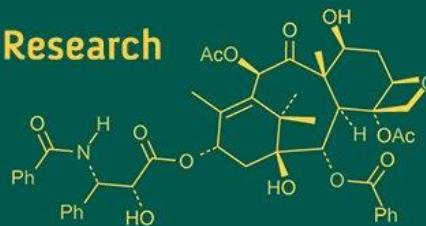
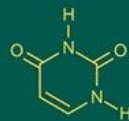
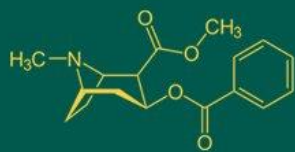


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***In vitro* evaluation of antagonistic activity of bacterial and fungal bioagents against fungal-nematode complexes in tomato**

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Abstract

Tomato (*Solanum lycopersicum*) is one of the most important vegetables of the Solanaceae family. The root-knot nematode (*Meloidogyne*), upon its infection, paves the way for the entry of pathogens, leading to the formation of a fungal-nematode complex in tomatoes. The native *Fusarium sp.* was isolated in the tomato field of MITCAT Farm. An *in vitro* study was carried out using two biocontrol agents, *Bacillus subtilis* (Bbv57) and *Pochonia chlamydosporia*, which have antagonistic properties against *Fusarium* and *Meloidogyne*. Dual culturing of bioagents followed by egg-hatching inhibition and juvenile mortality of nematodes was conducted. In this study, *Bacillus subtilis* (71.40%) gave maximum growth inhibition of *Fusarium*, whereas *Pochonia chlamydosporia* had the egg-hatching inhibition (85.0%), and juvenile mortality (85.0%) rate was observed to be high.

Keywords: *Bacillus subtilis*, *Pochonia chlamydosporia*, dual culturing, juvenile mortality

Introduction

Vegetables are the most important part of horticulture that is grown worldwide. Recently, the productivity of vegetables has tremendously increased for past last years. Vegetables are highly chosen by farmers because of its short duration, better yield, nutritious value and economic viability. India ranks second in vegetable production. Among various vegetable, Tomato (*Lycopersicon esculentum*) is widely cultivated in India (11.2%), ranking second position in area and production worldwide (Praveen kumar *et al.*, 2022) [14]. Tomato is being cultivated in 3.7 million hectares across the world with the production of about 100 million tons (FAOSTAT, 2024) [3]. In India the present area and production of tomato is 4.58 lakh ha and 74.62 lakh tones respectively, with productivity of 16.29 tons per hectare. Tomatoes can be grown in both field and protected condition. They are highly adapted to various climatic conditions temperate to hot and humid. They are usually sown during March-April in hill areas whereas June to November in plains (Fontana *et al.*, 2008) [11]. Even though India ranks second in tomato production, farmers are facing losses due to various biotic and abiotic stresses during cultivation. The abiotic factors affecting the yield include heavy rainfall, drought, salinity, osmotic shock and excess light incidence. The biotic factors leading to crop losses includes various insect pests, pathogens and nematodes (Aneta Gerszberg *et al.*, 2017) [4].

Among the biotic stress factors, plant parasitic nematodes are major biotic threat in successful crop cultivation. Based on data of AICRP on Nematodes, it has been concluded that nematodes alone cause 21.3% crop losses annually (Kumar *et al.*, 2022) [7]. The infestation of plant parasitic nematodes also leads to formation of mechanical wounds which pave way for the entry of pathogens into crop forming fungal-nematode complex and causing severe crop damage. Among various plant parasitic nematodes, Root knot nematodes (*Meloidogyne sp.*) are widespread soil-borne nematodes causing severe crop losses. *Meloidogyne entereolobii*, the guava root knot nematode emerging as a high threat in tomato cultivation due to its wide hosts range, distribution and ability to reproduce on tomato genotypes. This nematode alone can cause losses up to 65% in tomato cultivation. The affected crops become stunted, causes chlorosis and reduce yield by induction of root galls on host plants. In Tomato, the *Meloidogyne entereolobii* forms a fungal-nematode complex with the pathogen *Fusarium sp.* which causes wilt in tomato. *Fusarium* wilt and nematode colonization in tomato are often

associated, where the nematodes serving a pathway for the fungus to penetrate the plant roots (Shivam Maurya et al., 2019)^[17].

The pathogen enters the plant by the root tips and can survive in the soil for up to 30 years (Thangavelu et al., 2009)^[19]. Adopting of managing strategy for the control of fungal-nematode complex caused by *M. enterolobii* in tomato is being very difficult due to its morphological similarities with the other root-knot nematodes. Lack of knowledge on its infestation till the harvesting of crops is commonly prevailing among many farmers, until observing the clumpy root galls in the roots (Shwarz, 2019). In view of this, the current analysis aims to determine the most efficient invitro management approach for preventing fungal-nematode complex infestation in tomatoes.

Materials and methods

Isolation of *Fusarium* sp

Soil samples were taken from native tomato plants that were infected with *Fusarium*. The pathogen was isolated by serial dilution level of 10⁻³. The cultivated plates were incubated for seven days at 27-28°C (Malathi 2015)^[8]. After incubation the pathogen's morphology were identified and preserved for the further study.

Collection of Antagonistic bio-agents

From TNAU in Coimbatore, commercial talc formulations of bacterial and fungal bio-agents, namely *Bacillus subtilis* and *Pochonia chlamydosporia*, have been collected.

Culturing of bacterial bio-agent *Bacillus subtilis* and fungal bio-agent *Pochonia chlamydosporia*

The cultures of the strain Bbv57 *Bacillus subtilis* and the fungal bio-agent *Pochonia chlamydosporia* were prepared through serial dilution. For bacterial cultures, 10⁻⁵ dilution was plated on NA medium and incubated at 27°C for 24 hours. For fungal cultures, 10⁻³ dilution was plated on PDA medium and incubated at 27°C for 7 days. Post-incubation, cultures were confirmed via Gram staining and microscopic observation, followed by sub-culturing and further incubation. The grown cultures were then stored and used for further *In-vitro* analysis.

In-vitro dual culturing of *Fusarium* sp. growth inhibition by bacterial bio-agent *Bacillus* sp.

The cultured bio-agents *Bacillus* sp. was used against *Fusarium* sp. growth inhibition in dual culturing, potato dextrose agar was used as medium. The zone of inhibition was observed after 5 days of incubation at 28°C. An apparent inhibition zone around the colony was considered to be a positive result. For each isolates 6 replications were carried out. The inhibition rate was determined by the following formula,

$$\text{Inhibition rate (\%)} = C-T/C*100$$

C- Diameter of pathogen in control; T- Diameter of pathogen in treatment

In-vitro dual culturing of *Fusarium* sp. growth inhibition by fungal bio-agent *Pochonia chlamydosporia*

The cultured bio-agents *Pochonia chlamydosporia* was used against *Fusarium* sp. growth inhibition in dual culturing, potato dextrose agar was used as medium. The cultures

incubated at 25°C for 7days. Each isolates were replicated 6 times and the growth inhibition of the pathogen was observed. (Fig 1)

$$\text{Inhibition rate (\%)} = C-T/C*100$$

C- Diameter of pathogen in control; T- Diameter of pathogen in treatment

Collection of root samples and Extraction of nematode egg masses: Root and soil samples were collected randomly from the infected Tomato field at MITCAT farm by using khurfi and stored in polythene bags for further extraction and examination of nematodes. The root samples were washed to eliminate soil particles, then dissected with magnification to retrieve egg masses, which were monitored for active nematode stages.

Extraction of juveniles from soil sample

Approximately 250 cc of mixed soil samples were combined with 1000 ml of water and stirred to suspend nematodes. The mixture was decanted through a 20 mm sieve to remove heavy particles, then further filtered into a clean bucket and collected via angled sieve into a pan. After multiple washings up to a 325 mm sieve, the contents were placed in a petri dish with water for 24 hours. Nematodes were then collected and stored for further analysis (Fig 2).

In-vitro Screening of egg hatching and juvenile mortality test:

Egg masses of *Meloidogyne enterolobii* from infected tomato roots were hand-picked and surface sterilized with 0.1 per cent sodium hypochlorite (NaOCl) for ten seconds followed by five washings with sterile water. The surface sterilized egg masses were kept in water for hatching (Popal, 2020)^[13].

Egg hatching inhibition by the antagonistic bacterial and fungal bio-agents

The standard antagonistic bacterial suspension of Bbv57 strain of *Bacillus* sp. and fungal suspension of *Pochonia chlamydosporia* were diluted to the concentration of 25, 50, 75 and 100 percent respectively were poured in the Petri dishes. Five Egg masses were inoculated on each suspension and are incubated at 28±1°C for 24, 48, 72 and 96 hours. Egg masses were inoculated in sterile water acts as control and followed by inoculating egg masses to petriplate containing Velum prime acts as chemical control. Each treatment was maintained with 6 replications. They are evaluated under the aid of Stereo zoom microscope at determined period of time (Ramayabharathi et al., 2018)^[15]. The percent egg hatching was established by the following formula,

$$\text{Hatching (\%)} = \frac{\text{Number of hatched juveniles} \times 100}{\text{Number of hatched eggs} + \text{Number of unhatched eggs}}$$

Calculating the rate of juvenile mortality by the antagonistic bacterial and fungal bio-agents

The egg masses of *M. enterolobii* were kept in water for hatching. The fungal and bacterial suspension diluted to different concentrations viz, 25, 50, 75 and 100 percent respectively. The chemical control was maintained by inoculating J2 stages to Petri plates with Velum prime. The juveniles in distilled water used as control. Each treatment was carried out with 6 replications. They are further incubated at 28±1°C for 24, 48, 72 and 96 hours and mortality

rate were observed under stereomicroscope at specified period of time (Ramyabharathi et al., 2018). The mean% of J2 were estimated using formula (Devi et al., 2018) ^[2, 15],

$$\text{Percent mortality (\%)} = \frac{\text{Total number of dead juveniles} \times 100}{\text{Total number of juveniles}}$$

Statistical analysis

STAR, Statistical Tool for Agricultural Research was used to find out the significant difference through one way ANOVA ($P < 0.05$). Turkey's honestly significant difference test (Turkey's HSD) was used for multiple mean comparisons.

Results

The results of isolation and dual culturing *In-vitro* study for the effective management of fungal-nematode complex in tomato was studied.

Isolation of native *Fusarium* sp.

The wilt infected tomato field was chosen and the soil samples were collected for pathogen isolation. The isolation was performed by serial dilution followed by staining and sub-culturing of the pathogen. The isolated pathogen was observed under microscope and identified as *Fusarium* which produces three types of conidia: Macroconidia, microconidia and chlamydospores causing the wilt diseases in Tomato.

Isolation of bio-agents *Bacillus* sp. and *Pochonia chlamydosporia*

Commercial formulation of *Bacillus subtilis* (Bbv57) and *Pochonia chlamydosporia* were collected from TNAU, Coimbatore. Serially diluted and cultured *Bacillus* sp. and *Pochonia chlamydosporia* followed by the subculturing from the pure culture.

In-vitro Dual-culturing

Three treatments and six replications were fixed, first treatment was performed by streaking antagonistic *Bacillus subtilis* against *Fusarium* on both sides of the pathogen, second treatment was performed by placing *Pochonia chlamydosporia* against *Fusarium* and *Fusarium* alone act as control (Fig 1). The growth inhibition was observed from dual culturing, as the growth of pathogenic *Fusarium* causing wilt was highly inhibited by the antagonistic activity of Bbv57 strain of *Bacillus subtilis* (71.40%) than the fungal antagonistic *Pochonia chlamydosporia* where growth inhibition is (48.17%) (Fig 4a & 4b). From this, *In-vitro* study it was concluded that *Bacillus* sp. acts as virulent antagonistic against *Fusarium* sp.

In-vitro analysis of antagonistic activity of bio-agents on *Meloidogyne enterolobii*

Egg hatching inhibition by the antagonistic bio-agents

The egg hatching inhibition test was observed by using *Bacillus subtilis* (T₁), *Pochonia chlamydosporia* (T₂), Velum prime (T₃) at different concentrations which includes 25,50,75,100 percent and sterile distilled water (T₄) act as control. The eggs were observed at the regular time interval of 24 hours, 48 hours, 72 hours and 96 hours. From the results of egg hatching ability, eggs treated with *Pochonia chlamydosporia* reduced the egg hatching percentage up to 80.2, 76.8, 80.66, 85.0 percent followed by *Bacillus subtilis* reduced the egg hatching percentage up to 50.3, 59.5, 63.16

and 64.8 percent at the concentration of 25,50,75 and 100 percent respectively, after the exposure period of 96 hours (Table 1).

Here, the chemical and sterile water was kept as positive check. It followed the pattern chemical > *Pochonia chlamydosporia* > *Bacillus subtilis*. The results indicated that an increase in concentration and period of exposure leads to progressive decrease in egg hatching percentage. (Fig 3)

Effect of antagonistic bio-agents on juvenile mortality

The juvenile mortality test was done using *Bacillus subtilis* (T₁), *Pochonia chlamydosporia* (T₂) and Velum Prime chemical (T₃) at different concentrations which includes 25,50,75 100 percent respectively and sterile distilled water (T₄) as control. The juvenile mortality was observed at the regular time interval of 24 hours till 96 hours.

From the results of juvenile mortality, juveniles treated with *Pochonia chlamydosporia* showed high mortality percentage up to 40.8, 78.5, 83.1, 85.0 percent followed by *Bacillus subtilis* showed mortality up to 37.5, 62.8, 73.8 and 74.8 percent at the concentration of 25,50,75 and 100 percent respectively, after the exposure period of 96 hours. (Table 2). It followed the pattern chemical > *Pochonia chlamydosporia* > *Bacillus subtilis*. The results indicated that an increase in concentration and period of exposure leads to progressive increase in juvenile mortality percentage. By comparing the bio-agents, this study indicates that *Pochonia chlamydosporia* (T₂) acts as an effective antagonistic at the various concentrations against *Meloidogyne enterolobii* than the bacterial bio-agents (T₁). Discussion

Tomato (*Solanum lycopersicum*) is one of the most widely used and cultivated vegetable crops in India. The Root knot nematode, *Meloidogyne enterolobii* is one of the economically threats causing huge yield loss in tomato. Similarly, Mwangi et al., 2019 ^[10] states that the simultaneous infestation of Root knot nematode and *Fusarium* leads to the formation of a disease complex in Tomato. Management of this disease complex involves plant resistance, biological control agents and use of Neem-extracts. Additionally, Siddiqui et al., 2000 ^[18] notes that *Pseudomonas aeruginosa* alone or with *Purpureocillium lilacinum* had control over Root knot nematode and root infecting fungi under laboratory and field conditions. This chapter discusses the findings from this study analysis of various management techniques. Through the use of soil and root samples, the tomato plants were grown under greenhouse condition and isolation of pathogen and nematodes were made by serial dilution and modified Baerman's method respectively. Similarly, Patil et al., 2020 ^[12] demonstrated the isolation of *Fusarium* from soil by performing serial dilution and growing pathogen on Nash and Snyder's medium. Two bio-control agents such as *Bacillus subtilis* and *Pochonia chlamydosporia* were used in the *In-vitro* assay to determine the effective control over *Meloidogyne enterolobii* and *Fusarium* wilt of tomato. Similarly, Meena et al., 2020 ^[9] stated that for the control of wilt disease complex in tomato, bio-control agents were applied as both seed treatment and soil application. Hence the bio-agents were tested for their antagonistic activity against fungal-nematode complex by undergoing two *In-vitro* study. Dual-culturing was carried out against *Fusarium* pathogen to determine the virulent antagonistic bio-agents. Similarly, Jagadish et al (2018) ^[6] demonstrated dual culturing by different bio agents such as Tv-1, Th-1, Pl-1, Pc-1 and two

bacterial bio agents were evaluated for their mycelial inhibition of *Fusarium*. Also carried out egg hatching inhibition by different concentration of culture filtrates. Hence the mycelial inhibition of pathogen was observed from the plates with *Bacillus subtilis* (Bbv57). The other *in vitro* study was made to know effective antagonistic bio-agents for the control of nematodes. The egg hatching inhibition and juvenile mortality were performed at different concentration of culture filtrates of bio agents. Similarly, Huang et al., 2016^[5] concentration of biocontrol agents. Similarly, Das et al., 2022^[1] performed functional evaluation of cultural filtrates of *Bacillus subtilis* and *Pseudomonas fluorescence* on the mortality and hatching of *Meloidogyne javanica*. As same as,

virulence of four fungal bio-agents against Root knot nematode by egg inhibition and juvenile mortality, *Pochonia chlamydosporia* showed 53.0% of egg inhibition after 72 ha followed by 70.20% of juvenile mortality of root knot nematode.

From this study, it was concluded that *Pochonia chlamydosporia* act as virulent antagonist against *Meloidogyne enterolobii* by inhibition of egg hatching and increasing the death of nematodes. Using the results, the field study can be executed by the inoculation of pathogens and nematodes to determine the effective management technique to overcome the fungal-nematode complex in Tomato.

Table 1: Effect of bacterial and fungal bio-agents on egg hatching inhibition of *Meloidogyne enterolobii*

Egg hatching inhibition by bio-agents																	
Column1 S.No	Column2 Treatments	25%				50% Column650%Column8				75% Column11				100% Column12100% Column14			
		24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
1	<i>Bacillus</i>	24±2.3	38.2±1.8	38.2±0.3	50.3±1.5	34±2.3	44±2.3	54±2.3	59.5±0.9	46.6±0.6	51.3±0.6	58.83±0.6	63.16±2.4	54.3±1.5	60.8±1.3	62.8±0.9	64.8±1.5
2	<i>Pochonia</i>	31.8±0.6	40.2±0.9	60.3±1.5	80.2±1.2	42±2.2	56.2±2.1	66.8±1.5	76.8±1.5	52±2.2	62±2.2	71.3±2.1	80.66±1.7	59.5±1.2	70.5±1.2	94.5±1.0	85±2.3
3	Velum prime	92.2±1.8	93.2±1.8	96.7±0.6	96.8±0.6	94.5±0.9	93.3±0.9	97.8±0.6	97.3±1.2	93±1.2	94.2±0.6	98±0.3	98.33±0.5	93.3±0.9	92.7±0.9	98.5±0.3	98.8±0.0
4	Sterile distilled Water	0.0±0.0	0.0±0.0	0.5±0.7	1.2±1.2	0.0±0.0	0.0±0.0	0.7±0.7	0.3±0.3	0.0±0.0	0.0±0.0	1.33±1.2	1.66±1.2	0.0±0.0	0.0±0.0	1.2±0.9	1.7±1.2
5	CD (0.05)	2.71	2.29	2.37	2.39	3.46	2.85	2.44	2.7	2.96	2.83	3.46	2.41	2.73	2.73	2.31	2.41

Note: Data shown correspond to mean of three replicates± standard error. According to Turkey's honest significant difference (HSD) test, the same letter indicates data in each column is not significantly different ($P<0.05$).

Table 2: Effect of bacterial and fungal bio-agents on juvenile mortality of *Meloidogyne enterolobii*

Juvenile mortality by antagonistic bio-agents																	
Column1 S.No	Column2 Treatments	25%				50% Column650%Column8				75% Column11				100% Column12100% Column14			
		24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h	24h	48h	72h	96h
1	<i>Bacillus</i>	25±0.6	32.7±1.2	37.5±0.9	52.0±1.2	37.5±0.6	44.3±1.9	54.2±1.7	62.8±1.2	47.8±0.9	52.5±0.6	63.6±0.5	73.8±2.7	53.5±0.9	64.8±0.9	69.0±0.6	74.8±0.3
2	<i>Pochonia</i>	33.3±0.6	41.5±0.7	61.0±1.5	78.3±1.5	40.8±1.2	53.8±1.7	64.3±1.5	78.5±1.5	53.3±1.2	64.0±0.9	73.5±0.5	83.1±1.2	61.5±0.9	74.2±1.2	81.8±2.6	85.0±2.3
3	Velum prime	92±0.6	93.0±1.8	96.7±0.6	97.2±0.9	94.1±0.6	92.8±0.9	98.0±0.3	97.8±0.6	94.3±0.7	94.7±0.6	98.1±0.3	98.8±1.2	95.2±0.3	95.7±0.6	98.5±0.7	99.7±0.3
4	Sterile distilled Water	0.0±0.0	0.0±0.0	0.5±0.3	0.7±0.6	0.0±0.0	0.0±0.0	1.0±0.6	1.3±0.9	0.0±0.0	0.0±0.0	1.16±1.2	1.3±0.8	0.0±0.0	0.0±0.0	1.0±0.9	1.5±1.2
5	CD(0.05)	2.37	2.21	2.27	2.16	2.16	2.77	3.06	1.91	2.23	1.75	1.41	3.49	2.5	2.41	2.64	2.33

Note: Data shown correspond to mean of three replicates± standard error. According to Turkey's honest significant difference (HSD) test, the same letter indicates data in each column is not significantly different ($P<0.05$).

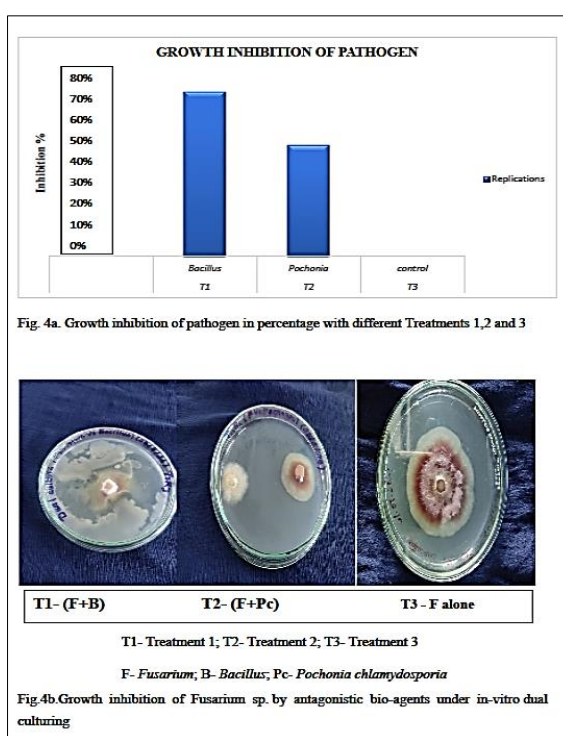
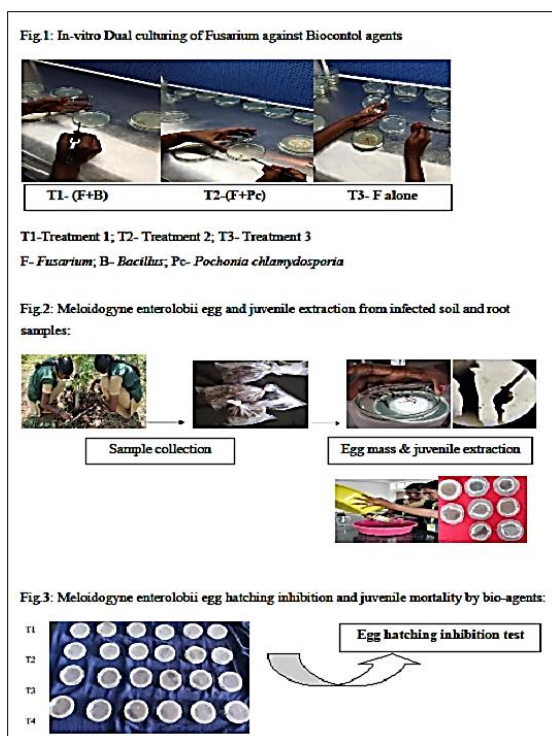
Conclusion: From this study, it was concluded that *Pochonia chlamydosporia* act as virulent antagonist against *Meloidogyne enterolobii* by inhibition of egg hatching and increasing the death of nematodes. Using the results, the field study can be executed by the inoculation of pathogens and nematodes to determine the effective management technique to overcome the fungal-nematode complex in Tomato.

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Conflict of interest

All authors declare that they have no conflicts of interest.



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