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EVALUATION OF INTEGRATED MANAGEMENT APPROACHES AGAINST CITRUS NEMATODE (*TYLENCHULUS SEMIPENETRANS*) IN PAKISTAN

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ABSTRACT

Present study was conducted to explore the nematicidal potential of different synthetic chemicals, biopesticides and antagonists against citrus decline. Effect of eleven chemicals, seven biopesticides and two antagonists on juvenile mortality was evaluated *in vitro*. Four concentrations (2S, S, S/2 and S/4) of each chemical were prepared on the basis of recommended dose for each chemical. Juvenile mortality of citrus nematodes was calculated after 24, 48 and 72 hour of exposure to chemicals. Rugby expressed maximum percentage of juvenile mortality at 2S, S, S/2 and S/4 concentration. Mortality percentage observed by Rugby, Furadan, Match and Cartap after 72 h at 2S concentration was (100, 100, 95.44, 88.23%) whereas at S/4 concentration mortality percentage was (76.32, 81.18, 62.15, 55.28%) respectively. Among biopesticides maximum percentage mortality observed by Proclaim and Cure after 72 hours at 2S concentration was (83.87, 80.44%) while at S/4 concentration it was (59.87, 57.38%) respectively. Cultural filtrates of two antagonist *Trichoderma harzianum* and *Trichoderma viridi* were evaluated at S, S/2 and S/4 concentration under lab conditions. Maximum mortality (88.42%) was observed when both antagonists were applied in combined treatment at S concentration after 72 hours of exposure. Two best performing chemicals, one biopesticide and one antagonist were evaluated under greenhouse against *T. semipenetrans* on *Citrus jambhiri* Lush (rough lemon) and their effect on plant growth and nutrient uptake was measured. Nitrogen uptake was measured by following micro Kjeldahl method. For phosphorus and potassium uptake absorbance of samples at 720 nm was measured with the help of spectrophotometer. Phosphorus was calculated by comparing standard curve already prepared while potassium uptake was assessed by flame photometer method after digestion. All the treatments were found significantly effective against citrus nematode but maximum plant height, stem diameter, number of leaves, root length, root weight, shoot weight, and number of feeder roots (90 cm, 2.4 cm, 102, 48.3 g, 12.1 g, 48.8 g and 70) as compared to control when Rugby, Furadan, Proclaim and *T. harzianum* were applied in combination. Similarly maximum uptake of Nitrogen (3.14) Phosphorous (2.44%) and Potassium (1.95%) was observed when all the treatments were applied in combination. The results of present study will be helpful in selecting the suitable chemicals for growers having problems of citrus nematodes in orchards.

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INTRODUCTION

Citrus orchards are infested with wide range of plant parasitic nematodes (PPN), but among these citrus nematode (*Tylenchulus semipenetrans*) is one of the key nematode pest which has the potential to cause huge losses in citrus yield worldwide (Sorribas *et al.*, 2008). It is a semi-endo-parasitic nematode of all citrus species which has ability to feed on deeper cortex cells. Due to its feeding on roots tree slowly losses its vigor and productivity which ultimately lead towards slow decline (Abd-Elgawad *et al.*, 2010). Annual yield loss due to *T. semipenetrans* ranges from 30-50 percent (Baines *et al.*, 1962). Depending upon infection, 10-30% yield losses have been reported due to citrus nematodes (Verdejo-Lucas and McKenry, 2004). Management of citrus nematodes is difficult as no single method provide adequate control (Verdejo-Lucas and McKenry, 2004). Use of few nematicides and fumigants has been restricted due to their health hazard effects on human, environment and non-target organisms (Rich *et al.*, 2004). However, these chemical are considered as dominant approach for managing the nematodes.

Synthetic chemicals should express a higher magnitude of nematode destruction in a short period of time and should have no phytotoxic effects. It is prerequisite to have information about level of nematode infestation to ensure adequate use of nematicides (Dubey and Trivedi, 2011). Lamberti *et al.* (2000) reported that application non-fumigant nematicides is safe as compared fumigants which are widely being used such as oxamyl, aldicarb, cadusafos, carbofuran and fenamiphos based nematicides.

Biopesticide include the products obtaining from natural sources such as microorganisms, plants, nematodes and insects (Gašić and Tanović, 2013; Bashir *et al.*, 2020). Based on origin and nature these fall into different categories including botanicals, antagonists, pheromones, growth promoters and predators (Semeniuc *et al.*, 2017). However due to the presence of high components of bioactive compounds microorganism and plants are the main source of biopesticides (Nefzi *et al.*, 2016; Hyder *et al.*, 2020) which can be applied in organic farming practices against enemies and minimize the use of chemical insecticides (Shishir *et al.*, 2015; Bibi *et al.*, 2017).

Bio-control agents (BCAs) can be used for the improvement of crop production within existing sources to avoid the problems introduced by chemical pesticides and nematicides (Khan *et al.*, 2014). The use of antagonists to suppress the soil borne pathogens is of immense significance (Zaitoun *et al.*, 2015; Cigdem and Kivanc, 2005). The main reason for applying these beneficial organisms is their ability to establish, colonize and survive in the rhizosphere for effective bio-control (Graham, 2004). *Pseudomonas fluorescens* has promising biocontrol potential to manage *Phytophthora* spp. of citrus (Gade and Armarkar, 2011), but when it is applied by integrated application with fungicides, it proved most effective against root rot of citrus (Koche, 2011). The fundamental principle of IDM is any potential management strategy that may prove environmental friendly and economically feasible through which pesticide treatment is reduced by combination with other non-chemical means (Singh *et al.*, 2012; Ali *et al.*, 2014).

Rehman *et al.* (2006) studies the effect of different chemicals against nematodes on sunflower and found that that Cadusaphos (Rugby) was most effective followed by Unihypo and Carbofuran (Furadan 3 G). Singh (2004) investigated that application of Carbofuran and phorate suppressed the nematode population on ten year old Rough lemon (*Citrus jambhiri* Lush) under field conditions. Effect of different biopesticides such as abamectin, emamectin, and biosal was investigated against *Meloidogyne incognita* (Kofoid and White) on tomato (Ullah *et al.*, 2015).

Khan *et al.* (2017) evaluated bio-protectant ability of neemex (Azadirachtin) and mycorrhizal fungus (*Glomus mosseae*) against invasion and development of *M. incognita*. It was observed that combined application of neemex and *G. mosseae* were most effective and gave maximum inhibition in development of nematodes. A little work has been done so for in the use of different chemicals and bio products against citrus nematode (*T. semipenetrans*. In public domain true nematicides are not available therefore present study was designed to exploit the nematicidal potential of bio and synthetic chemicals against *T. semipenetrans*. Also due to complications and difficulties it is not possible to manage the disease in orchards by focusing only on a single component alone and there is a dire need to integrate all the components. For sustainable and eco-friendly management of disease it is necessary to apply biocontrol agents in IDM frameworks. Therefore, current study is designed to evaluate synthetic chemicals, biopesticides and antagonists against citrus decline disease.

MATERIALS AND METHODS

Isolation of nematodes from soil and root samples

The isolation of nematodes was performed by using Whitehead and Hemming tray method (Whitehead and Hemming, 1965). In this technique each sample was put into a bowl and mixed to separate the roots and debris from soil. Soil texture was made uniform by grinding the coarse soil particles. 100 ml soil sample was measured by using measuring cylinder and was spread on the tissue paper that was attached to the perforated plastic dish which was fixed in the plastic tray containing water in it. It was adjusted in the way that water in the tray hardly touches the tissue paper and tray was covered with the help of plastic lid and was placed in an incubator for 48 hours. Nematodes during this time period came out and were settled in the base of the tray. The water suspension having nematodes were poured into a beaker and were subjected for the counting of juveniles. After the settling of nematodes in the bottom of beaker the supernatant was discarded and the remaining concentrated suspension was transferred to another beaker for further studies. Extraction of nematodes from root samples was performed by using the Baermann funnel technique (McKenry and Roberts, 1985). Feeder roots from each sample were initially washed carefully with tap water and were cut into the portion of small pieces. One gram of root sample from each composite sample was taken and spread on tissue paper that were attached in perforated sheet in the funnel which was fixed with rubber tubes enclosed by clamp at posterior portion. The water present in the funnel just hardly touched the surface of tissue paper and nematodes moved from roots into the rubber tubes and were settled in the bottom of tubes. After 48 hours' clamps were opened and water containing nematodes was poured into beakers. The water suspension containing nematodes was used for counting juveniles. For 3-4 hours' nematode suspension was allowed to settle and excess of supernatant was discarded. The concentrated water suspension was transferred into separate beaker for further investigations.

Preparation of inoculum

Preparation of Nematode (*Tylenchulus semipenetrans* Cobb.) inoculum

The citrus nematode (*T. semipenetrans* Cobb.) was multiplied on six month old seedlings of rough lemon (*Citrus jambhiri*) in pots. The rootstock of *C. jambhiri* was grown in pots with standard soil mixture 1:1:1 (sand, silt, clay). After two weeks of transplanting, pot were inoculated @ 2500 Juveniles/pot and temperature was maintained 27 ± 2 ℃ .The juveniles of citrus nematodes were isolated from soil and roots as described previously. The juveniles from these culture plants and extracted during survey were used for further experimental studies.

In vitro evaluation of bio and synthetic chemicals against *T. semipenetrans*

For the management of nematode elven synthetic chemicals (Rugby, Carbofuran, Match Cartap, Confidor, Arrivo, Movento, Actara, Steward, Polo and Regent) and seven bio-pesticides (Proclaim, Cure, Radient, Astra, Neemix, Timer and Spintor) were evaluated *in vitro*. The nematicidal potential of various nematicides was assessed by their impact at various concentrations (2S, S, S/2 and S/4) on larval mortality of *T. semipenetrans* after 24, 48 and 72 hours of incubation. For this purpose, 0.5 ml of nematodes suspension containing 50 freshly hatched juveniles was poured into petri plates and 5ml of nematicides was added in it with the help of pipette. The plates were then placed at 25 ℃ where dead and surviving nematodes were measured under microscope after 12, 24 and 48 hours. To confirm the death of nematodes they were transferred into sterilized distilled water and the nematodes which did not regain their motility when probed were considered as dead (Mahmood *et al.*, 1979; Abbasi *et al.*, 2008) and which gained motility were considered as alive (El-Rokiek and El-Nagdi, 2011). The percent larval mortality was calculated by using the formula described by Abbott (1925);

$$\text{Percent juvenile mortality} = \frac{\text{No. of juvenile killed}}{\text{Total no. of juveniles}} \times 100$$

All the bio-pesticides were evaluated by using the same procedure with subsequent concentrations. Petri plates having distilled water and juveniles of *T. semipenetrans* were considered as control.

Effect of culture filtrates on larval mortality of *Tylenchulus semipenetrans*

The assay was performed by using the suspension freshly hatched second stage nematodes at the concentration of 100 juveniles/ml that were mixed with

10 ml of cultural filtrates in a petri plate while control was maintained by adding one ml of J_2 in distilled water. Each treatment was replicated three times which were kept at 25°C and the numbers of live and dead nematodes were calculated under Stereomicroscope (Olympus SZ 61) at 40X after 24, 48 and 72 hours of treatment (Osei *et al.*, 2011). The straight shape and immobile nematodes were considered as dead and the mortality percentage of each treatment was recorded by following the equation given below:

$$\text{Mortality (\%)} = \frac{(C1 - C2)}{C1} \times 100$$

While, C_1 is the number of live nematodes juveniles in control treatments and C_2 is the number of live nematodes juvenile counted in other treatments (Li *et al.*, 2005).

Plant material and soil preparation

All the disease free citrus rootstocks of *Citrus jambhiri* Lush. were collected from Citrus Nursery Sanitation Laboratory, Institute of Horticultural Sciences, University of Agriculture, Faisalabad, Pakistan. Soil was prepared before inoculation of fungal and nematode pathogens. Sandy loam soil was prepared by mixing sand (70%), silt (21%), clay (6%) and organic matter (3%). Then the soil was spreaded on wooden bench in the form of thin layer for drying. After that, uniform soil was separated by removing stones and plant debris. All plant husbandry practices were carried out throughout the study to maintain plants healthy. Before any experimental trial, plants with equal size of six month age were selected. Plants were carefully watered so that to avoid leaching of nematodes from soil and to prevent from drying in soil.

Management of *T. semipenetrans* in greenhouse

All the pots were inoculated with freshly hatched 4000 juveniles/pot by making 4-6 holes in each near the root zone. After inoculation with nematodes plants were inoculated with nematicides, biopesticides, and *T. harzianum* alone and in different combinations. Two nematicides (Rugby and Furadan), one biopesticide (Proclaim) and one antagonist (*T. harzianum*) which performed best during *in vitro* studies were selected to evaluate in greenhouse. Pots were arranged in Completely Randomized Design (CRD). Samples were harvested after three months of application of treatments. Samples were collected in polythene bags and labelled with date and name of treatments. Then these were brought into lab and stored. All the treatments were replicated five times. The treatment combinations evaluated against *T. semipenetrans* were as follows;

T_1 = Rugby
T_2 = Furadan
T_3 = Proclaim
T_4 = *T. harzianum*
T_5 = Rugby + Furadan
T_6 = Rugby + Proclaim
T_7 = Rugby + *T. harzianum*
T_8 = Furadan + Proclaim
T_9 = Furadan + *T. harzianum*
T_{10} = Proclaim + *T. harzianum*
T_{11} = Rugby + Furadan + Proclaim
T_{12} = Rugby + Furadan + *T. harzianum*
T_{13} = Furadan + Proclaim + *T. harzianum*
T_{14} = Rugby + Furadan + Proclaim + *T. harzianum*
T_{15} = Healthy control (Distilled water)
T_{16} = Diseased control (*T. semipenetrans*)

Five replications of each treatment were maintained. Data of growth parameters including Plant height (cm), stem diameter (cm), number of leaves, root weight (g), shoot weight (g), root length (cm), number of feeder roots was recorded three months after inoculation.

Determination of biochemical changes

Preparation of samples

Leaf samples of rough lemon rootstock were harvested and dried in oven for 48 hours at 70 °C. After that samples were grounds by using mortar and pestle samples were grounded. Then 100 mg of dried samples were boiled in 10 ml of 1.4N HNO_3 by using hotplate (TH-550; Advantec, Tokyo, Japan) for 30 minutes at 100 °C. Suspension was diluted 250 times with distilled water and analyzed for the determination of Nitrogen (N), phosphorus (P) and potassium (K) by following the method described by Bhargava and Raghupathi (1995).

Determination of total nitrogen

Total Nitrogen in each sample was measured by following micro Kjeldahl method (Kjeldahl, 1883). A known amount of oven dried sample (WI) was taken in a Kjeldahl flask with long neck and five gram of digestion mixture (K_2SO_4, $CuSO_4$ and 25 mL of concentrated H_2SO_4) was added into it. Samples were boiled in

digestion hood first at low temperature and then vigorous boiling was performed to clear the contents in solution. After cooling, distilled water was added in 250 mL volumetric flask to dilute the solution. 10 mL solution was transferred into micro Kjeldahl distillation apparatus and distilled in 10 mL of 40% NaOH solution. Ammonia was collected in a beaker having 2% of boric acid solution containing 2 drops of methyl red as an indicator. After that titration of solution was done against 0.1 N H_2SO_4 to light pink mark. At the end percentage of nitrogen was measured by applying the following formula (Kjeldahl, 1883);

$$\% \text{ of Nitrogen} = \frac{0.1 \text{ N H2SO4 x } 0.0014 \text{ x } 250}{\text{WI x } 100} \times 100$$

Determination of phosphorus and potassium

Sample solution of 0.1 mL obtained after digestion was collected in volumetric flask and 8.7 ml of distilled water with 1 mL of ammonium molybdate reagent was added into it. Solution was mixed by gently shaking the flask and 0.4 mL of aminonephthol sulphonic acid was added into it. Absorbance of samples was measured at 720 nm with the help of spectrophotometer with distilled water as blank in place of sample. The concentration of phosphorus was calculated by comparing the absorbance with standard curves prepared already and concentration of potassium was measured by flame photometer after digestion of samples (Fisk and Subbarow, 1925).

Statistical analysis

Data obtained from experiments was subjected to Analysis of variance (ANOVA). To determine the significant differences, least significant design (LSD) was applied. All the statistical tests were performed by using SAS/STAT statistical software (Institute, 1990).

RESULTS

***In vitro* evaluation synthetic chemicals against *T. semipenetrans* after 24, 48 and 72 hours**

Synthetic chemicals *i.e.* Rugby, Furadan, Match, Cartap, Arrivo, Movento, Actara, Confidor, Steward, Polo, and Regent were evaluated against citrus nematode (*T. semipenetrans*) at 2S, S, S/2 and S/4 concentrations. All the treatments were significantly different from each other ($p \leq 0.05$). Maximum Juvenile (J_2) mortality (88.13%) was calculated by Rugby followed by Furadan (83.92%), Match (71.34%), Cartap (65.22%), Arrivo (61.35), Movento (58.10%), Actara (55.15%), Confidor (50.42%), Steward (43.93%), Polo (40.71%) and Regent (25.1%) as shown in Figure 1.

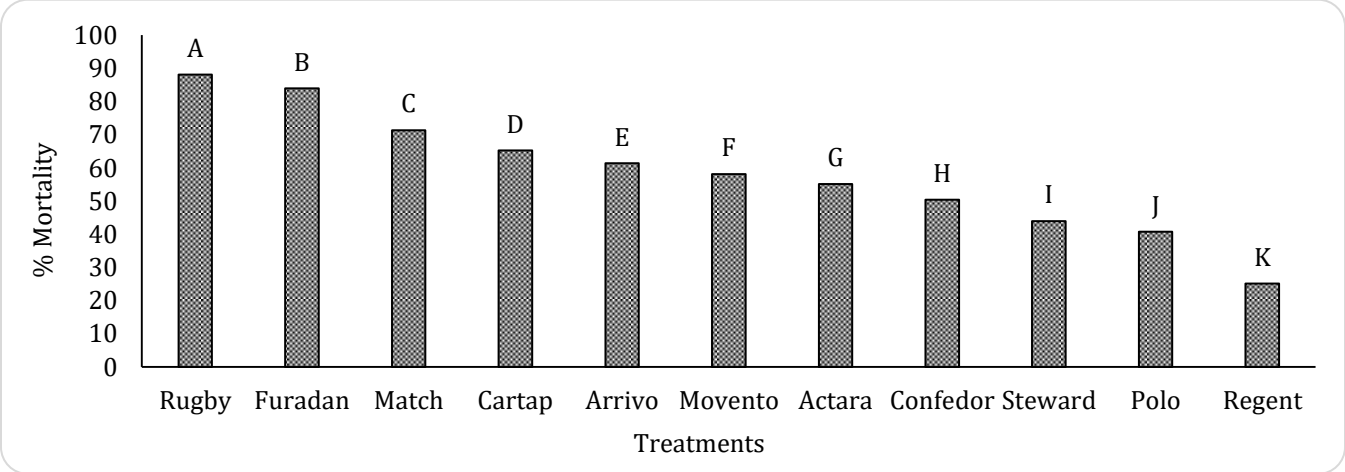

Figure1. Effect of synthetic chemicals on mortality (%) of *T. semipenetrans.*

Rugby expressed maximum percentage of juvenile mortality at 2S, S, S/2 and S/4 concentration. After 24 h maximum juvenile mortality was found in Rugby, Furadan, Match and Cartap (100, 92.42, 82.93,71.32) % at 2S concentration while at S/4 concentration percentage of mortality was (61.26, 53.35, 37.84, 39.82) respectively. After 48 h maximum juvenile mortality in Rugby, Furadan, Match and Cartap was (100, 96.54, 89.66, 82.16) % at 2S concentration while at S/4 concentration mortality was (69.15, 74.54, 50.17, 47.08) % respectively. Mortality percentage observed after 72 h at 2S concentration was (100, 100, 95.44, 88.23) % whereas at S/4 concentration

mortality percentage was (76.32, 81.18, 62.15, 55.28) % respectively. However, minimum mortality at 2S concentration was observed by Regent (26.27, 33.68, 40.1) % while at S/4 concentration it was (14.96, 18.24, 22.2) % after 24, 48 and 72 h respectively. Juvenile's mortality was greatly influenced by treatment's concentration and time of exposure. Maximum J_2 mortality was calculated at 2S and S concentrations as compared to S/2 and S/4. J_2 mortality also increased with exposure of time in all treatments (Table 1).

Table. 1: *In vitro* evaluation of synthetic chemicals against *T. semipenetrans* after 24, 48 and 72 hours

Treatments	Dose	Juvenile Mortality (%)		
		24 h	48 h	72 h
Rugby	2S	100 A	100 A	100 A
	S	100 A	100 A	100 A
	S/2	75.67 OPQ	80.62 KL	94.65 B
	S/4	61.26 ab	69.15 TU	76.32 OP
Furadan	2S	92.42 C	96.54 B	100 A
	S	85.67 GH	91.16 CD	94.56 B
	S/2	69.21 STU	82.27 IJK	86.22 FG
	S/4	53.35 hi	74.54 PQ	81.18 JK
Match	2S	82.93 IJ	89.66 DE	95.44 B
	S	77.42 NO	80.28 KLM	86.65 FG
	S/2	51.27 jkl	67.42 UVW	74.92 PQ
	S/4	37.84 yzA	50.17 klm	62.15 ab
Cartap	2S	71.32 R	82.16 IJK	88.23 EF
	S	62.14 ab	78.77 LMN	84.18 HI
	S/2	45.78 qrs	59.23 cde	68.67 U
	S/4	39.82 wxy	47.08 opq	55.28 gh
Arrivo	2S	69.36 RSTU	78.55 MN	85.12 GH
	S	60.17 bcd	66.24 VWX	68.2 UV
	S/2	47.93 nop	57.83 e	61.2 abc
	S/4	39.12 wxyz	50.33 klm	52.18 ijk
Movento	2S	64.2 YZ	71.19 RS	75.36 PQ
	S	57.64 ef	62.37Za	70.88 RST
	S/2	45.54 qrs	51.98 ijk	64.49 XY
	S/4	38.78 xyz	44.65 rs	50.2 klm
Actara	2S	58.26 de	68.56 U	73.65 Q
	S	53.17 ij	61.28 ab	66.14 WXY
	S/2	46.2 pqr	49.87 lmn	55.72 fg
	S/4	37.58 zA	42.06 tuv	49.36 lmn
Confidor	2S	53.77 ghi	68.38 U	71.19 RS
	S	48.34 mno	59.07 de	62.21 Za
	S/2	39.23 wxyz	42.28 tu	46.6 opqr
	S/4	31.08 E	39.17 wxyz	43.82 st
Steward	2S	46.63 opqr	59.14 de	68.2 UV
	S	40.84 uvw	45.86 qr	51.29 jkl
	S/2	35.33 BC	39.25 wxyz	44.65 rs
	S/4	27.86 FG	31.86 DE	36.28 AB
Polo	2S	41.14 uvw	50.76 kl	55.45 g
	S	33.19 D	46.22 pqr	50.38 kl
	S/2	30.8 E	39.88 wx	45.33 qrs
	S/4	27.78 FG	32.18 DE	35.51 BC
Regent	2S	26.27 FG	33.68 CD	40.1 vwx
	S	20.83 H	25.84 G	33.3 D
	S/2	16.67 IJ	21.12 H	28.1 F
	S/4	14.96 J	18.24 I	22.2 H

Mean sharing similar letter do not significantly differe from each other. $p \leq 0.05$

Effect of synthetic chemicals on mortality (%) of *T. semipenetrans*

***In vitro* evaluation of Biopesticides against *T. semipenetrans* after 24, 48 and 72 hours**

Biopesticides *i.e* Proclaim, Cure Radient, Astra, Neemix, Timer and Spintor were evaluated against citrus nematode (*T. semipenetrans*) at 2S, S, S/2 and S/4 concentrations. All the treatments were significantly different from each other ($p \leq 0.05$). Maximum juvenile (J_2) mortality (65.03%) was calculated by Proclaim followed by Cure (61.89%), Radient (57.42%), Astra (49.57%), Neemix (41.68%), Timer (32.24%), and Spintor (18.22%) as shown in Figure 2.

Proclaim exhibited maximum percent juvenile mortality at 2S, S, S/2 and S/4 concentration. Maximum percentage of juvenile mortality was found in Proclaim at 2S, S, S/2 and S/4 concentration (83.87, 75.38, 63.74, 59.87) followed by Cure (80.44, 72.15, 61.22, 57.38), Radient (78.08, 69.19, 57.78, 51.94), Astra (74.49, 66.4, 50.38, 44.82), Neemix (60.33, 56.18, 42.33, 36.67), Timer (52.48, 44.27, 36.51, 28.53) and Spintor (30.19, 25.78, 17.6, 13.98) after 72 h.

After 24 h juvenile mortality in Proclaim was highest at 2S, S, S/2 and S/4 concentration was (70.54, 67.74, 55.12, 45.9) followed by Cure (67.56, 61.28, 50.16, 44.24), Radient (59.1, 55.39, 47.66, 42.38), Astra (50.28, 46.84, 36.62, 29.54), Neemix (44.23, 39.84, 26.48, 20.08), Timer (32.15, 29.54, 18.67, 15.3) and Spintor (21.45, 18.92, 11.64, 8.32) % respectively. Similarly, after 48 h maximum percentage of juvenile mortality was observed in Proclaim (76.35, 71.85, 59.25, 50.78) followed by Cure (73.55, 69.42, 55.09, 50.26), Radient (68.74, 58.31, 52.15, 48.32), Astra (62.48, 58.32, 41.14, 33.6), Neemix (56.75, 51.28, 34.92, 31.18), Timer (41.92, 38.22, 27.67, 21.53) and Spintor (26.81, 16.18, 14.88, 12.96) at 2S, S, S/2 and S/4 concentrations respectively. Treatment's concentration and time of exposure influenced juvenile mortality significantly where maximum mortality was calculated at 2S and S concentrations as compared to S/2 and S/4 concentrations. Mortality was also increased with time of exposure (Table 2).

Evaluation of cultural filterates of antagonistic fungi against *T. semipenetrans*

Cultural filtrates of *T. harzianum*, *T. viridi* and combination of both fungus (*T. harzianum* and *T. viridi*) were evaluated against *T. semipenetrans*. All the treatments (T), their concentrations (C) and exposure period (T) were significantly different from each other at $P \leq 0.05$. Interaction among treatments and concentrations (TxC) showed that combined treatment of both antagonistic fungi caused maximum mortality percentage (83.80, 77.48, and 67.75%) followed by *T. harzianum* (65.02, 60.20, and 54.83%) and *T. viridi* (43.25, 39.96, and 37.84%) at S, S/2 and S/4 concentrations respectively (Table 3). Similarly, interaction between treatment and time (TxT) revealed that maximum mortality was showed after 72 h in combined treatment of both antagonistic fungi followed by alone treatment of *T. harzianum* and *T. viridi* respectively (Table 4). Similarly, interaction between treatments, concentration and time showed that at S/4 concentration all antagonists expressed minimum percentage of mortality after 24, 48 and 72 h as compared to S/2 and S concentrations (Table 5).

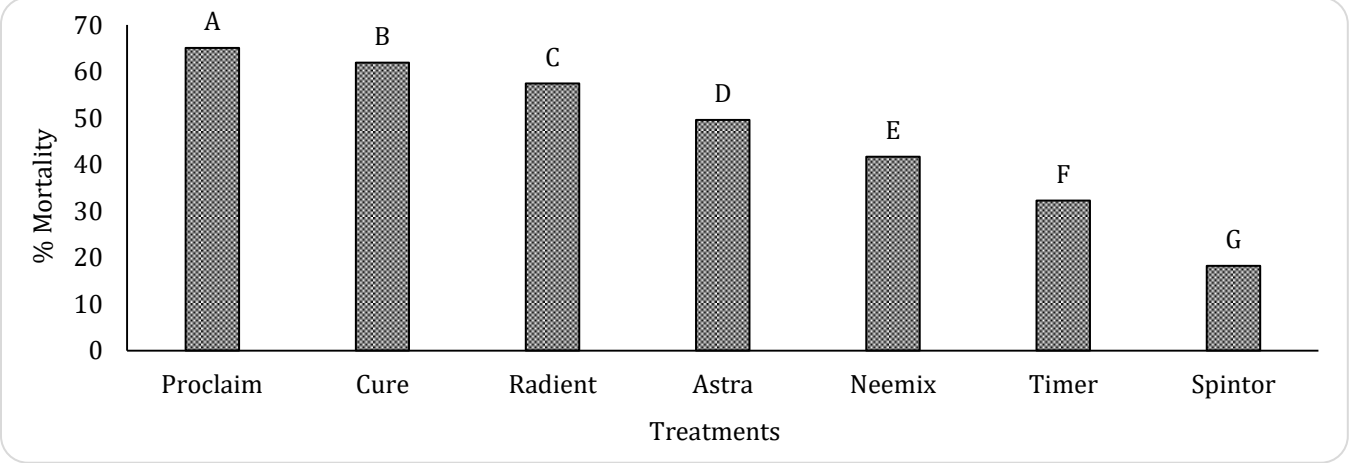

Figure 2. Effect of bio-pesticides on mortality (%) of *T. semipenetrans.*

Table 2: *In vitro* evaluation of Biopesticides against *T. semipenetrans* after 24, 48 and 72 hours.

Treatments	Dose	Juvenile Mortality (%)		
		24 h	48 h	72 h
Proclaim	2S	70.54 HI	76.35 CD	83.87 A
	S	67.74 JKL	71.85 GH	75.38 DE
	S/2	55.12 V	59.25 PQR	63.74 M
	S/4	45.9 ab	50.78 WXY	59.87 OPQ
Cure	2S	67.56 KL	73.55 FG	80.44 B
	S	61.28 NO	69.42 IJ	72.15 GH
	S/2	50.16 Y	55.09 V	61.22 NO
	S/4	44.24 b	50.26 Y	57.38 STU
Radient	2S	59.1 PQRS	68.74 IJK	78.08 C
	S	55.39 V	58.31 QRST	69.19 IJK
	S/2	47.66 Za	52.15 WX	57.78 RSTU
	S/4	42.38 c	48.32 Z	51.94 WXY
Astra	2S	50.28 Y	62.48 MN	74.49 EF
	S	46.84 Za	58.32 QRST	66.4 L
	S/2	36.62 fg	41.14 cd	50.38XY
	S/4	29.54 klm	33.6 hi	44.82 b
Neemix	2S	44.23 b	56.75 TUV	60.33 OP
	S	39.84 de	51.28 WXY	56.18 UV
	S/2	26.48 op	34.92 gh	42.33 c
	S/4	20.08 qr	31.18 jk	36.67 fg
Timer	2S	32.15 ij	41.92 c	52.48 W
	S	29.54 klm	38.22 ef	44.27 b
	S/2	18.67 rs	27.67 mno	36.51 fg
	S/4	15.3 uv	21.53 q	28.53 lmn
Spintor	2S	21.45 q	26.81 nop	30.19 kl
	S	18.92 rs	16.18 tu	25.78 p
	S/	11.64 x	14.88 uv	17.6 st
	S/4	8.32 y	12.96 wx	13.98 vw

Table 3. Effect of cultural filtrates of antagonists and their concentrations % mortality of *T. semipenetrans*

Treatments	% Mortality		
	S	S/2	S/4
T.H + Ts	65.02 d	60.20 e	54.83 f
T.V +Ts	43.25 g	39.96 h	37.84 i
T.H. + T.V.+ Ts	83.80 a	77.48 b	67.75 c
Control (Ts)	1.73 j	1.73 j	1.73 j

T.H= *Trichoderma harzianum*, **T.V**= *Trichoderma viride*, **Ts**= *Tylenchulus semipenetrans*

Table 4. Effect of cultural filtrates of antagonists and their exposures on % mortality of *T. semipenetrans*

Treatments	% Mortality		
	24 h	48 h	72 h
T.H + Ts	57.01 f	59.44 e	63.59 d
T.V + Ts	36.79 i	39.54 h	44.72 g
T.H. + T.V + Ts	71.63 c	76.41 b	81.04 a
Control (Ts)	1.25 k	1.95 j	2 j

T.H= *Trichoderma harzianum*, **T.V**= *Trichoderma viride*, **Ts**= *Tylenchulus semipenetrans*

Table 5. Effect of cultural filtrates of antagonists, their exposure and concentration on % mortality of *T. semipenetrans*

Treatment	Concentration	Time		
		24	48	72
T.H + Ts	S	62.85 g	65 f	67.21 e
T.H + Ts	S/2	56.95 i	59.65 h	64 fg
T.H + Ts	S/4	51.25 k	53.69 j	59.55 h
T.V + Ts	S	40.04 o	43.07 n	46.65 l
T.V + Ts	S/2	36.53 q	38.56 p	44.79 m
T.V + Ts	S/4	33.80 r	36.99 q	42.74 n
T.H + T.V + Ts	S	78.89 c	83.16 b	89.37 a
T.H + T.V + Ts	S/2	72.26 d	78.04 c	82.14 b
T.H + T.V + Ts	S/4	63.74 g	68.02 e	71.50 d
Control (Ts)		1.25	1.95	2

T.H= *Trichoderma harzianum*, **T.V**= *Trichoderma viride*, **Ts**= *Tylenchulus semipenetrans*

Management of *T. semipenetrans* under greenhouse conditions

Effect of synthetic chemicals, bio-pesticides and *T. harzianum* alone and in different combinations was evaluated against *T. semipenetrans* in growth and development of rough lemon under greenhouse conditions. Rugby, Furadan, Proclaim and *T. harzianum* were applied alone and in different combinations for the management of citrus nematode. All the treatments varied significantly from each other at (P≤0.05). All the treatments and their combinations reduced the effect of pathogen by improving plant growth. Among growth parameters maximum plant height (90 cm), stem diameter (2.4 cm), number of leaves (102), root length (48.3 cm), root weight (12.1 g), shoot weight (48.8 g) and number of feeder roots (70) as compared to diseased and healthy control was observed in combined treatment of both synthetic chemicals, bio-pesticide and *T. harzianum* (Rugby+ Furadan + Proclaim+*T. harzianum*). However, when applied alone, Rugby was found best in reducing pathogen by improving plant growth as shown (Table 6).

Table 6: Effect of combined and individual application of synthetic chemical, bio-pesticides, *T. harzianum* on growth and development of Rough lemon against *T. semipenetrans*

Treatment	PH (cm)	SD (cm)	NL	RL(cm)	RW(g)	SW(g)	NFR
Rugby	70.5 f	2.2 abc	73 g	30.1 g	8.1 cd	25 f	48.2 fg
Furadan	64.4 g	2.2 abc	63.6 i	26.6 h	7.8 cd	20.1 hi	43 h
Proclaim	56 i	2.1 bc	59.6 j	21.5 j	7.6 d	18 j	46 gh
T.H	59.1 h	2.1 bc	67.8 h	22.3 ij	8.2 cd	21 h	50 fg
Ru+Fu	79.9 d	2.2 abc	87 c	39 d	9 c	28.2 e	56.2 de
Ru+Pro	73 f	2.2 abc	80 e	32.9 f	8.3 cd	23 g	51.6 ef
Ru+T.H	76 e	2.3 ab	86 cd	35 e	8.6 cd	30.1 d	56 de
Fu+Pro	71.1 f	2.1 bc	83.8 d	26 h	7.7 d	25 f	51.2 f
Fu+T.H	72.6 f	2.1 bc	80.6 e	37.1 d	9 c	29 de	49 fg
Pro+T.H	66.7 g	2 c	80 e	24 i	8.5 cd	20.2 h	47.4 fgh
Ru+Fu+Pro	84.2 c	2.3 ab	92 b	41.1 c	10.5 b	36.3 c	60.6 cd
Ru+Fu+T.H	87 b	2.4 a	93.8 b	43 c	11 ab	40.2 b	63 bc
Fu+Pro+T.H	89.1 ab	2.3 ab	87.4 c	45.2 b	11.2 ab	38 c	66 ab
Ru+Fu+Pro+T.H	90 a	2.4 a	102 a	48.3 a	12.1 a	48.8 a	70 a
Diseased	42 j	1.7 d	52.8 k	18.4 k	5.3 e	9.6 k	59.4 cd
Healthy	55 i	2.2 abc	77 f	29.6 g	8.5 cd	18.4 ij	46 gh

Ru=Rugby, **Fu=**Furadan, **Pro=**Proclaim, **T.H.** *Trichoderma harzianum*, **PH=**Plant height, **SD=**Stem diameter, **NL=**Number of leaves, **RL=**Root length, **RW=**Root weight, **SW=**Shoot weight, **NFR=**Number of feeder roots

Effect of nematicides, bio-pesticide and antagonist on macronutrients against *T. semipenetrans*

Effect of nematicides, bio-pesticide and antagonist on nitrogen (%) against *T. semipenetrans*

Effect of nematicides (Rugby, Furadan), bio-pesticide (Proclaim) and antagonist (*T. harzianum*) alone and in various combinations on nitrogen percentage of Rough lemon under greenhouse conditions. All the treatments varied significantly from each other at (P≤0.05). Maximum concentration of nitrogen (3.14%) was measured in combined treatment of both nematicide, bio-pesticide and antagonist (Rugby+ Furadan+ Proclaim+ *T. harzianum*) followed by Rugby+ Furadan + *T. harzianum* (2.98%), Rugby+ Furadan + Proclaim (2.80%), Furadan+ *T. harzianum* (2.80%), Rugby + *T. harzianum* (2.72%), + Furadan + Proclaim (2.68%), Furadan + Proclaim + *T. harzianum* (2.62%), Rugby + Furadan (2.58%), Rugby (2.50%), Proclaim + *T. harzianum* (2.48%), Rugby + Proclaim (2.40%), Furadan (2.38%), *T. harzianum* (2.18%), Proclaim (2.02%) as compared to diseased (1.51%) and healthy (1.92%) control (Figure 3).

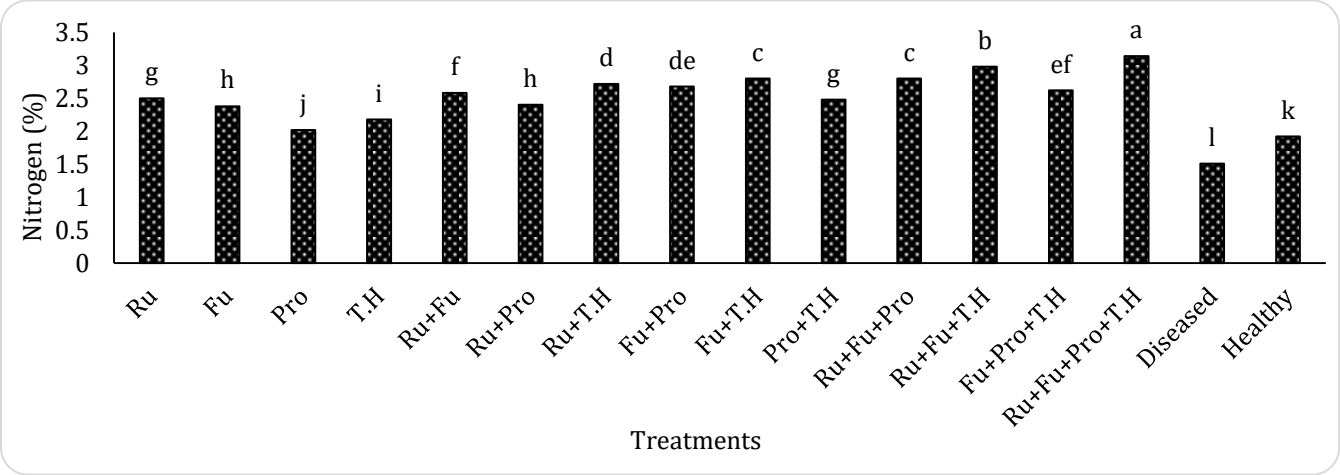

Figure 3. Effect of synthetic chemicals, bio-pesticides and *T. harzianum* on Nitrogen level of Rough lemon against *T. semipenetrans*; **Rug**= Rugby, **Fu**= Furadan, **Pro**= Proclaim, **T.H**= *T. harzianum*

Effect of nematicides, bio-pesticide and antagonist on phosphorus (%) against *T. semipenetrans*

Effect of nematicides (Rugby, Furadan), bio-pesticide (Proclaim) and antagonist (*T. harzianum*) alone and in various combinations on phosphorus percentage of Rough lemon under greenhouse conditions. All the treatments varied significantly from each other at (P≤0.05). Maximum concentration of phosphorus (2.44%) was measured in combined treatment of both nematicide, bio-pesticide and antagonist (Rugby+ Furadan+ Proclaim+ *T. harzianum*) followed by Rugby+ Furadan + *T. harzianum* (2.26%), Furadan + Proclaim + *T. harzianum* (2.13%%), Rugby+ Furadan + Proclaim (1.89%), Rugby + Furadan (1.80%) Furadan+ *T. harzianum* (1.72%), Rugby + *T. harzianum* (1.64%), Rugby (1.62%), Rugby + Proclaim (1.52%), Furadan + Proclaim (1.48%), Furadan (1.44%), Proclaim + *T. harzianum* (1.39%), *T. harzianum* (1.28%), Proclaim (1.02%) as compared to diseased (1.40%) and healthy (1.72%) control (Figure 4).

Effect of nematicides, bio-pesticide and antagonist on potassium (%) against *T. semipenetrans*

Effect of nematicides (Rugby, Furadan), bio-pesticide (Proclaim) and antagonist (*T. harzianum*) alone and in various combinations on potassium percentage of Rough lemon under greenhouse conditions. All the treatments varied significantly from each other at (P≤0.05). Maximum concentration of potassium (1.95%) was measured in combined treatment of both nematicide, bio-pesticide and antagonist (Rugby+ Furadan+ Proclaim+ *T. harzianum*) followed by Rugby+ Furadan + *T. harzianum* (1.85%), Rugby+ Furadan + Proclaim (1.81%), Furadan + Proclaim + *T. harzianum* (1.73%), Rugby + Furadan (1.68%) Furadan+ *T. harzianum* (1.61%), Rugby + *T. harzianum* (1.53%), Rugby + Proclaim (1.49%), Furadan + Proclaim (1.41%), Rugby (1.38 %), Proclaim + *T. harzianum* (1.29%), Furadan (1.22%), *T. harzianum* (1.12%), Proclaim (1.02%) as compared to diseased (1.12%) and healthy (1.4%) control (Figure 5).

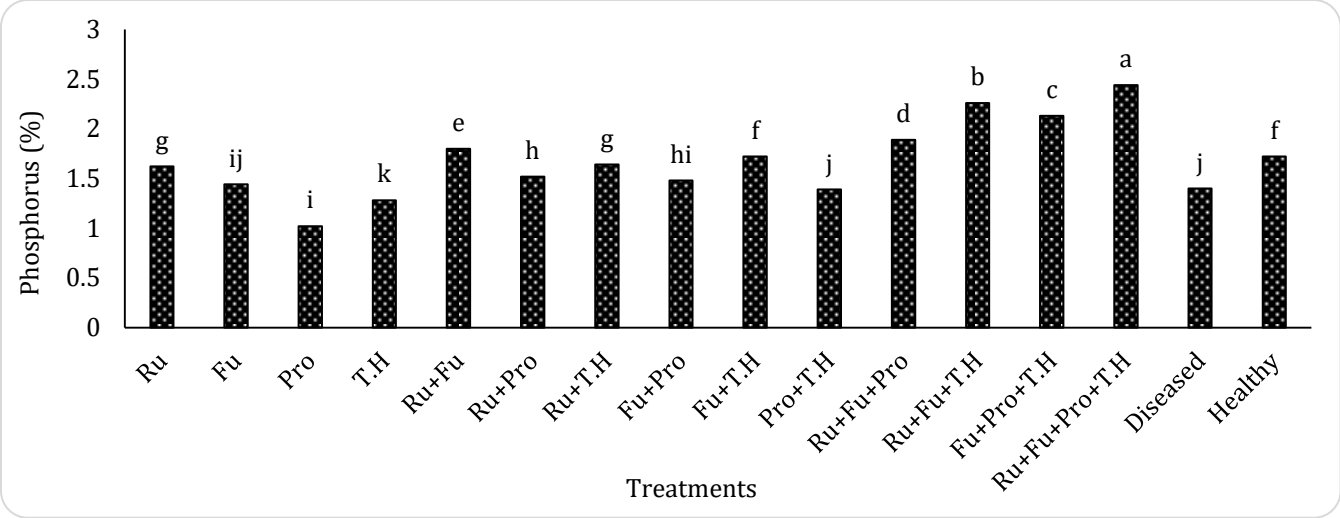

Figure 4. Effect of synthetic chemicals, bio-pesticides and *T. harzianum* on Phosphorus level of Rough lemon against *T. semipenetrans*; **Ru=** Rugby, **Fu=** Furadan, **Pro=**Proclaim, **T.H=** *T. harzianum*

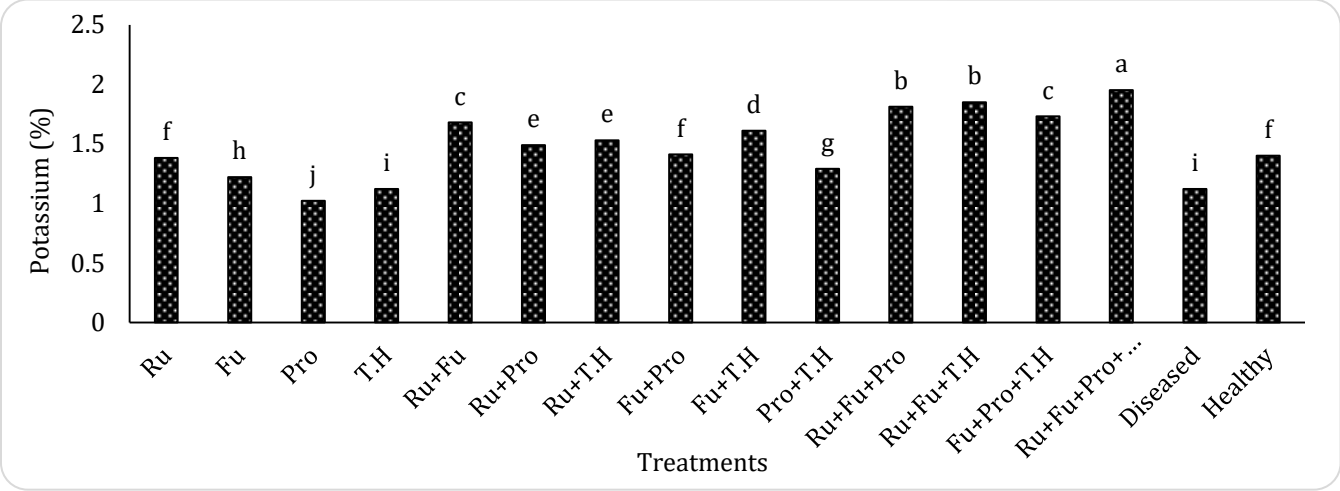

Figure 5. Effect of synthetic chemicals, bio-pesticides and *T. harzianum* on Potassium level of Rough lemon against *T. semipenetrans*; **Ru=**Rugby, **Fu=**Furadan, **Pro=**Proclaim, **T.H=** *T. harzianum*

DISCUSSION

Different synthetic chemicals and bio-pesticides were evaluated for their nematicidal potential against *T. semipenetrans* under *in vitro* conditions. All the tested chemicals caused different levels of J_2 mortality at various concentrations. Nematicidal potential of chemicals against nematodes was reported by several scientists (Cayrol *et al.*, 1993; Safdar *et al.*, 2012). Rugby followed by Furadan, Match and Cartap were found very effective against nematodes *in vitro.* Nematicidal action of these chemicals was attributed to their various mechanisms of actions. Rugby and Cartap belongs to organophosphate while Furadan belongs to Carbamate group. Rugby and Caratp as belongs to organophosphate group, their nematicidal activity was due to the inactivation of acetylcholinesterase which is an important enzyme in the nervous system of nematodes. As locomotion of nematodes depends upon motor neurons and interneurons which act by using neurotransmitter acetylcholine whose activity is stopped by inactivation of acetylcholinesterase (Johnson and Stretton, 1987; Ali *et al.*, 2019). Similarly, Match (Lufenuron) act by limiting the chitin production in nematodes, as a result of which larvae could not develop hard outer covering and its internal organs exposed after hatching and molting (Meola *et al.*, 1999).

Biopesticides also reduced the population of *T.*

semipenetrans by increasing the mortality percentage of nematodes. Bio chemicals are not harmful to human beings as these have less residual effect as compared to synthetic chemicals, easily decompose; effective in small quantities with lower risks. Use of bio chemicals for the management of nematodes is effective and eco-friendly practice. Proclaim and Cure were found most effective in reducing the population of nematodes *in vitro*.

For the control of plant pathogens which limit the yield of crop, farmers mostly depend on the use of synthetic chemicals. Non-judicious use of these chemicals is harmful for both environment and human beings. To replace these chemicals, the alternative method for the control of plant diseases is the use of biological control agents. The most suitable microorganisms used as bio-control agents are those which grow in the rhizosphere. These can be the potential ecofriendly and cost effective strategy for farmers. *Trichoderma* species are known as promising bio-control agents as they lower the disease incidence of fungal pathogens such as *Fusarium* through various mechanism including mycoparasitism, ability to compete for food and space, antibiosis and activation of defense system (Dubey and Trivedi, 2011).

Nutrients have different type of effects in the development of diseases. Status of plant's nutrients has close relationship with pathogen which is dynamic and complex (Vandermeer *et al.*, 2010; Ploetz, 2006; Desaeger *et al.*, 2004) and hence proper nutrient management can reduce the severity of number of diseases. Morphological and histological properties and structure are determined by the nutritional status of a plant, which controls the pathogen entry, penetration rate and pathogenesis. Different species of pathogens and plant interact with each other undergo different soils and environmental conditions. Plants acquire all nutrients from soil, they commonly provide most of the nutrients required for growth of pathogen. So it is quite possible that different nutrients may distinctly influence virulence/avirulence of pathogen as well as susceptibility/resistance of the host. Plant which obtains proper nutrition with all essential elements undergoes a less disease development. Nutrition uptake is essential process that affects the growth of plant and disease development have remarkable effect on plant's nutritional status as described by (Bhaduri *et al.*, 2014; Spann and Schumann, 2010; Dordas, 2008; Mishra and Gupta, 2012; Qifei *et al.*, 2003).

For the management of *T. semipenetrans* cultural filtrates of T*. harzianum* and *T. viridi* were evaluated at different concentrations to access the mortality of nematode. It was observed that combined application of *T. harzianum* and *T. viridi* cultural filtrates caused highest nematode mortality as compared to alone application of each antagonist. However, among both fungi when applied alone *T. harzianum* gave maximum nematode mortality as compared to *T. viridi* alone aplictaion. Cultural filtrates of various fungi have the ability to produce toxic substances against nematodes. Trichoderma species produce different antibiotics like trichoderin, trichodermol A and harzianolide. Various molecules are such as enzymes, VOCs and pentyl α-pyrone (Samson *et al.*, 1996) and these compounds damage the cuticle of nematodes. *T. harzianum* produce different kind of enzymes including glucanase, chitinase and protease which are responsible for digesting the cuticle of nematode and destroy the integrity of cell wall resulting in death of nematode (Huang *et al.*, 2004). Also hyphae of Fungal species build a physical barrier by growing along the roots of host plant. Direct parasitism of nematodes by Trichoderma requires successful penetration of nematode cuticle which is observed by the production of lytic enzymes (Spiegel *et al.*, 2004). Mechanisms besides direct antagonism, *Trichoderma* spp. include production of, fungal metabolites and induced resistance (Samuels, 1996; Goswami *et al.*, 2008). Casas-Flores and Herrera-Estrella (2007); Moosavi and Zare (2020) reported that *Trichoderma harzianum* has been an effective ecofriendly control method for the management of nematodes. Citrus nematode (*T. semipenetrans*) is found to be a sever threat to citrus industry. Management of citrus nematode can be achieved by integration of nematicides, biopesticides and antagonistic fungi.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests.

AUTHORS CONTRIBUTIONS

All the authours have contributed equally in the manuscript.