Biological Control 157 (2021) 104569

Contents lists available at ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Bio-management of soil borne pathogens infesting cucumber (*Cucumis sativus* L.) under protected cultivation system

Satyendra Singh *, Rekha Balodi

ICAR-National Research Centre for Integrated Pest Management, New Delhi 110012, India

HIGHLIGHTS

- Biological control is a key component for IPM under protected cultivation farming system.
- Consortia of bio-agents for management of soil borne disease.
- Bio-agents reduces pesticide risks under protected environment.
- Help to develop bio-agent based IPM module for protected crops.

ARTICLE INFO

Keywords:
Biocontrol agents
Cucumber
Protected cultivation
Soil borne pathogen

ABSTRACT

Trichoderma harzianum, *Bacillus subtilis* and *Pseudomonas fluorescens* were tested separately and together under *in vivo* for their ability to supress soil borne pathogens i.e. *Meloidogyne incognita, Fusarium oxysporum* and *Rhizoctonia solani* infesting cucumber (*Cucumis sativus* L.) cv. Magicstar in two consecutive trials (2018–2020) under protected cultivation system. The initial mean population of *M. incognita, F. oxysporum* and *R. solani* was recoded 1.72 J_2/g soil, 1.3 × 10^3 cfu/g soil and 7.8 propagules/50 cc soil respectively. Freshly prepared bio-agents were used as soil and drench application. Tested bio-agents were mixed with FYM and vermicompost for fortification before soil application, whereas for drench application, they were mixed with water at the rate of 5 ml/l. The suppressive effect of bio-agents was significantly ($P > 0.05$) greatest with combined bio-agents (*T. harzianum* + *B. subtilis* + *P. fluorescens*) application treatment which caused greatest reduction of *M. incognita, F. oxysporum* and *R. solani* multiplication which was up to 90% at the end of subsequent second season trial with significant enhancement in plant health. The eggs of *M. incognita* obtained from plants treated with all tested bio-agents showed least hatching (20%) when subjected to bioassay test after termination of experiments. Fluensulfone did not affect multiplication of *F. oxysporum* and *R. solani* in soil but establish the reduction in shoot and root disease severity in the plant system. Tested bio-agents were successfully established in the rhizosphere of cucumber plants which showed compatibility among them, hence may be ideal companions to develop consortia against soil borne diseases under protected cultivation system.

1. Introduction

Protected cultivation technology is relatively, a new technology and popular among farmers/growers globally. As per an estimate the total area covered under protected cultivation in India is approximately 30000 ha (Shweta et al., 2014), which is increasing rapidly, however, this technology is still in its infancy stage in India compared to 405000 ha throughout the world (Reddy, 2016). Among crops grown under protected cultivation system, cucumber (*Cucumis sativus* L.) is an important vegetable worldwide and is the second most popular crop planted in green houses (Mao et al., 2017) and due to its short duration farmers/growers are cultivating cucumber twice a year under naturally ventilated protected structure in India. Due to the low cost, higher quality products and income per unit area (Nimbrayan et al., 2018) these are very popular among farmers but high temperature and humidity conditions in these structures round the year make cucumber crop more vulnerable to soil borne diseases (Shishido, 2011). Among soil borne pathogens, *Meloidogyne* spp., *Fusarium* spp. and *Rhizoctonia* spp. cause extensive losses to this crop in both, open field cultivation as well as under protected cultivation system (Mao et al., 2017). *Meloidogyne*

* Corresponding author at: ICAR-NCIPM, LBS Building, IARI (PUSA) Campus, New Delhi 12, India.
E-mail addresses: satyendra.singh1@icar.gov.in (S. Singh), rekha.balodi@icar.gov.in (R. Balodi).

https://doi.org/10.1016/j.biocontrol.2021.104569
Received 3 November 2020; Received in revised form 20 January 2021; Accepted 16 February 2021
Available online 23 February 2021

spp. attacks nearly every crop and has been reported to cause an annual loss of Rs. 547.5 million INR in cucurbits (Jain et al., 2007) in India. *M. incognita*, is the most common and wide spread species which is number one enemy of cucumber crop and caused yield loss up to 69.2% (Krishnaveni and Subramanian, 2002; Singh et al., 2018). It also increases the severity of the diseases by predisposing the plant roots, to wilt and root rot fungal pathogens (Singh and Goswami, 2001; Mallaiah et al., 2014; Meena et al., 2015; Patil et al., 2017) causing synergistic effect and in most of the cases, even the destruction of the whole plant (Pandey et al., 2003).

Fusarium wilt caused by *Fusarium oxysporum* and root rot caused by *Rhizoctonia solani* are other serious pathogens of cucumber plants. *Fusarium* wilt has been reported to be one of the most destructive diseases in commercial cucumber production (Hu et al., 2010). The yield losses caused by this fungus on cucumber is recorded 70–100% globally (Egel and Martyn, 2007) whereas in India, it may cause crop losses to the tune of 10–80% (Bharat and Sharma, 2014). Also, *R. solani*, that lives in the soil in the form of sclerotia and does not reproduce asexual spores (Baker, 1970, Strashnov et al., 1985), still represents one of the most difficult problem to be managed both in nurseries and fields (Lamichhane et al., 2017) under greenhouses. There is no detailed and precise estimation of losses in monetary terms, however, the losses due to incidence of damping off and root rot disease caused by *R. solani* may vary from 5 to 80% (Lamichhane et al., 2017).

The chemical pesticides have important role in modern agricultural developments to increase in production by supressing the agricultural pests and still these chemicals are the first choice of growers to grow more food for escalating population. But frequent and indiscriminate use of these chemical pesticides raises a number of ecological disturbances, human health hazards, and depletion in beneficial microorganisms in the soil and ozone layer depletion and also lead to development of pesticide resistance (Panth et al., 2020).

As per an estimate about 0.1% of these chemicals reach to the target pest and rest of the used chemicals caused damage to environment (Ashraf and Zuhaib, 2013). In many parts of the world, one of the control strategy for soil borne pathogens was application of methyl bromide (MeBr), before the implementation of the Montreal Protocol in 1986 to protect ozone layer (Bell, 1996). The main targets of MeBr are weeds, root-knot nematode, wilt and root rot and damping diseases. After the ban on MeBr, metham sodium, dazomat, cador, oxamyl, fenamiphos, cadusafos and ethoprop were among the most popular soil fumigants used to counter soil borne pathogens (Giannakou et al., 2002, Sharma et al., 2008a, Sharma et al., 2008b). Other chemicals i.e. chlorpyriphos, carbosulfan, carbofuran etc. were also used alone or in combinations with bio-agents by several researchers (Nagesh and Reddy, 2005; Sharma et al., 2008a, Sharma et al., 2008b; Singh, 2013, 2019). However, these alternative methods either have inconsistent results (Keinath and Batson, 2000) or are less effective than MeBr (Gerik and Hanson, 2011). In addition, presence of residue of these pesticides drew due attention which can be found in a great variety of every day foods and ground water (Gunnell et al., 2007; Burnett and Welford, 2007) and also detected from human breast milk samples (Pirsaheb et al., 2015). All these demerits of synthetic chemical pesticides and their exclusion from agricultural production system served as motivation to identify suitable alternative that are both economically feasible and environment friendly (Desaeger et al., 2008) which could be an effective method to develop natural suppression of soil borne pathogens (Mazzola, 2004). Introduction of antagonists in the soil microenvironment has resulted in an efficient method for biological control of nematodes (Singh and Mathur, 2010a), wilt and root rot diseases (Pertot et al., 2015).

It is well known that rhizosphere comprises many fungi, bacteria, insects, nematodes and other microbes, it is very important to understand interactions among microorganisms to develop a soil health management strategy instead of focussing on individual disease causing species (Panth et al., 2020). In order to select potentially best antagonistic agents, it is necessary to test them *in vitro* and later *in vivo* and under field conditions. *Trichoderma harzianum* (Accession no. MT734519), *Bacillus subtilis* (Accession no. W008011) and *Pseudomonas fluorescens* (NCIPM/PCPF/01) microorganisms were selected after their noteworthy performance against *M. incognita*, *F. oxysporum* and *R. solani* under *in vitro* studies and confirmation of biochemical characteristics to produce disease supressing chemicals. These bio-agents also showed their compatibility against each other when subjected to bioassay tests (Singh et al., 2020). Evaluation of these bio-agents to supress multi disease causing organisms (*M. incognita*, *F. oxysporum* and *R. solani*) under protected cultivation is lacking in the literature, however, not many concerted investigations have been undertaken to manage concomitant infestation of these soil borne pathogens under protected cultivation system.

Therefore, present investigation aims at highlighting the urgent need for a new concept in agriculture involving drastic reduction in the use of chemical pesticides under protected cultivation system. In view of this, present research was carried out in two consequent seasons to evaluate the biocontrol potential of *T. harzianum*, *B. subtilis* and *P. fluorescens* on development of *M. incognita* and multiplication of *F. oxysporum* and *R. solani* in soil as well in cucumber roots. This focuses on the management of major soil borne pathogens associated with cucumber crop and to develop a consortium of antagonistic microbes against soil borne diseases and complex diseases involving *M. incognita, F. oxysporum* and *R. solani* on cucumber under protected cultivation system.

2. Materials and methods

2.1. Experimental location and layout, good agronomical practices, treatments and observations

Two different *in vivo* trials were conducted under protected cultivation system for the management of various soil borne pests of cucumber i.e. *M. incognita*, *F. oxysporum* and *R. solani* respectively, using *B. subtilis*, *P. fluorescens* and *T. harzianum* separately and in combination. During survey (2016–18) heavy infestation of *M. incognita*, *F. oxysporum* and *R. solani* were recorded from capsicum, cucumber and tomato crops grown under protected structures (poly houses). It is noted that the crops grown under protected structures aged more than two years were greatly affected by concomitant infestation of these soil borne pathogens. To overcome this heavy infestation, two consecutive field trials were conducted during 2018–2020. In this research article the results of *in vivo* studies presented and discussed, for which 30 cm dia. pots were filled with 15 kg of infested soil from infested field (experimental site) located at village-Jainpur, district-Sonipat (Haryana), India at coordinates of 29.0678° N and 77.1277° E. The pots were kept in between main cucumber (Cv. Multistar) crops in randomised design in strips in a manner that they get water and nutrients easily through drip irrigation system. Each pot was transplanted with twenty five day old cucumber (Cv. Multistar) seedling grown separately in pro trays containing sterilized coco peat mixture.

The initial mean population of *M. incognita, F. oxysporum* and *R. solani* was 1.72 J_2/g soil, 1.3×10^3 cfu/g soil and 7.8 propagules/50 cc soil respectively at the time of transplanting of cucumber seedlings in pots during first season trial. The soil of the experimental site was neutral to slightly alkaline, with pH 7.3. Electrical conductivity ranged from 0.28 dS m^{-1}, soil organic carbon was recorded as 0.57% while the bulk density of the soil was 1450 kg m^{-3}. Silt, sand and clay contents were 14.3, 66.4 and 19.3%, respectively. Soil was fertilised with nitrogen (N), phosphorus (P) and potash (K) at 20, 25 and 20 kg 4000 m^2 (0.4 ha) using urea, single super phosphate (SSP) and muriate of potash (MOP), respectively as basal dose. Besides this, 80 kg calcium ammonium nitrate, 125 kg super phosphate, 33 kg muriate of potash and 40 kg of sulphate of potash per ha was also applied at the time of preparing the field before filling of pots with field soil. Other application of fertilizers (N:P:K) i.e. 12:61:0, 0:52:34, 13:0:45 at 2 kg per irrigation at 5 days interval and magnesium sulphate, calcium nitrate and boron at 3 kg/

season was applied through drip irrigation covering 0.4 ha area including experimental pots. The experiments comprises of six treatments: T1- Control (untreated); T2- *T. harzianum* alone (2.0×10^6 cfu/g); T3- *B. subtilis* (1.0×10^8 cfu/g); T4- *P. fluorescens* (1.0×10^8 cfu/g); T5- T2 + T3 + T4 and T6- Fluensulfone (480 EC) (Nematicide) at 7.0 kg/ha (0.7 g/pot) as Control. Fluensulfone is a member of 1, 3-thiazoles, an organochlorine pesticide, a sulfone, an organofluorine pesticide and an olefinic compound has a role as a nematicide and an agrochemical (NCBI, 2020). No chemical fungicide was used in the present study, as the *M. incognita* is the prime cause to damage cucumber plants and provide entry points to fungal pathogens by predisposing the host which are considered as secondary pathogens. The dose of bio-agents was calculated on the area basis of pots (0.94 m^2) at 10 kg and or litre/ha (i.e. 1.10 g or ml/pot as the case may be) were used. Freshly prepared liquid formulation of bio-agents (*T. harzianum*-15 day old culture, *B. subtilis* and *P. fluorescens*-5 day old culture) were used through drip irrigation and/or by drenching at 5 ml/l water. For soil application, well decomposed farm yard manure (FYM) and vermicompost was fortified with first half dose of all bio-agents before amending the pots, and rest half dose of *T. harzianum* was applied manually in three equal doses directly to root system in soil by drenching, whereas *B. subtilis* and *P. fluorescens* was applied through drenching after 10 days of transplanting at 10 days interval. All treatments were repeated in second season trial without disturbing the soil and replicated four times. The experiments were terminated after four months of transplantation and observations were made on plant growth parameters (shoot length, shoot weight and root weight), multiplication of nematode in terms of number of galls and egg masses/root system, eggs/egg mass and soil population of *M. incognita* J_2 and for fungal pathogens, shoot and root disease severity and population of reproductive units in soil were recorded.

For all experiments, at harvest, the severity of Fusarium wilt on cucumber plants was recorded by shoot and root disease of each plant. Whole plants were removed from pots and thoroughly washed under running tap water to remove all attached soil particles. In case of *F. oxysporum*, shoot disease was assessed on a 0 – 5 severity rating scale, where: 0 = plant well developed, no disease symptoms; 1 = plant slightly stunted; 2 = plant stunted and yellowing; 3 = plant severely stunted and/or wilting; 4 = majority of leaves of the plant wilted or dead; 5 = plant dead and root disease was also assessed on a 0–5 disease severity scale, where: 0 = root well developed, no discolouration; 1 = < 25% root discolouration; 2 = 26 to 50% root discolouration; 3 = 51 to 75% root discolouration; 4 = > 75% root discoloration and 5 = all root discoloured (rotted). Plant dead (Fang et al. 2011). In case of *R. solani,* root disease severity was assessed on soil-free plants on scales from 0 to 5, where 0 = no lesions, clean roots; 1 = small lesion on root; 2 = necrosis of up to 30%; 3 = necrosis covering 31–60% of the root; 4 = necrosis covering 61–99% of the root; 5 = completely severed root (Khangura et al., 1999). Besides these multiplication and establishment of tested bio-agents (population estimation in terms of cfu/g soil) and percent egg hatching of *M. incognita* was also recoded after harvest of each experiment. Treatment impact on cucumber plants in terms of pathogenicity etc. was also compared with controls with all tested microorganisms and *M. incognita*.

2.2. Isolation and identification of nematode and fungal pathogens

2.2.1. Isolation and identification of root-knot nematode, M. incognita

Root knot nematode, *M. incognita* initially detected in bell pepper, cucumber and tomato crops under protected environment was identified mainly by the perineal patterns present with the mature females (Hartman and Sasser, 1985). For which, infected roots of tomato and cucumber were washed in running tap water and then mature females were handpicked from galls using forceps. To estimate nematode populations, the soil samples were processed immediately after collection by using Cobb's sieving and gravity method followed by Baerman funnel extraction (Southey, 1986). The number of galls and egg masses per root system of each treatment were counted with the help of a magnifying glass. The number of eggs per egg mass in each treatment was calculated under stereoscopic binocular microscope using a counting dish by taking an average of five egg masses of similar size. For separation of eggs from egg masses 4.0% sodium hypochlorite solution was used. For estimation of *M. incognita* population density in soil, an average of five counts was taken initially and at the end of each trial. The reproductive factor (Rf) was calculated by dividing final population (P_f) with the initial population (P_i) (Rf = Pf/Pi). It is the ratio of final population densities at the end of the crop to the pre-planting population densities and expressed as the ability of the plant to reproduce the nematode.

2.2.2. Isolation and identification of F. oxysporum and R. solani

F. oxysporum and *R. solani*, both fungal pathogens were aseptically isolated from the sick plant roots of bell pepper, cucumber and tomato crops grown under protected cultivation fields where trials were conducted. Infected plant tissues were rinsed twice in water and then sterilised with 5% sodium hypochlorite for 5 min, washed with sterilized distilled water, prior to be cut aseptically into 1.0 cm pieces. Four to five pieces were placed onto each potato dextrose agar (PDA) medium plates. After 7 days of incubation at 25 ± 2 °C, isolates were identified on the basis of their cultural characteristics and the morphology of their vegetative and reproductive structures produced on different culture media according to different keys of identifications given by Nelson et al., 1983 and Agrios, 2004 for *F. oxysporum* and Yamamoto and Uchida, 1982 and Agrios, 2004 for *R. solani*. Observations on microbial growth were taken periodically after 24 h onwards, thus, fungal colonies appeared, were isolated and purified by repeatedly sub-culture techniques and maintained at 4 °C in refrigerator. The reproductive units in terms of colony forming unit per g (cfu/g) was estimated in case of *F. oxysporum* before giving treatment and at the end of each trial, whereas, number of propagules/50 cc soil was estimated in case of *R. solani* before giving treatment and at the end of each trial.

Isolation of fungi from soil was made through serial dilution plating technique and serial dilution were made up to 10^{-7}, for which 0.2 ml suspension of various dilutions were transferred on sterilized Petri plates containing PDA medium and spread it uniformly by using of glass spreader. For extraction of *R. solani* form soil, sieves were used as in routinely used for nematode extractions. For which, debris obtained from 50 cc soil, retained on a 0.425 mm sieve was collected and stored in a glass beaker after rinsed with water, the debris was filtered using filter paper and then equal amount of debris was rinsed in to 2% water agar medium in Petri plates which was replicated three times. All plates were incubated in a BOD incubator at 25 ± 2 °C for 24 h. The emerged colonies in plates were examined and counted under binocular microscope using combination of incident and transmitted light for estimation of colonies.

2.3. Isolation of bio-control agents

2.3.1. Isolation and identification of T. harzianum

Trichoderma species were isolated from soil by serial dilution plating technique using *Trichoderma* selective medium (TSM) as per method described in 2.2.2. The isolates were then purified on TSM plates using repeated sub-culture technique and maintained at 4°C. *T. harzianum* was identified based on mycological keys described by Barnett and Hunter (1972). Initially biocontrol potential of five isolates of *T. harzianum* (1–5) were tested *in vitro* against root-knot nematode, *M. incognita*. On the basis of high antagonistic activity against *M. incognita*, one isolate *T. harzianum* (Accession no. MT734519) was further tested against *F. oxysporum* and *R. solani* (Singh et al., 2020). The spore load of *T. harzianum* was estimated in diluted samples using a haemocytometer and maintained 2.0×10^6 cfu/g throughout the study period.

2.3.2. Isolation and identification of B. subtilis and P. fluorescens

B. subtilis and *P. fluorescens* were also isolated by serial dilution plating technique using Nutrient agar (NA) and/or Pseudomonas Fluroscein Agar media (PFA) as per method described under 2.2.2. The colonies appeared in Petri plates with yellow-green and blue white pigments were detected and marked individually and observed under UV light with the help of an ultra violet (UV) Trans illuminator. Colonies were then picked up carefully with sterilized loop and transferred on to fresh King's B medium. The pure culture was obtained through repeated sub-culture technique and maintained at 4°C in refrigerator and identified on the basis of gram staining reaction (Claus, 1992) and the manual for the identification of Medical Bacteria (Cowan and Steel, 1965). The spore load of both bacterial bio-agents was estimated in diluted samples using haemocytometer and maintained at 1.0×10^8 cfu/ml throughout the study period.

2.3.3. Statistical analysis

The experimental design was randomized block design and pots were kept in strips so that remain within the reach of drip irrigation. Each treatment was replicated four times and repeated twice during 2018–2020. The original data on plant growth parameters and nematode multiplication were square root transformed to normalize the distribution. The data for two consecutive experiments on plant growth parameters and multiplication of *M. incognita, F. oxysporum* and *R. solani* were analysed and subjected to ANOVA separately using SPSS ver.16. A test for homogeneity of variances was conducted for pooling the data, since it showed difference, the data presented separately for both trials. Similarly, average mean with standard error of mean of four replication was calculated. Reproductive factor (R.F) was also calculated by dividing final population (P_f) by initial population (P_i) ($R.F = P_f/P_i$). Duncan's multiple range test (DMRT) was used to determine significant difference ($P < 0.05$) between test antagonists.

3. Results

3.1. Plant growth

Root-knot nematode, *M. incognita*, wilt fungus, *F. oxysporum* and root rot fungus, *R. solani* had suppressive effect on plant growth parameters on cucumber cv. Magicstar (Table 1), all three pathogens under concomitant infestation caused significant ($P > 0.05$) reduction in shoot length, shoot weight and root weight compared to treated plants. All the treated plants showed greater length and weight of shoot and roots of cucumber plants compared to control. In general, application of *B. subtilis, P. fluorescens* and *T. harzianum* individually did not differ significantly ($P < 0.05$) with fluensulfone treated plants, whereas the treatment that received combined application of all three bio-agents showed significantly ($P > 0.05$) greater shoot length and weight and root weight compared to other treatments and control in both season trials (Table-1). Among bio-agents, these treatments can be ranked in descending order as follows: *T. harzianum* + *B. subtilis* + *P. fluorescens* > *P. fluorescens* > *B. subtilis* > *T. harzianum*. On the other hand, the maximum root weight was recorded under control treatment due to presence of big and amalgamated root galls on the root system. Significantly ($P > 0.05$) greater shoot length (up to 42%) and shoot weight (47%) was recorded in the treatment, which was amended with *T. harzianum* + *B. subtilis* + *P. fluorescens* followed by fluensulfone (up to 33% each in shoot length and weight) compared to un-inoculated control at the end of second season's experiments. It is interesting to note that the root weight was recorded insignificant between control and the treatment which received combined application of bio-agents during both trials (Table 1). Data augmented that no negative impact of applied bio-agents was noted on the cucumber plants health.

3.2. Suppression of root-knot nematode, M. incognita

Under untreated control *M. incognita* formed big and amalgamated root galls. All bio-agents and fluensulfone treatment found to supress significantly ($P > 0.05$), the multiplication of *M. incognita* in roots as well in soil compared to control (Table 2). During this study fluensulfone 480 EC was kept as control to compare the performance of bio-agents in reducing the concomitant infestation of soil borne pathogens i.e. *M. incognita, F. oxysporum* and *R. solani*.

Data presented in Table 2 revealed that the suppressive effect of all bio-agents separately and in combination increased gradually with the time or we can say it was higher in the subsequent second season trial.

Table 1
Effect of *Trichoderma harzianum, Bacillus subtilis, Pseudomonas fluorescens* and fluensulfone on plant growth parameters of cucumber cv. Magicstar under concomitant infestation caused by root-knot nematode, *Meloidogyne incognita,* wilt fungus, *Fusarium oxysporum* and root-rot fungus, *Rhizoctonia solani* under protected cultivation.

Treatments	Effect of bio-agents on plant growth parameters of cucumber cv Magicstar					
	First season trial (September to December 2018)			Second season trial (January to April 2019)		
	Shoot length (cm)	Shoot weight (g)	Root weight (g)	Shoot length (cm)	Shoot weight (g)	Root weight (g)
Untreated check (control)	112.5 **[0.0]** (10.65 ± 0.25)^c	90.0 **[0.0]** (9.52 ± 0.36)^c	113.5 **[0.0]** (10.69 ± 0.23)^a	96.5 **[0.0]** ** *(9.86 ± 0.26)^c	84.0 **[0.0]** (9.21 ± 0.30)^d	114.5 **[0.0]** (10.74 ± 0.24)^a
Trichoderma harzianum	131.5 **[+14.4]** (11.50 ± 024)^b	112.0 **[+19.6]** (10.62 ± 0.22)^c	85.5 **[−24.7]** (9.30 ± 0.14)^b	139.5 **[+30.8]** (11.85 ± 0.18)^b	121.8 **[+31.0]** (11.07 ± 0.19)^b	93.5 **[−25.1]** (9.81 ± 0.28)^b
Bacillus subtilis	133.3 **[+15.6]** (11.58 ± 0.17)^b	129.5 **[+30.5]** (11.34 ± 0.82)^b	81.0 **[−28.6]** (9.05 ± 0.23)^b	140.0 **[+31.2]** (11.82 ± 0.15)^b	126.0 **[+33.3]** (11.27 ± 0.05)^b	91.5 **[−25.1]** (9.62 ± 0.05)^b
Pseudomonas fluorescens	135.0 **[+16.7]** (11.66 ± 0.15)^b	114.5 **[+21.4]** (10.73 ± 0.36)^c	81.5 **[−28.2]** (9.08 ± 0.23)^b	141.5 **[+31.8]** (11.94 ± 0.10)^b	124.0 **[+32.3]** (11.17 ± 0.03)^b	90.5 **[−25.1]** (9.23 ± 0.33)^b
T. harzianum + *B. subtilis* + *P. fluorescens*	152.0 **[+26.0]** (12.37 ± 0.11)^a	151.0 **[+40.4]** (12.32 ± 0.30)^a	103.0 **[−9.3]** (10.18 ± 0.31)^a	167.5 **[+42.4]** (12.81 ± 0.09)^a	161.0 **[+47.8]** (12.72 ± 0.18)^a	112.0 **[−2.2]** (10.63 ± 0.14)^a
Fluensulfone	137.5 **[+18.2]** (11.77 ± 0.17)^b	120.0 **[+25.0]** (11.00 ± 0.17)^c	80.0 **[−29.5]** (8.99 ± 0.27)^b	143.0 **[+32.5]** (11.94 ± 0.19)^b	125.0 **[+32.8]** (11.22 ± 0.14)^b	89.5 **[−25.1]** (9.01 ± 0.18)^c
CD at 0.05	0.50	1.33	0.62	0.68	0.70	0.66

Note:
*Figures presented in parentheses [ ] and bold are percent increase (+) or decrease (−) over untreated check.
**Figures presented in parentheses () are square root transformed value ± Standard Error.
Means in each column with different superscript letters differ significantly ($P < 0.05$).

Table 2
Effect of *Trichoderma harzianum, Bacillus subtilis, Pseudomonas fluorescens* and Fluensulfone on root-knot nematode, *Meloidogyne incognita* multiplication infesting cucumber cv. Magicstar under protected cultivation.

Treatment	Effect of bio-agents on root-knot nematode, *Meloidogyne incognita* multiplication							
	First season trial (September to December 2018)				Second season trial (January to April 2019)			
	Number of galls/root system	Number of egg mass/ root system	Number of eggs/egg mass	Number of J_2/ 100 cc soil	Number of galls/root system	Number of egg masses /root system	Number of eggs/egg mass	Number of J_2/ 100 cc soil
Untreated check (control)	60.0 *[0.0] **(7.79 ± 0.34)[a]	60.8 [0.0] (7.85 ± 0.19)[a]	168.3 [0.0] (13.01 ± 0.21)[a]	352.5# {+51.1} [0.0] ǂ{2.1} (18.79 ± 0.30)[a]	96.0 [0.0] (9.86 ± 0.39)[a]	98.3 [0.0] (9.89 ± 0.14)[a]	172.0 [0.0] (13.14 ± 0.28)[a]	594.0 {+71.1} [0.00] {3.5} (24.39 ± 0.30)[a]
Trichoderma harzianum	34.5 [−42.5] (5.95 ± 0.21)[bc]	35.5 [−41.6] (6.03 ± 0.21)[b]	81.5 [−51.6] (9.16 ± 0.46)[c]	90.0 {−47.77} [−74.5] {0.5} (9.54 ± 0.11)[c]	38.0 [−42.4] (6.23 ± 0.26)[b]	43.0 [−37.0] (6.62 ± 0.24)[b]	63.6 [−63.0] (8.22 ± 0.46)[d]	69.8 {102.6} [−88.3] {0.6} (8.39 ± 0.71)[b]
Bacillus subtilis	38.0 [−36.7] (6.23 ± 0.24)[b]	33.8 [−44.4] (5.89 ± 0.14)[b]	122.8 [−27.0] (11.12 ± 0.24)[b]	96.00{−44.3} [−72.8] {0.6} (9.85 ± 0.14)[c]	36.3 [−45.1] (6.09 ± 0.20)[b]	40.3 [−41.0] (6.41 ± 0.21)[b]	127.0 [−26.2] (11.34 ± 0.28)[b]	68.3 {−60.0} [−88.5] {0.4} (8.32 ± 0.17)[b]
Pseudomonas fluorescens	37.6 [−37.1] (6.19 ± 0.38)[b]	35.3 [−41.9] (6.02 ± 0.15)[b]	130.5 [−22.4] (11.46 ± 0.16)[b]	111.5 {−32.9} [−68.37] {0.7} (10.60 ± 0.22)[b]	35.0 [−46.9] (5.94 ± 0.51)[b]	43.3 [−36.6] (6.64 ± 0.19)[b]	123.0 [−28.5] (11.11 ± 0.42)[b]	83.5 {−51.5} [−85.9] {0.5} (9.19 ± 0.17)[b]
T. harzianum + *B. subtilis* + *P. fluorescens*	25.8 [−57.1] (5.15 ± 0.27)[c]	15.8 [−74.1] (4.06 ± 0.29)[c]	70.0 [−58.4] (8.40 ± 0.43)[c]	58.8 {−65.9} [−83.3] {0.3} (7.72 ± 0.27)[d]	18.0 [−72.7] (4.31 ± 0.36)[c]	12.5 [−81.7] (3.65 ± 0.24)[c]	53.3 [−69.0] (7.34 ± 0.38)[d]	24.5 {−85.8} [−95.9] {0.1} (5.03 ± 0.28)[c]
Fluensulfone	3.8 [−93.6] (2.07 ± 0.40)[d]	1.5 [−97.5] (1.54 ± 0.25)[d]	163.5 [−2.8] (12.82 ± 0.61)[a]	26.0 {−84.9} [−92.6] {0.2} (5.17 ± 0.29)[e]	7.5 [−88.6] (2.91 ± 0.11)[b]	4.0 [−94.1] (2.21 ± 0.21)[d]	167.0 [−2.9] (12.95 ± 0.37)[a]	20.0 {88.4} [−96.6] {0.1} (4.54 ± 0.36)[c]
CD at 0.05	0.76	0.66	0.96	0.68	1.07	1.06	0.98	0.82

Note: Initial population of *Meloidogyne incognita* at the time of transplanting for first season crop – 172.3 J_2/100 cc soil; *Figures presented in parentheses [ ] and bold are percent increase (+) or decrease (−) over untreated check; **Figures presented in parentheses () are square root transformed value ± Standard Error;); #Figures presented in parentheses { } are percent increase (+) or decrease (−) over initial population (172.3 J_2/100 cc soil); ǂ Figures presented in parentheses { } and bold are reproductive factor (R.F. = P_f/P_i), where R. F.-Reproductive factor, P_f -Final population and P_i - initial population; Means in each column with different superscript letters differ significantly ($P > 0.05$).

Maximum suppression of number of galls/root system, number of egg masses/root system, number of eggs/egg mass and soil population/100 cc soil was recorded with Fluensulfone followed by combined application *T. harzianum* + *B. subtilis* + *P. fluorescens* treatment under both season's trials. During first season, fluensulfone caused significantly ($P > 0.05$) higher reduction in number of root-knot galls, number of egg masses per root system and number of J_2/100 cc soil up to 93, 97 and 92% in comparison to combined application of *T. harzianum* + *B. subtilis* + *P. fluorescens* by up to 57, 74 and 83% respectively compared to control. But at the end of second season trail, it is clear from Table 2 that the suppression of *M. incognita* J_2 soil population under combined treatment (95.9%) and fluensulfone (96.6%) treated soil was insignificant at $P < 0.05$. It is also notable that the number of egg masses/root system formed under fluensulfone treated soil were very few but the number of eggs/egg mass were not affected and did not differ statistically at $P > 0.05$ compared to control. The bio-agents treated roots caused significant reduction in number of eggs/egg mass compared to control and fluensulfone treatment. Reproductive factor for *M. incognita* was also calculated, which was 2.1 at the end of first season trial and subsequently reached up to 3.5 at the end of second season trial under control treatment. Under treated soil it was in the range of 0.2–0.7 with lowest in fluensulfone treatment (0.2) followed by combined application of bio-agents treatment (0.3) which was significant during first season trial but insignificant at the end of second season trial and reduced up to 0.1 in both fluensulfone and combined bio-agents treatments.

3.3. Suppression of shoot and root diseases caused by F. oxysporum and R. solani

Data presented in Table 3 showed that application of bio-agents and fluensulfone caused significant ($P > 0.05$) reduction in shoot and root disease severity caused by both *F. oxysporum* and *R. solani* compared to control. In soil without any treatment (control), plants showed the most severe disease with the highest shoot and root disease rating 3.31 and 3.06 respectively in case of *F. oxysporum* whereas it was recoded 3.19 in case of *R. solani* during first season crop, which was subsequently higher (4.13, 4.33 and 3.63 respectively) with next season crop. In general, the effect of individual bio-agents, *T. harzianum* or *B. subtilis* or *P. fluorescens* and fluensulfone was insignificant in reducing the shoot and root disease caused by *F. oxysporum* and *R. solani*. The combined application of tested bio-agents (*T. harzianum* + *B. subtilis* + *P. fluorescens*) treatment caused significantly ($P > 0.05$) greatest reduction in shoot disease, root disease caused by *F. oxysporum* and root-rot disease caused by *R. solani* up to 84, 63 and 68% respectively at the end of first season trial and this reduction was recorded greatest (98, 100 and 93% respectively) in subsequent second season trial compared to control. It is also notable that suppression of *M. incognita* infestation by any means, also reduced shoot and root disease severity caused by *F. oxysporum* and *R. solani* (Table 2 and 3) as in case of fluensulfone treated plants where shoot and root disease severity caused by *F. oxysporum* and root disease severity caused by *R. solani* was reduced up to 68, 81 and 65% respectively at the end of second season compared to control. No root symptoms of root disease caused by *F. oxysporum* and *R. solani* was recorded with treatment that received *T. harzianum* + *B. subtilis* + *P. fluorescens* together.

3.4. Suppression of F. oxysporum and R. solani in soil

The initial population of reproductive units of both fungi was recorded insignificant among all treatments. An incessant proliferation in reproductive units of both fungi in soil was recorded under control

Table 3
Effect of *Trichoderma harzianum, Bacillus subtilis, Pseudomonas fluorescens* and fluensulfone on disease severity of Fusarium wilt and root rot disease caused by *Fusarium oxysporum* and *Rhizoctonia solani* respectively on cucumber cv. Magicstar.

Treatment	Disease severity of Fusarium wilt caused by *F. oxysporum*				Disease severity of root rot caused by *R. solani*	
	Shoot disease severity		Root disease severity		Root disease severity	
	First season trial	Second season trial	First season trial	Second season trial	First season trial	Second season trial
Untreated check (control)	3.31* **[0.00]** # (2.08 ± 0.52)**[a]	4.13 **[0.00]** (2.26 ± 0.04)[a]	3.06# (61.25)‡ {51.80 ± 5.79}![a] **[0.00]**	4.33 (82.50) {65.53 ± 2.49}[a] **[0.00]**	3.19 (63.75) {53.20 ± 4.29}[a] **[0.00]**	3.63 (72.50) {58.10 ± 4.60}[a] **[0.00]**
Trichoderma harzianum	1.63 **[−50.94]** (1.62 ± 0.50)[b]	1.25 **[−69.70]** (1.49 ± 0.09)[bc]	1.63 (32.50) {34.66 ± 1.99}[b] **[−46.94]**	0.69 (13.75) {18.74 ± 6.70}[b] **[−83.33]**	1.25 (25.00) {29.17 ± 5.06}[b] **[−60.78]**	0.75 (15.00) {21.97 ± 3.87}[b] **[−79.31]**
Bacillus subtilis	1.38 **[−58.49]** (1.54 ± 0.05)[b]	0.63 **[−84.85]** (1.27 ± 0.06)[c]	1.25 (25.00) {28.83 ± 5.64}[b] **[−59.18]**	0.56 (11.25) {18.56 ± 4.02}[b] **[−86.36]**	1.75 (35.00) {35.99 ± 3.96}[b] **[−45.10]**	1.25 (25.00) {29.47 ± 3.90}[b] **[−65.52]**
Pseudomonas fluorescens	1.56 **[−52.83]** (1.60 ± 0.04)[b]	0.94 **[−77.27]** (1.39 ± 0.08)[bc]	1.88 (37.50) {37.70 ± 1.92}[b] **[−38.78]**	0.62 (12.50) {20.17 ± 2.93}[b] **[−84.85]**	1.63 (32.50) {33.88 ± 6.09}[b] **[−49.02]**	1.21 (24.12) {27.22 ± 7.45}[b] **[−66.72]**
T. harzianum + *B. subtilis* + *P. fluorescens*	0.50 **[−84.91]** (1.22 ± 0.04)[c]	0.06 **[−98.48]** (1.03 ± 0.03)[d]	1.12 (22.50) {28.13 ± 2.24}[b] **[−63.27]**	0.00 (0.00) {0.00 ± 0.00}[c] **[100.00]**	1.00 (20.00) {26.10 ± 3.36}[b] **[−68.63]**	0.25 (5.00) {11.07 ± 3.91}[c] **[−93.10]**
Fluensulfone	1.56 **[−52.83]** (1.59 ± 0.06)[b]	1.31 **[−68.18]** (1.51 ± 0.11)[b]	1.13 (22.50) {28.13 ± 2.24}[b] **[−63.27]**	0.75 (15.00) {21.83 ± 4.29}[b] **[−81.82]**	1.44 (28.75) {32.02 ± 4.01}[b] **[−54.90]**	1.25 (25.00) {29.47 ± 3.90}[b] **[−65.52]**
CD at 0.05	0.15	0.23	10.68	9.82	14.76	13.99

Note: *0–5 rating scale where 0 = plant well developed, no disease symptom and 5 = dead plant. Different letters in the same column indicate significant differences according to Duncan' test (P > 0.05).
**Figures presented in parentheses () are square root transformed value.
#0–5 rating scale where 0 = plant well developed, no disease symptom and 5 = dead plant.
‡Figures presented in parentheses () are original percent value.
! Figures presented in parentheses { } are angular transformed value.
#Figures presented in parentheses [ ] and bold are percent decrease (-) over control.
Means in each column with different superscript letters differ significantly (P > 0.05).

treatment, which was 42 and 58% greater in *F. oxysporum* and *R. solani* respectively at the end of second season crop than that of their initial population. Significantly lower population of reproductive units of both fungi, *F. oxysporum* and *R. solani* was recorded in all treated plants compared to control except fluensulfone treatment which did not differ significantly (P < 0.05) compared to control. Fluensulfone reduced reproductive units of *F. oxysporum* and *R. solani* only up to 11 and 15% respectively in the first season crop and 12 and 23% in subsequent second trial and recorded less effective in reducing the soil population of *F. oxysporum* and *R. solani* compare to tested bio-agents (Table 4).

T. harzianum alone caused greatest reduction i.e. 84 and 76% in the population of *F. oxysporum* and *R. solani* respectively which was insignificant with combined application of bio-agents (*T. harzianum* + *B. subtilis* + *P. fluorescens*) treatment which dropped the population of both fungi up to 79 and 76% respectively (Table 4) at the end of first trial. Subsequently, at the end of second season trial, this suppression in reproductive units of *F. oxysporum* in soil reached up to 100% with treatments having *T. harzianum* either alone or in combination with tested bacterial isolates. It is notable in the present investigation that suppression (up to 100%) of *R. solani* reproductive units in soil was recorded with all bio-agents treated plants at the end of second season trial except *B. subtilis* (98%) where it was recorded in traces (Table 4).

Table 4
Effect of bio-agents, *Trichoderma harzianum, Bacillus subtilis* and *Pseudomonas fluorescens* on multiplication of *Fusarium oxysporum* and *Rhizoctonia solani* in the soil at different time intervals.

Treatment	Number of colony forming units (cfu) of *Fusarium oxysporum* per gram soil (x 10^3)			Number of colony/propagules per 50 cc soil of *Rhizoctonia solani* (propagules/50 cc soil)		
	Initial population	At the end of first season trial	At the end of second season trial	Initial population	At the end of first season trial	At the end of second season trial
Untreated check (control)	1.23 (1.49 ± 0.05)*	1.73 **[0.00]** # (1.65 ± 0.07)[a]	2.13 **[0.00]** (1.76 ± 0.09)[a]	7.00 (2.81 ± 0.16)	13.00 **[0.00]** (3.71 ± 0.27)[a]	17.00 **[0.00]** (4.22 ± 0.23)[a]
Trichoderma harzianum	1.25 (1.50 ± 0.06)	0.28 **[−84.06]** (1.12 ± 0.08)[d]	0.00 **[100.00]** (1.00 ± 0.00)[c]	6.00 (2.60 ± 0.28)	3.00 **[−76.92]** (1.98 ± 0.17)[b]	0.00 **[100.00]** (1.00 ± 0.00)[c]
Bacillus subtilis	1.23 (1.49 ± 0.04)	0.85 **[−50.72]** (1.36 ± 0.07)[b]	0.30 **[−85.88]** (1.14 ± 0.06)[c]	10.00 (3.30 ± 0.18)	6.00 **[−53.85]** (2.61 ± 0.25)[b]	0.33 **[−98.09]** (1.15 ± 0.05)[c]
Pseudomonas fluorescens	1.45 (1.56 ± 0.09)	0.75 **[−56.52]** (1.32 ± 0.04)[bc]	0.18 **[−91.76]** (1.08 ± 0.03)[c]	8.00 (2.99 ± 0.15)	5.00 **[−61.54]** (2.43 ± 0.19)[b]	0.00 **[100.00]** (1.00 ± 0.00)[c]
T. harzianum + *B. subtilis* + *P. fluorescens*	1.30 (1.51 ± 0.09)	0.35 **[−79.71]** (1.16 ± 0.05)[cd]	0.00 **[100.00]** (1.00 ± 0.0)[c]	7.00 (2.80 ± 0.23)	3.00 **[−76.92]** (1.92 ± 0.32)[b]	0.00 **[100.00]** (1.00 ± 0.00)[c]
Fluensulfone	1.25 (1.49 ± 0.07)	1.53 **[−11.59]** (1.59 ± 0.05)[a]	1.85 [−12.94] (1.69 ± 0.05)[b]	9.00 (3.10 ± 0.37)	11.00 [−15.38] (3.45 ± 0.19)[a]	13.00 **[−23.53]** (3.71 ± 0.25)[b]
CD at 0.05	NS	0.19	0.15	NS	0.65	0.40

Note: Means in each column with different superscript letters differ significantly (P > 0.05).
*Figures presented in parentheses () are square root transformed value.
#Figures presented in parentheses [ ] and bold are percent decrease (−) over control.

3.5. Multiplication of bio-agents, T. harzianum, B. subtilis and P. fluorescens and their effect on egg hatching of M.incognita

The results presented in Table 5 showed successful establishment of the bio-agents as data depicts a substantial proliferation in colony counts which was lower in first season trial but greater at the end of second season crop over initial inoculated population. An increase in cfu/g was recorded up to 97% (range 72–97%) during first season crop, which reached up to 99% at the end of second season crop trial. The proliferation of bio-agents was greatest in combined bio-agents application compared to individual application of either of the tested bio-agents. At the termination of each trial, egg hatching test was also performed with eggs collected from each treatment separately and results presented in Table 5. Eggs, thus obtained from treated plant roots showed significant ($P > 0.05$) inhibition in hatching behaviour compared to control except the eggs those obtained from fluensulfone treated plant roots under both season's trials which were recorded insignificant. It was recorded insignificant ($P < 0.05$) among the treatments that received individual bio-agents alone, however, combined application of bio-agents showed greatest egg inhibition under both season trials. In general, among bio-agents, these treatments can be ranked in descending order based on the results obtained from first season's trial as follows: *B. subtilis* + *P. fluorescens* + *T. harzianum* > *P. fluorescens* > *T. harzianum* > *B. subtilis* whereas, finally at the end of second season trial, *P. fluorescens* replaced with *T. harzianum* followed by *B. subtilis* and *P. fluorescens*. *P. fluorescens* which was second in the ranking at the end of first trial, add nothing and exhibit similar number (57%) of egg inhibition during second season trial. Finally, in the treatment that received all bio-agents together, egg inhibition was greatest (80%), however, insignificant with *T. harzianum* (75%) and *B. subtilis* (71%). Eggs collected from fluensulfone treated plant roots hatched up to 87% and insignificant with control in both seasons trial (Table 5).

4. Discussion

M. incognita, *F. oxysporum* and *R. solani* are associated with cucumber crop causing disease in the root system. These soil borne pathogens share the same habitat in the rhizosphere, disrupt the vascular system of the host plant and interfere with physiological processes involved in water and nutrient uptake (Ayala-Donas et al, 2020). To suppress these pathogens, innovative solutions involving bio-agents are in high demand. In most of the cases, bio-agents provide environmental friendly management than traditional chemical pesticides. Fungal and bacterial bio-agents could represent non-chemical practices to control pathogens, as they are closely associated with the plant system. Here we studied that naturally occurring local strains of *T. harzianum, B. subtilis* and *P. fluorescens* isolated from rhizosphere can reduce concomitant infestation of *M. incognita*, *F. oxysporum* and *R. solani* pathogens on cucumber under protected cultivation system. Disease suppressive effects of tested bio-agents improved the cucumber plant health. This indicates that the enhancement in plant biomass was partly due to decline in *M. incognita*, *F. oxysporum* and *R. solani* infestation and partly to improved soil fertility by addition of bio-agents fortified FYM and vermicompost with good agronomical practices. In the past, several researchers proposed that a possible means of increasing consistency and efficacy of bio-agents agents was to apply combinations of these bio-agents, who achieved successful management of either of *M. incognita*, *F. oxysporum* or *R. solani* individually or disease complexes involving these pathogens on various vegetable crops under *in vivo* and/or field conditions (Dubey et al., 2007; Singh and Singh, 2012; Singh, 2013, 2019; Singh et al, 2013; Abo-Elyousr et al., 2014), that is why the combined application of antagonistic fungus, *T. harzianum* and bacterial antagonists (*B subtilis* and *P. fluorescens*) was demonstrated to have greater potential for increased activity and many more effectively colonize the rhizosphere of cucumber plants under protected cultivation system. The finding of the present investigation showed that root weight under combined bio-agents treatment did not differ statistically even without root galling as in case of control where root weight was maximum due to big and amalgamated root galls. The root weight increased in the treatment that received all tested bio-agents in combination, partly due to high rate of suppression of pathogens and diseases caused by them and partly due to bio-agents positive contribution to increase in plant biomass in terms of root weight shoot weight.

In most of the work published by various researchers, initial testing of biological control agents or their combinations were carried out under lab control conditions on artificial medium and it is always not true that

Table 5
Multiplication of bio-agents, *Trichoderma harzianum, Bacillus subtilis* and *Pseudomonas fluorescens* in soil at different time intervals and percent egg hatching of *Meloidogyne incognita* at the end of both trials.

Treatment		Population of Bio-agents (cfu/g soil) at different time intervals (x 10^3)				Percent egg hatching of *M. incognita* at the end of season (s)	
		Initial		At the end of first season trial	At the end of second season trial	At the end of First season trial	At the end of Second season trial
		Before inoculation	After inoculation				
Untreated check (control)		0.00	0.00	0.00 **{0.00}**	0.00 **{0.00}**	88.00 **[0.00]** * (70.68 ± 3.90) ǂ[a]	90.00 **[−0.00]** (72.74 ± 4.02)[a]
Trichoderma harzianum		0.00	0.06	1.03 **{+94.19}**	2.47 **{+97.57}**	38.00 **[−56.82]** (37.93 ± 2.85)[bc]	22.00 **[−75.11]** (27.67 ± 2.97)[bc]
Bacillus subtilis		0.00	0.30	1.27 **{+76.38}**	2.83 **{+89.39}**	40.00 **[−54.55]** (39.15 ± 2.28)[b]	26.00 **[−71.11]** (30.19 ± 3.88)[bc]
Pseudomonas fluorescens		0.00	0.30	1.07 **{+72.03}**	3.60 **{+91.67}**	37.00 **[−57.95]** (37.36 ± 2.64)[bc]	38.00 **[−57.78]** (37.95 ± 2.61)[b]
T. harzianum + *B. subtilis* + *P. fluorescens*	*T. harzianum*	0.00	0.06	1.57 **{+96.17}**	3.00 **{+98.00}**	24.00 **[−72.73]** (29.20 ± 1.99)[c]	19.00 **[−80.00]** (25.35 ± 3.38)[c]
	B. subtilis	0.00	0.03	1.00 **{+97.01}**	3.10 **{+99.03}**		
	P. fluorescens	0.00	0.03	1.20 **{+97.50}**	2.90 **{+98.97}**		
Fluensulfone		0.00	0.00	0.00 **{0.00}**	0.00 **{0.00}**	87.00 **[−1.14]** (69.70 ± 3.77)[a]	84.00 **[−6.67]** (67.06 ± 3.75)[a]
CD at 0.05		–	–	–	–	9.22	11.11

Note: Means in each column with different superscript letters differ significantly ($P > 0.05$).
*Figures presented in parentheses [ ] and bold are percent decrease over control.
ǂ Figures presented in parentheses () are angular transformed value.
#Figures presented in parentheses { } and bold are percent increase (+) over initial population (after inoculation).

bio-agents showing the greatest inhibition or biocontrol potential under *in vitro* are best biocontrol agents (Weller, 1988), hence they must performed with conditions close to natural infestation in which the part or entire host are used (Garcia et al., 2020). The success of antagonistic fungi and/or bacteria is mainly depend upon their establishment in the rhizosphere and ability to colonise the target pests (Singh and Mathur, 2010b) for which they require energy to grow and colonise in the soil prior to parasitization and/or predation (Mankau, 1981, Singh and Mathur, 2010b). In this work, we found that *T. harzianum, B. subtilis* and *P. fluorescens* multiplied well in soil, which was re-isolated and estimated 99% more (cfu/g) population of bio-agents at the end of second season trial compared to initial inoculum at the time of transplanting of first year trial. As present investigation recommends the fortification of FYM and vermicompost with *B. subtilis*, *P. fluorescens* and *T. harzianum* prior to application in the soil, that is why the organic matter present in FYM and vermicompost not only helps to provide energy to bio-agents for survival and proliferation, but also provide additional nutrients to the plants, resulted in good plant health throughout the cropping seasons during present investigation. This is also supported by the findings, during this work, in which application of bio-agents not only cause inhibition of *M. incognita* egg hatching, but also suppress reproductive units of *F. oxysporum* and *R. solani* in soil and reduce the shoot and root disease severity caused by them. Secondly, foliar spray of bio-agents through drenching may keep away important pests and pathogens from cucumber plants. It is also documented that the formation of biofilms by plant growth promoting bacteria (PGPR) is associated with root colonization (Garcia et al., 2020) in which they communicate to each other to perform in a coordinated way and has been related to the induction of resistance (Choudhary and Johri, 2009). In general, it is also observed that attack of cutting and sucking pests was in traces on plant that received bio-agents through drenching compared to control(s), besides that the trials were conducted in between the main cucumber crop, however, this was not the part of present investigation.

Several other workers demonstrate earlier that how mycelium of *Trichoderma* can behave like a matrix used by *Bacillus* and *Pseudomonas* to adhere and form biofilms on the surface of pathogenic fungi and can contribute to the migration of bacteria as helpers for their movement (Garcia et al., 2020; Triveni et al., 2012; Warmink et al., 2011). These microorganisms could have a synergistic action (Triveni et al., 2012) and participate in biodegradation, plant growth promotion, mineralization etc. in the rhizosphere, and significantly contribute to increase plant health and vigour (Kostov et al., 2009). As evident from the data, fluensulfone did not affect multiplication of *F. oxysporum and R. solani* in soil but found to reduce shoot and root disease severity in the plant system. It suggests that fluensulfone checked the *M. incognita* which is primary pathogen and already been proved that it provides entry to secondary pathogens (*F. oxysporum* and *R. solani* in the present case) by predisposing the host roots for entry of fungal pathogens (Khan and Sharma, 2020; Onkendi et al., 2014: Back et al., 2002 Singh and Goswami, 2001). The lower disease severity in the plant system could be due to availability of lesser entry points at initial stage of crop growth in fluensulfone treated plants.

In this study we observed direct correlation between pathogens inhibition in soil and plant system and proliferation of tested bio-agents under greenhouse assay. The reduction in disease in the plant system was associated with reduction in *M. incognita*, *F. oxysporum* and *R. solani* meaning that *T. harzianum*, *B. subtilis* and *P. fluorescens* treatment has a direct effect on pathogen activity. These characteristics make them ideal candidate to be employed as a consortium in an agroecosystem to control multi-diseases caused by *M. incognita*, *F. oxysporum* and *R. solani* under protected cultivation system.

5. Conclusion

The present investigation was carried out to develop a consortium of potential bio-agents of different origin (*T. harzianum, B. subtilis* and *P. fluorescens*) which may be very useful in the disease management caused by *M. incognita*, *F. oxysporum* and *R. solani* under protected cultivation system. Hence, on the basis of these studies, we concluded that tested bio-agent's consortia have great promise, as an effective component in management of soil borne diseases of cucumber. Thus, these soil borne pathogens are considered a major limitations to greenhouse cucumber production and require more research efforts for durable and sustainable management technologies. Further, research is needed to establish practical methods for growers to apply *T. harzianum, B. subtilis* and *P. fluorescens* in the field. However, mode of action behind bio-agents with good potential to manage these diseases is still the subject of research to untie the facts of disease control. Acceptable investigation in this area to avoid chemical pesticides could be a major development in the improvement of health of various economically important crops grown under protected structures and also a step further to protect environment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Authors are thankful to Director, NCIPM, New Delhi for providing necessary help and facilities during course of study and preparation of manuscript.

References

Abo-Elyousr, K.A.M., Sobhy, A.H., Abdel-Rahim, I.R., 2014. Isolation of *Trichoderma* and evaluation of their antagonistic potential against *Alternaria porri*. J. Phytopathol. 162, 567–574.

Agrios, G.N., 2004. Plant pathology, 4th edn. Elseiver, Amsterdam, p. 236.

Ashraf, S., Zuhaib, M., 2013. Fungal biodiversity: A potential tool in plant disease management. In: Management of microbial resources in the environment. Springer, Dordrecht, pp. 69–90. https://doi.org/10.1007/978-94-007-5931-2.

Ayala-Donas, A., Cara-Garcia, M., Talavera-Rubia, M., Verdejo-Lucas, S., 2020. Management of soil borne fungi and root-knot nematodes in cucurbits through breeding for resistance and grafting. Agronomy 10, 1641; doi:10.3390/agronomy10111641. www.mdpi.com/journal/agronomy.

Back, M.A., Haydock, P.P.J., Jenkinon, P., 2002. Disease complexes involving plant pararsitic nematodes and soil borne pathogens. Pl. Pathol. 51, 683–697.

Baker, K.F., 1970. Types of *Rhizoctonia* diseases and their occurrence. In: Parmeter, J.R. (Ed.), *Rhizoctonia solari*: Biology and Pathology. University of California Press, Berkeley. CA, USA, pp. 125–148.

Barnett, H.L., Hunter, B., 1972. Illustrated genera of imperfect fungi. Burgess publishing company, Minnesota.

Bell, C.H., 1996. Alternative physical methods and emission reduction. In The Methyl Bromide Issue; Bell, C.H., Price, N., Chakrabarti, B. (Eds.), John Wiley and Sons: West Sussex, UK, 1996; pp. 323–329.

Bharat, N.K., Sharma, J., 2014. Occurrence of *Fusarium* wilt of tomato under protected cultivation in Himachal Pradesh. India. Int. J. Bio. Stress Manag. 5, 285–287.

Burnett, M., Welford, R., 2007. Case study: coca-cola and water in India: episode 2. Corporate Soc. Responsib. Env. Mgmt. 14, 298–304. https://doi.org/10.1002/csr.157.

Choudhary, D.K., Johri, B.N., 2009. Interactions of *Bacillus* spp. and plants - With special reference to induced systemic resistance (ISR). Microbiol. Res. 164, 493–513. https://doi.org/10.1016/j.micres.2008.08.007.

Claus, D., 1992. A standardized Gram staining procedure. World J. Micribiol. Biotechnol. 8, 451–452.

Cowan, S.T., Steel, J.K., 1965. Manual for the identification of medical bacteria. Cambridge University Press, London.

Desaeger, J.A., Seebold, K.W., Csinos, A.S., 2008. Effect of application timing and method on efficacy and phytotoxicity of 1, 3-D, chloropicrin and metam-sodium combinations in squash plasticulture. Pest Manag. Sci. 64, 230–238. https://doi.org/10.1002/ps.1503. PMID:18181144.

Dubey, S.C., Suresh, M., Singh, B., 2007. Evaluation of *Trichoderma* species against *Fusarium oxysporum* f. sp. *ciceris* for integrated management of chickpea wilt. J. Bio. Cont. 40, 118–127.

Egel, D.S., Martyn, R.D., 2007. *Fusarium* wilt of watermelon and other cucurbits. The Plant Health Instructor. https://doi.org/10.1094/PHI-I-2007-0122-01.

Fang, X.L., Phillips, D., Li, H., Sivasithamparam, K., Barbetti, M.J., 2011. Comparisons of virulence of pathogens associated with crown and root diseases of strawberry in Western Australia with special reference to the effect of temperature. Sci. Horti. 131, 39–48.

Garcia, L.F.I., Almario, A.G., Cotes, A.M., Velandia, C.A.M., 2020. *Trichoderma virens* GI006 and *Bacillus velezensis* Bs006: a compatible interaction controlling *Fusarium* wilt of cape gooseberry. Sci. Rep. 10, 6857. https://doi.org/10.1038/s41598-020-63689-y.

Gerik, J.S., Hanson, B.D., 2011. Drip application of methyl bromide alternative chemicals for control of soilborne pathogens and weeds. Pest Manag. Sci. 67, 1129–1133.

Giannakou, I.O., Sidiropoulus, A., Athanasiadou, D.P., 2002. Chemical alternatives to methyl bromide for control of root-knot nematodes in greenhouses. Pest Manag. Sci. 58, 290–296.

Gunnell, D., Eddleston, M., Phillips, M.R., Konradsen, F., 2007. The global distribution of fatal pesticide self-poisoning: systematic review. BMC Public Health 7, 357. https://doi.org/10.1186/1471-2458-7-357.

Hartman, K. M., Sasser, J. N., 1985. Identification of Meloidogyne spp. On the basis of differential host test and perineal pattern morphology. In An advance treatise on Meloidogyne Vol. II: Methodology. Eds. Barker, K. R., Karter, C. C. and Sasser, J. N. North Carolina State University Graphics, US. Pp. 223.

Hu, J., Lin, X., Wang, J., Shen, W., Wu, S., Peng, S., Mao, T., 2010. Arbuscular mycorrhizal fungal inoculation enhances suppression of cucumber *Fusarium* wilt in greenhouse soils. Pedosphere 20, 586–593.

Jain, R.K., Mathur, K.N., Singh, R.V., 2007. Estimation of losses due to plant parasitic nematodes on different crops in India. Ind. J. Nematol. 37, 219–221.

Keinath, A.P., Batson, W.E., 2000. Evaluation of biological and chemical seed treatments to improve stand of snap bean across the southern U.S. Crop Prot. 19, 501–509.

Khan, M.R., Sharma, R.K., 2020. Fusarium-nematode wilt disease complexes, etiology and mechanism of development. Ind. Phytopathol. 73, 1–14. https://doi.org/10.1007/s42360-020-00240-z.

Khangura, R.K., Barbetti, M.J., Sweetingham, M.W., 1999. Characterization and pathogenicity of *Rhizoctonia* species on canola. Plant Dis. 83, 714–721.

Kostov, O., Vasileva, V., Kaloianova, N., 2009. Industrial, ecological and agricultural importance of biocontrol agents based on *Trichoderma* species. Ecol. Fut. 8, 28–37.

Krishnaveni, M., Subramanian, S., 2002. Assessment of yield loss in cucumber (*Cucumis sativus* L.) due to *Meloidogyne incognita*. Proc. of National Symposium on Biodiversity and Management of Nematodes in Cropping Systems for Sustainable Agriculture. Jaipur, India 2002, 88–89.

Lamichhane, J.R., Durr, C., Schwanck, A.A., Robin, M., Sarthou, J., Cellier, V., Messean, A., Aubertot, J., 2017. Integrated management of damping off disease. A review. Agron. Sustain. Dev. 37 (10) https://doi.org/10.1007/s13593-017-0417-y.

Mallaiah, B., Muthamilan, M., Prabhu, S., Ananthan, R., 2014. Studies on interaction of nematode, *Pratylenchus delattrei* and fungal pathogen, *Fusarium incarnatum* associated with crossandra wilt in Tamil Nadu. India. Curr. Biotica 8, 157–164.

Mankau, R., 1981. Microbial control of nematodes. In Plant-Parasitic Nematodes eds. Zuckerman, B.M., Rohde, R.A., New York: Academic 3, pp. 475-94.

Mao, L., Jiang, H., Zhang, L., Zhan, Y., Sia, M.U., Yu, H., Cao, A., 2017. Replacing methyl bromide with a combination of 1,3-dichloropropene and metam sodium for cucumber production in China. PLoS ONE 12 (11), e0188137. https://doi.org/10.1371/journal.pone.0188137.

Mazzola, M., 2004. Assessment and management of soil microbial community structure for disease suppression. Ann. Rev. Phytopathol. 42, 35–59.

Meena, S.K., Ramyabharathi, S.A., Raguchander, T., Jonathan, E.I., 2015. *Meloidogyne incognita* and *Fusarium oxysporum* interaction in Gerbera. Afr. J. Microbiol. Res. 9, 1281–1285.

Nagesh, M., Reddy, P.P., 2005. Management of carnation and gerbera to control the root-knot nematode *Meloidogyne incognita* in commercial polyhouse. Nematol. Medit. 33, 157–162.

National Center for Biotechnology Information, 2020. PubChem Compound Summary for CID 11534927, Fluensulfone. Retrieved December 8, 2020 from. https://pubchem.ncbi.nlm.nih.gov/compound/Fluensulfone.

Nelson, P.E., Toussoun, T.A., Marasas, W.F.O., 1983. *Fusarium* species: An Illustrated manual for identification. University Park, Pennsylvania State University Press, p. 193.

Nimbrayan, P.K., Chauhan, R.S., Mehta, V., Bhatia, J.K., 2018. A review on economic aspect of protected cultivation in India. In: Research trends in Horticulture Science. AkiNik Publishers, Delhi, India, pp. 43–59.

Onkendi, E.M., Kariuki, G.M., Marais, M., Moleleki, L.N., 2014. The threat of root-knot nematode (*Meloidogyne* spp.) in Africa: a review. Pl. Pathol. 63, 727–737.

Pandey, R., Kalra, A., Gupta, M. L., Sharma, P., 2003. Phytonematodes: major pest of MAPs. In Proceedings of first National Interactive Meet on Medicinal and Aromatic Plants (Mathur, A. K., (Ed.). CIMAP, Lucknow, India. 188-197.

Panth, M., Hassler, S.C., Gurel, F.B., 2020. Methods for management of soil borne diseases in crop production. Agric. 10 (16), 1–21. https://doi.org/10.3390/agriculture10010016.

Patil, J., Goel, S.R., Yadav, S., 2017. Bio-management of cucumber wilt complex by root-knot nematode, *Meloidogyne incognita* and *Fusarium oxysporum* f.sp. *cucumerinum* in poly house under protected cultivation. J. Pure App. Microbiol. 11, 1909–1917.

Pertot, I., Alabouvette, C., Esteve, E. H., Franca, S., 2015. Focus group in soil borne diseases: The use of microbial biocontrol agents against soil borne diseases. In EIP-AGRI Focus Group on Soil-Borne Diseases: Final Report; Claude, A., Clarkson, J., de Cara, F.M., de Caravalho Franca, S., Debode, J., Elorrieta, M., Furlan, L., Grand, A., Hinarejos Esteve, E., Kos, J., et al., Eds.; EIP-AGRI: Brussels, Belgium, pp. 3–5.

Pirsaheb, M., Limoee, M., Namdari, F., Khamutian, R., 2015. Organochlorine pesticides residue in breast milk: a systematic review. Med. J. Islam Repub. 29, 228.

Reddy, P.P., 2016. Sustainable crop protection under protected cultivation. Springer Singapore. https://doi.org/10.1007/978-981-287-952-3.

Sharma, H.K., Pankaj, Lal, J., 2008a. Effect of chemicals on root-knot nematode, *Meloidogyne incognita* infecting tomato under polyhouse cultivation. Ind. J. Nematol. 38, 124–125.

Sharma, H.K., Pankaj, Singh, B., 2008b. Protected cultivation and nematode problem. Ind. J. Nematol. 39, 1–8.

Shishido, M., 2011. Plant disease management in protected horticulture. HortRes. 65, 7–18.

Shweta, Bhatia, S.K., Malik, M., 2014. Protected Farming. Popular Kheti 2, 74–79.

Singh, S., 2013. Integrated approach for the management of root-knot nematode, *Meloidogyne incognita* on eggplant under field conditions. Nematol. 15, 747–757.

Singh, S., 2019. Integrated approach for management of root-knot nematode (*Meloidogyne incognita*) in bitter gourd (*Momordica charantia*). Ind. J. Agri. Sci. 89, 71–76.

Singh, S., Balodi, R., Meena, P. N., Singhal, S. 2020. Biocontrol activity of *Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens* against *Meloidogyne incognita*, *Fusarium oxysporum* and *Rhizoctonia solani*. (Unpublished).

Singh, S., Goswami, B.K., 2001. Studies on the management of disease-complex caused by root-knot nematode, *Meloidogyne incognita* and wilt fungus, *Fusarium oxysporum* on cowpea by neem cake and carbofuran. Ind. J. Nematol. 31, 122–125.

Singh, S., Mathur, N., 2010a. In vitro studies of antagonistic fungi against the root-knot nematode, *Meloidogyne incognita*. Biocont. Sci. Tech. 20, 275–282.

Singh, S., Mathur, N., 2010b. Bio-control of root-knot nematode, *Meloidogyne incognita* infesting tomato. Biocont. Sci. Tech. 20, 865–874.

Singh, S., Pandey, R.K., Goswami, B.K., 2013. Biological control activity of *Purpureocillium lilacinum* strains in managing root-knot disease of tomato caused by *Meloidogyne incognita*. Biocont. Sci. Tech. 23, 1469–1489.

Singh, S., Singh, A.P., Sellaperumal, S., 2018. Incidence and population density of plant parasitic nematodes infesting vegetable crops and associated yield losses in Eastern Uttar Pradesh. Ind. J. Nematol. 48, 198–202.

Singh, S., Singh, R.K., 2012. Development of an integrated approach for managing root-knot disease of chili (*Capsicum annum* L.) under field condition. Rus. J. Nematol. 20, 65–73.

Southey, J.F., 1986. Laboratory Methods for Work with Plant and Soil Nematodes. Ministry of Agriculture, Fisheries and Food HMSO, London, p. 202.

Strashnov, Y., Slad, Y., Sivan, A., Rudich, Y., Chet, I., 1985. Control of *Rhizoctonia solani* fruit rot of tomatoes by *Trichoderma harzianum* Rifai. Crop Protec. 4, 359–364.

Triveni, S., Prasanna, R., Saxena, A.K., 2012. Optimization of conditions for *in vitro* development *of Trichoderma viride*-based bioflms as potential inoculants. Folia Microbiol. 57, 431–437. https://doi.org/10.1007/s12223-012-0154-1.

Warmink, J.A., Nazir, R., Corten, B., Elsas, J.D., 2011. Hitchhikers on the fungal highway: The helper effect for bacterial migration via fungal hyphae. Soil Biol. Biochem. 43, 760–765. https://doi.org/10.1016/j.soilbio.2010.12.009.

Weller, D.M., 1988. Biological control of soil borne plant pathogens in the rhizosphere with bacteria. Ann. Rev. Phytopathol. 26, 379–407.

Yamamoto, D.T., Uchida, J.Y., 1982. Rapid nuclear staining of *Rhizoctonia solani* and related fungi with acridine orange and with safranin O. Mycol. 74, 145–149.