ng genomic DNA. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with ITS1 and ITS4 primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The PCR reaction was carried out with an initial denaturation temperature of 95◦C for two minutes followed by 35 cycles of 30 seconds at 94◦C, 56 ◦C for 1 min, and 72 ◦C for 2 min and ended with a final extension set at 72 ◦C for 8 min. Later, aligner software was used to generate consensus sequences of the PCR amplicon from forward and reverse sequence data (Singha *et al.,* 2016). The ITS nucleotide sequence was used to carry out BLASTN (Basic Local Alignment Search Tool for Nucleotide Sequences) with the NCBI Genbank database and alignment of the obtained first ten sequences was done by using the multiple alignment software program Clustal W and the phylogenetic tree was constructed using MEGA 11 software (Kumar *et al.,* 2018).

Assessment of the *F. oxysporum* f.sp. *lycopersici* induced disease severity in tomato in presence of nematode: Tomato seeds (ArkaVikas) were sown in one kg earthen pots filled with the sterilized pot mixture (1:1:1 ratio of red soil, FYM, and sand) at the rate of 3 seeds per pot and after 15 days of growth, the seedlings were thinned to one per pot. *M. incognita* second-stage juveniles (J_2) were inoculated at the rate of 2 J_2 /g soil near to the rhizosphere region and FOL multiplied on sorghum grains were added into each pot at the rate of 5g/kg soil as per to the treatment details mentioned in Table 3. Each treatment was replicated five times in a Completely Randomized Design. Plants grown without inoculum of fungal pathogens and nematodes served as control.

The experiment was terminated at 30 days after imposition of treatments. The cumulative effect after post-inoculation of nematode and fungus was observed in terms of the plant growth indices (shoot length, root length, shoot weight (fresh and dry), and root weight (fresh and dry), percent wilt incidence and nematode root population.

Statistical analysis: Data collected in the pot culture studies were pooled and analyzed according to Gomez and Gomez (1984) using SAS 9.3. The pooled data was subjected to analysis of variance (ANOVA) and least significant difference (LSD) tests for mean comparison at $P \leq 0.001$(Sokaland Rohlf, 1995).

Results and discussion

Identification of root-knot nematode by perineal pattern: Microscopic observations of a perineal pattern of the nematode female revealed characteristic cuticular markings with high squarish dorsal arch flattened at the top, which is the characteristic pattern observed in *Meloidogyne incognita* (Fig. 1).

Virulence of *Fusarium* isolates based on percent germination and wilt incidence: Among the five *Fusarium* isolates tested (TF-1, TF-2, TF-3, TF-4, and TF-5), least germination percentage (32.50) and maximum wilt incidence (85%) was observed in the plants inoculated with TF-4 isolate of *Fusarium* sp. followed by TF-5 (Table 1). The isolate, TF-1 recorded maximum germination percent (62.50) and least wilt incidence (20.0%). Based on the above results, the isolate TF-4 was designated as the most virulent isolate among all the tested isolates infecting tomato cv. Arka Vikas.

Morphological identification of *Fusarium* sp.: The colony morphology of the most virulent *Fusarium* isolate, TF4 revealed a white fluffy colour colony on the front and purplish-white colour colony on the reverse of the Petri plate. Macroconidia and microconidia of the fungi were depicted in Fig.2. Macroconidia were sickle-shaped and multi-celled usually varied from three

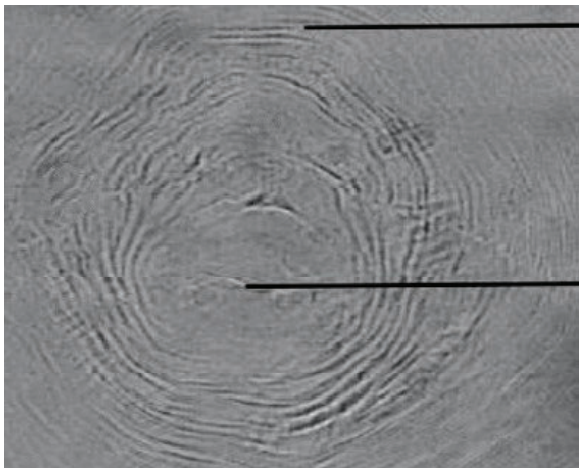

Fig.1. Perineal pattern of root-knot nematode (*Meloidogyne incognita*, female)

Table 1. Identification of virulent *Fusarium* isolates and their effect on germination percentage and wilt incidence in tomato (cv. Arka Vikas)

Isolates	Germination (%)*	Wilt incidence*
TF-1	62.50 (52.25)	20.0 (26.55)
TF-2	57.50 (49.30)	25.0 (29.87)
TF-3	60.0 (50.74)	22.50 (28.21)
TF-4	32.50 (34.70)	85.0 (67.47)
TF-5	40.0 (39.15)	75.0 (60.08)
Control	100 (90.0)	0 (0)
CD (0.5%)	4.303	4.831

*Values in the parentheses are arc-sin transformed. Each treatment was replicated four times in CRD.

to five in number divided by transverse septa whereas the microconidia are oval or globose to fusiform and often one or two-celled. Chlamydospores arise from the mycelial hyphae and appear as thick-walled structures.

Molecular identification of *Fusarium* isolate, TF4 : The ITS regions (ITS1 and ITS4) sequence amplified with fungal specific ITS1-ITS4 primers had a 484 bp amplicon. *Fusarium* sequence analysis of the ITS regions of the nuclear-encoded rDNA when compared with sequences from National Center for Biotechnology Information (NCBI) database using BLASTN showed a significant alignment of 99.79% with the *Fusarium oxysporum* f.sp. *lycopersici*. The ITS region of rDNA sequences was submitted to NCBI GenBank with the accession number as shown in Table 2. ITS region of the *Fusarium* isolate was aligned with consensus region using the Clustal W program and a phylogenetic tree (Fig. 4) was constructed using the neighbourhood joining method using MEGA 11.

Table 2. ITS-based characterization of the virulent *Fol* isolate, TF-4

Isolate	Species	Identified length (bp)	Identity	Accession number
TF4	*Fusarium oxysporum* f.sp. *lycopersici*	484	99.79%	OR905851

Effect of FOL infestation on plant growth parameters of tomato in presence of root-knot nematode: The co-inhabitation effect of FOL and *Meloidogyne incognita* in tomato in terms of plant growth parameters was depicted in the Table 3. Maximum reduction in the shoot length (27.46 cm) was observed in the sequential inoculation of nematode (24 h) prior to the fungus, followed by concomitant inoculation of nematodes and fungus (29.54 cm), and sequential inoculation of fungus (24 h) prior to nematode (32.90 cm). Similarly, the highest reduction in root

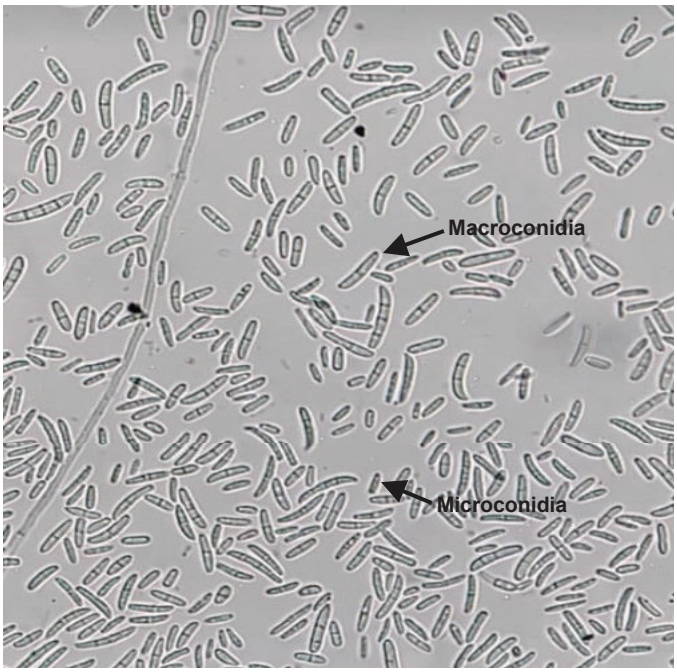

Fig. 2. Micro and macroconidia of *Fusairum* sp. TF-4

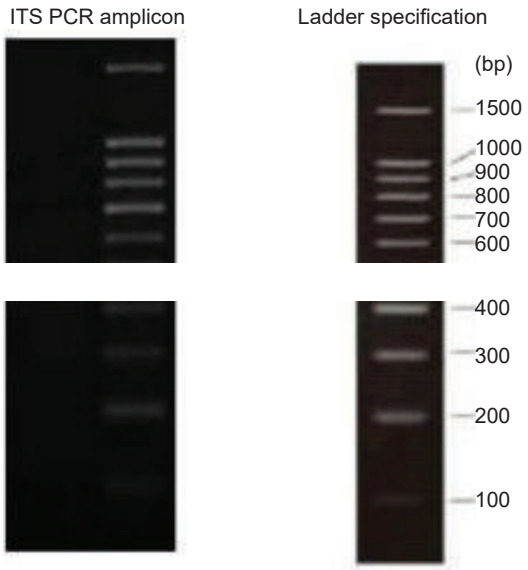

Fig. 3. Amplification of conserved ribosomal regions of *Fusarium* sp., TF-4 using the primers (ITS 1 and ITS 4)

length was observed in the sequential inoculation of nematode (24 h) prior to the fungus (19.50 cm), followed by concomitant inoculation (20.72cm) and sequential inoculation of fungus (24 h) prior to nematode (21.68 cm).

The shoot weight (wet) was lowest in sequential inoculation of nematode followed by fungus (8.99 g). In a similar manner, the recorded root weights were the lowest in the case of treatments where nematode preceded fungus (1.61 g) and the same treatment recorded minimum shoot (dry) (1.27 g) and root (dry) (0.69 g) weight. All the treatments negatively impacted all the plant growth parameters; however, the highest reduction in growth parameters was observed in the sequential inoculation of nematodes followed by fungi (Fig. 5 and 6). Similar results in Okra on plant growth parameters, where in *M. incognita* was inoculated 21 days prior to the fungus, *F. oxysporum* (Agbaglo *et al.,* 2020). Kayani *et al.* (2017) reported a negative correlation between nematode inoculum levels and the growth and yield parameters of cucumber.

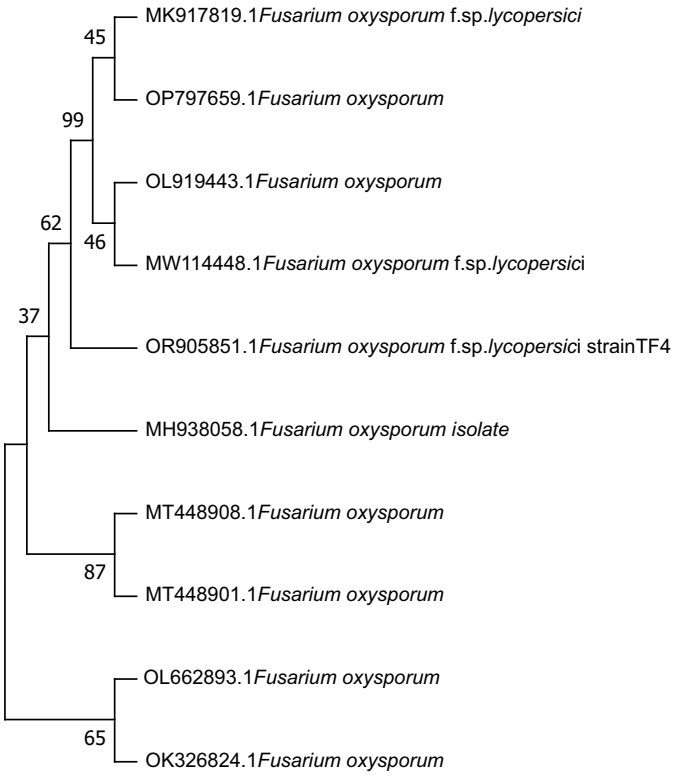

Fig. 4. Phylogenetic tree constructed based on nucleotide sequence of ITS region of conserved ribosomal DNA of *Fusarium* sp.

The present findings were also in confirmation with studies of Ramalingam *et al.* (2019), where both concomitant inoculation of *Fusarium* and *M. incognita* and the sequential inoculation of *M. incognita* prior to *Fusarium* drastically reduced plant growth of tomato.

Fig. 5. Effect of *F. oxysporum* f.spp. *lycopersici- M. incognita* interaction complex on plant growth parameters of tomato cv. Arka Vikas

Wilt incidence of FOL in presence of *Meloidogyne incognita*: Maximum wilt incidence was exacerbated in nematode-fungal interaction as sequential inoculation of nematode followed by fungus or concomitant inoculation of *Fusarium* and *M. incognita* and the overall plant damage was more severe when both pathogens were present. The percent wilt incidence was scored 30 days post inoculation of FOL and *M. incognita,* where, chlorosis and wilting symptoms beyond third leaves were observed in the sequential inoculation of nematode followed by fungus and their concomitant inoculation of *Fusarium* and *M. incognita* where in the wilt incidence of 72.50% and 67.0%, respectively was recorded and imperatively proving the role of nematode as predisposing factor to the fungus (FOL) infestation. Several researchers reported interactions between root-knot nematode species and soilborne pathogens like *Fusarium oxysporum* in cotton (Back *et al.*, 2002) and tomato (Regmi *et al.,* 2022). Similar reports were stated in the interaction of root-knot nematodes with *Fusarium* and *Verticillium* wilt in cotton (Katsantonis *et al.*, 2003). The presence of *M. incognita* enhanced susceptibility of watermelon to *Fusarium* wilt was reported by Hua *et al.* (2019). Sequential inoculation of fungus followed by nematode and the fungus alone treatment has shown chlorosis symptoms only in the second and third leaves beside the loss of primary leaves and scored minimum wilt incidence of 54.50 and 54.00 %, respectively. Least infection of *Fusarium* wilt was observed in the plants inoculated with FOL alone (54.00 %). This might be due to the absence of pre-disposal action by the nematodes, which created an easy entry point by the fungus. The authors confirmed that the wounds caused by nematodes paved way for the easy infection by the fungus. In addition, nematodes were found to affect the plant tissues and make them a better substrate for fungal growth and reproduction, thus increasing wilt incidence. Similar findings were observed in cotton by Walker *et al.* (1998), where they noted a significant rise in seedling mortality, root necrosis, and a lower number of bolls in the presence of *M. incognita*, so rendering the pathogen *Thielaviopsis basicola* acute damage. Abdel-Momen and Starr (1998) reported an increase in the root colonization of peanut pod rot pathogen *R. solani* in the presence of *M. javanica*.

Estimation of nematode gall indices: The number of galls were recorded to be highest in nematode alone (168/root) treatment followed by sequential inoculation of nematode prior to fungus (141/root) and concomitant inoculation (113.75/root) (Table 3).

Table 3. Interaction effect of *Fusarium oxysporum* f. sp. *lycopersici* with *Meloidogyne incognita* on plant growth parameters, percent wilt and root population of nematode in tomato cv. ArkaVikas

Treatment	Shoot length (cm)	Root length (cm)	Shoot fresh wt. (g)	Shoot dry wt. (g)	Root fresh wt. (g)	Root dry wt.(g)	Galls/ root	Females/ plant	Egg mass/ plant	Fungal wilt (%)
T1: Nematode (N) alone	34.68	22.96	19.53	1.97	2.03	1.36	168 (12.97)	175(13.26)	166(12.92)	0(0)
T2: Fungus (F) alone	35.50	24.16	17.36	1.90	1.89	1.27	0 (1)	0 (1)	0(1)	54.0(47.28)
T3: N + F	29.54	20.72	11.64	1.49	1.69	0.89	113.75 (10.35)	153 (12.40)	112 (10.62)	67.0 (54.93)
T4: N-F	27.46	19.50	8.99	1.27	1.61	0.69	141 (11.54)	172 (13.15)	165 (12.88)	72.5 (58.44)
T5: F-N	32.90	21.68	12.53	1.78	1.70	1.05	79 (8.71)	112 (10.62)	78 (8.88)	54.5 (47.57)
T6: Control	42.52	27.80	24.98	4.81	2.96	1.69	0 (1)	0 (1)	0 (1)	0 (0)
CD (0.05)	2.557	2.649	2.062	0.697	0.69	0.413	3.23	0.201	0.154	3.507

*Values in the parentheses represent transformed data (wilt index – arc sin transformation; Galls, females, and egg mass- square root transformation) to homogenize error variances. Each treatment was replicated five times in CRD. N+F: Concomitant inoculation of nematode and fungus; N-F: Sequential inoculation of nematode prior (24h) to fungus; F-N: Sequential inoculation of fungus prior (24h) to nematode.

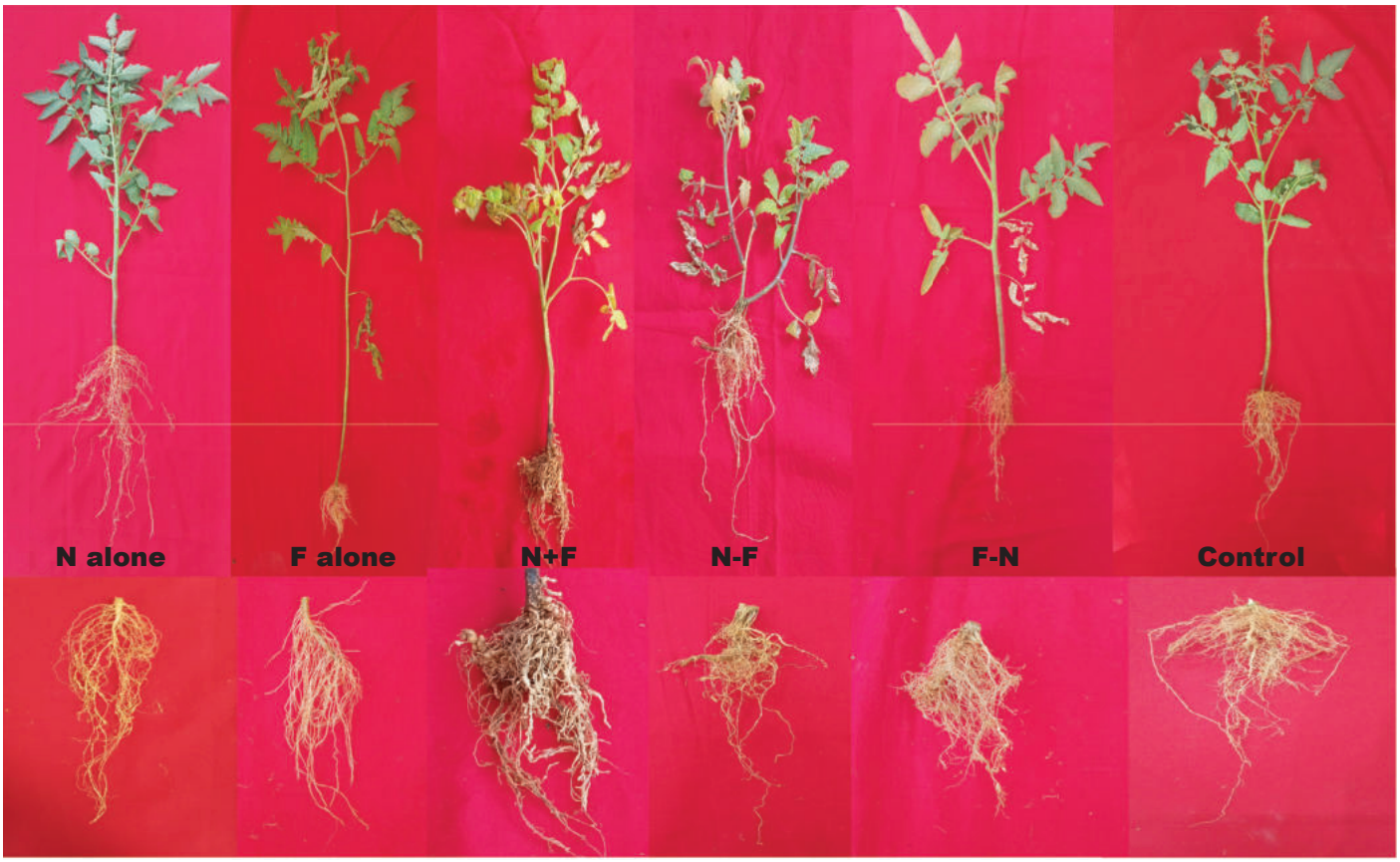

Fig. 6. Effect of *F. oxysporum* f.sp. *lycopersici- M. incognita* complex on wilting and root galling in tomato cv. Arka Vikas

In the context of nematode females, highest count was recorded in nematode alone treatment (175), followed by sequential inoculation of nematode prior to fungus (172) and concomitant inoculation (153). Similarly, egg mass count was recorded to be maximum in nematode alone treatment (166), followed by sequential inoculation of nematode followed by fungus (165) and concomitant inoculation (112). The results coincide with the studies by Roy *et al.* (2022) on the interaction effect of *Fusarium oxysporum* f.sp. *ciceri* and *Meloidogyne incognita* on chickpea and reported the maximum population of nematode in root when nematode were inoculated alone compared to concomitant inoculation treatment and the sequential inoculation of nematode followed by fungus. The reduction of the nematode population was due to the adverse effects of metabolites of fungus on the juveniles of *M. incognita.* In addition to fungal disruption of nematode feeding sites, plants affected by disease complexes are prone to early senescence and death, which may prevent the nematode from completing its life cycle, leading to reduced reproduction of the nematode female population (Kumar *et al.,* 2017).

In summary, the results of the current study indicated the changes in plant growth parameters, nematode gall index and *Fusarium* percent wilt incidences when both the pathogens were inoculated individually, sequentially, and concomitantly. The results confirmed that the nematodes predispose the host plants to early infection by the fungus and intensify the disease severity. At the same time, the presence of fungal pathogens inversely impacts the reproduction and damage levels of the nematodes. When these two pathogens act synergistically, they create a negative impact on the plant growth parameters and reduce the overall yield of the crop.

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