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Rhizophere engineering with beneficial microbes for growth enhancement and nematode disease complex management in gherkin (*Cucumis anguria* L.)

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ABSTRACT

Major studies on root knot nematode (*Meloidogyne incognita*) tend to focus on the nematode alone, but with not much attention to its synergistic association with bacterial or fungal pathogens co-inhabiting the crop rhizosphere. Nematodes pave the way for easy entry of these pathogens inside the host roots, forming a disease complex that causes enormous yield loss. Greater knowledge on the interaction of fungal and nematode pathogens is required to find better solutions for sustainable management of the nematode diseases complex. Hence, this research was designed to study the interaction between *M. incognita* and *Fusarium oxysporum* f. sp. *melonis* and evaluate the consortia formulation of biocontrol agents (BCA) *Trichoderma viride* IIHR TV-2 and *Bacillus subtilis* IIHR BS-21 against nematode-fungal diseases complex in gherkin (cv. Ajax). Nematode-fungus interaction studies revealed that inoculation of *M. incognita* 15 days earlier to fungus showed maximum wilt incidence (96.81%) than fungus alone (85.29%), revealing their synergistic association. Under *in vitro*, cell free culture filtrates of *T. viride* IIHR TV-2 and *B. subtilis* IIHR BS-21 showed significant antagonism ovicidal, larvicidal and fungicidal action. Both the BCA strains were found compatible with each other (91.84%). Under field conditions, seed treatment (20 ml kg^{-1}) and subsequent soil application of vermicompost (2 t ha^{-1}) enriched with BCA consortia (5 l) before planting, followed by monthly application at 1 t ha^{-1} showed maximum increase in the plant growth parameters and reduction in nematode population in soil (66.37%), roots (62.45%) and per cent wilt incidence (42.56%) compared to untreated plants. Marketable gherkin yields also increased by 34.42% over the control. These results emphasize the efficacy of vermicompost enriched with microbial consortia in managing pathogen complexes and can be included as promising component in integrated nematode and disease management for gherkin and other horticultural crops.

1. Introduction

Gherkin (*Cucumis anguria* L. Cucurbitaceae) popularly known as Burr gherkin or Pickle gherkin has been introduced into India in 1989 as a profitable cash crop and is mainly exported to other foreign countries (Venuprasad et al., 2013). Gherkin is grown for its edible vegetable, but it is majorly preferred in the form of pickling. In India, gherkin is grown throughout the year whereas in other major producing countries like Hungary, Madagascar and Mexico, it is grown three months in a year. Hence it has a vast scope as a commercial crop in India. It has important chemical constituents such as cucurbitacin B, cucurbitacin D and cucurbitacin G which are used in traditional treatment of different diseases (Gill et al., 2014), and cures constipation, indigestion and jaundice (Chadha, 2001).

Root-knot nematodes (*Meloidgyne* spp.) are obligate sedentary endoparasites and the most destructive pests in a range of horticulture crops (Gill and Mcsorley, 2011). Gherkin is highly susceptible to *M. incognita* which causes the yield losses to the tune of 38–43% (Nagesh et al., 2005). *M. incognita* infection results in variation in the levels of amino acids, organic compounds and chlorophyll content in the host plant (Sikora and Schuster, 2000; Freire and Bridge, 1985; Ferraz et al., 1988). Heavy nematode infestation causes stunting, yellowing, delay in flowering, fruit drop in immature stage and reduction in the root biomass (Kingland, 2001; Pandey et al., 2003).

Root knot nematodes also interact with other soil borne fungal and bacterial pathogens and result in nematode-pathogen disease-complex causing huge economic loss to the crops (Back et al., 2002; Rao et al., 2017). Atkinson (1892) was the first to document the interaction between the root knot nematodes and *Fusarium oxysporum* and their synergistic effect resulted in severe wilt of cotton. Interaction of nematode and *Fusarium* wilt was documented in several host crops, including

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alfalfa (Griffin, 1986); beans (France and Abawi, 1994); banana (Jonathan and Rajendran, 1998); coffee (Bertrand et al., 2000); chickpea (Kumar et al., 1988); lentil (De et al., 2001); tomatoes (Suleman et al., 1997); betelvine (Jonathan et al., 1997) and muskmelon (Bergeson, 1975). Patel et al., 2000 reported greater wilt incidence due to diseases complex in the chickpea caused by *F. oxysporum* f. sp. *ciceri* and *M. incognita* compared to individual pathogens alone.

Use of chemical nematicides and fungicides though widely used by farmers, are not economically feasible, and cause adverse impact on environment and human health (Adam et al., 2014). Microbial BCAs are recommended globally for effective management of diseases and pests as they are safe to humans and non-target organisms, amenable to individual applications and suitable for integrated pest and disease management approaches (Rao et al., 2015).

Since a multitude of pests and pathogenic microbes **co-**inhabit in agricultural ecosystems, use of single BCA might not be effective against different pathogens and in all kinds of soil environment. Hence, there is a paradigm shift in current research towards microbial consortia (or combination of microbes) for the management of soil borne pathogens. Diverse microbes in consortia possess desired characteristics like high rhizosphere competency, nutrient mobilization activities, direct antagonism against pathogens and inducing systemic resistance (ISR) in host plants against soil as well as foliar pathogens (Sarma et al., 2015).

M. incognita and *Fusarium oxysporum f.sp. vasinfectum* disease complex in okra (Rao et al., 2014). The mechanism of action of *B. subtilis* against nematodes and pathogenic fungi was elucidated through antibiotic production (Kavitha et al., 2012), direct antagonism on plant pathogens (Asaka and shoda, 1996) and induced plant resistance (Ongena et al., 2007). *Trichoderma viride,* a beneficial fungal antagonist, demonstrated its efficacy against nematode-Fusarium wilt disease complex in crossandra (Ramakrishnan et al., 2014). *T. viride* revealed direct parasitism on *M. incognita* eggs and juveniles through secretion of protease and chitinase enzymes (Sharon et al., 2001; Suarez et al., 2004).

The success of BCA in field conditions largely depends on suitable delivery mechanism which ensures its viability and perpetuation post-application in fields. This could be achieved successfully by enriching BCAs in organic amendments like Farm Yard Manure or neem cake or vermicompost (Latha et al., 2011; Sowmya et al., 2012;). Application of Vermicompost enhanced with BCA enhanced the yield and reduced the nematode diseases complex in carrot (Rao et al., 2017). Currently, gherkin growers mainly rely on chemical fungicides and nematicides for managing diseases, and worldwide no attempt is made so far for biological management of nematode disease complex in gherkin. Keeping this in view, the present work is focused to develop suitable formulations with a consortium of highly effective and compatible biocontrol agents (*B. subtilis* IIHR-21 and *T. viride* IIHR TV-2), modulate proper delivery mechanism and evaluate their effect on the management of nematode disease complex in gherkin.

2. Materials and methods

2.1. Nematode culture maintenance

Pure culture of root knot nematode *M. incognita* was maintained on tomato plants (cv. Arka Samrat) grown in sterilized loamy sand soil in earthen pots under the screen house conditions at Block VI, ICAR – Indian Institute of Horticultural Research (IIHR), Bengaluru. The identity of female *M. incognita* was confirmed based on their perineal cuticular pattern (Sasser and Carter, 1982). The mature egg masses were handpicked from the infected roots and allowed to hatch in distilled water for 36 h at 28 ± 2 °C. They were observed for emergence of second stage juveniles (J2) under stereoscopic microscope (Motic, Hong-kong). These nematodes (J_2) were counted per ml of suspension in three replicated aliquots and 1000 J_2 per pot were inoculated for all respective treatments.

2.2. Maintenance of BCAs and preparation of formulation

BCA strains of IIHR *T. viride* (IIHR TV-2, NCBI- KC403962.1) and *B. subtilis* (IIHR BS-21, NCBI- KX233724) were regularly cultured and maintained in the Nematology laboratory, ICAR-IIHR, Bengaluru. The fungal bio-agent was cultured using potato dextrose media whereas the *B. subtilis* was cultured using Nutrient broth. The fungal pathogen, *Fusarium oxysporum f.* sp. *melonis* was obtained from Division of Plant Pathology, ICAR-IIHR, Bengaluru.

Liquid formulations of BCAs were prepared as per the procedure of Rao (2015). The population in terms of colony forming units (CFU) was maintained for *B. subtilis* IIHR BS-21 (8×10^8 ml^{-1}) and *T. viride* IIHR TV-2 (9×10^8 ml^{-1}) in their individual formulations. In consortia formulation, the population was 6×10^8 CFU ml^{-1} and 7×10^8 CFU ml^{-1} of *B. subtilis* IIHR BS-21 and *T.viride* IIHR TV-2, respectively.

2.3. Enrichment of vermicompost

Five litres each of *B. subtilis* BS-21(1% A.S) and *T. viride* TV-2(1% A.S) were enriched in 5 tons of vermicompost (Kempmann Bioorganics LLP, Bengaluru with organic carbon -11.98%, nitrogen-0.78%, phosphorus-0.15%, and potassium 0.34%) placed in separate heaps and treated with respective formulations (Yang et al., 2011). Five tons of vermicompost was added to five litres of BCA respect to the treatments and mixed thoroughly. Enriched vermicompost were maintained at 25–30% moisture and incubated at optimum temperature (27–30 °C) under shade for 15 days, manually turned every day (Rao et al., 2017).

After enrichment, the colonies of *B. subtilis* IIHR BS-21 and *T. viride* IIHRTV-2 in the mixture were enumerated by serial dilution on Bacillus - selective media (Kinsella et al., 2009; Luo et al., 2010; Turner and Backman, 1991) and Trichoderma - selective media (Josie et al., 2003; Yang et al., 2011), respectively. The population of *B. subtilis* IIHR BS-21 ranged from 10^4 to 10^6 CFU g^{-1} and *T. viride* IIHR TV-2 ranged from 10^5 to 10^7 CFU g^{-1} of vermicompost.

For further confirmation, morphological and biochemical tests were conducted for *B. subtilis* IIHR BS-21 and *T. viride* IIHR TV-2. After 24 h, the colonies were studied for their morphological characteristics such as shape, spore formation, gram staining and subjected to biochemical tests such as susceptibility to catalase, citrate hydrolysis, motility, indole production, nitrate reduction, penicillin, Voges-Proskauer and production of hydrogen sulphide (Amin et al., 2015). The fungal colonies were observed for their conidia characters such as conidia, conidiophores and conidia shape (Sriram et al., 2013).

2.4. Preparation of Cell free extract from bioagents

B. subtilis IIHR BS-2 and *T. viride* IIHR TV-2 were inoculated in 100 ml of Nutrient Broth and Potato Dextrose Broth, respectively and incubated at 26 ± 2 °C on a rotary shaker at 150 rpm for 36 h for bacterial cultures and 120 h for fungal cultures. The cells were harvested by centrifugation at 10,000 rpm for 15 min and supernatant was filtered through the sterile Whatmann filters (0.45µ for fungi and 0.22µ for bacteria). Undiluted cell free extract was labelled as 100% concentration and further diluted with distilled water to 75%, 50%, and 25% for further bioassays.

2.5. In vitro – ovicidal assay

Ovicidal action of various treatments was evaluated by slightly modifying the procedure of Su and Mulla (1998). Approximately, hundred eggs of *M. incognita* were carefully placed into respective petridishes (3.5 cm dia) containing cell-free culture filtrate of bio-agents at different concentrations as 100, 75, 50 and 25%. Distilled water and media without bio-agents were treated as untreated control and sterile broth, respectively. Five replicates were maintained for precision results. The petri-dishes were incubated at room temperature 26 ± 2 °C

for 5 days. The hatched juveniles were counted microscopically under Stereo zoom microscope (Motic SMZ 168 series) every day up to 5 days. The whole experiment was repeated thrice.

The rate of percentage inhibition of egg hatching was assessed by following formula, 100- Number of hatched eggs/total number of eggs x 100.

2.6. In vitro larvicidal assay

Freshly hatched second stage juveniles (~100 J_2) in distilled water suspension were added to 3 ml of cell free culture filtrate of bio-agents of 100, 75, 50 and 25% concentrations. Treatments were replicated 5 times and the petri dishes were incubated at 26 ± 2 °C. Distilled water and media without bio-agents were treated as a control and media control. Number of dead and live second stage juveniles was observed microscopically under Stereo Zoom microscope (Motic SMZ 168 series) for 5 days, and percentage mortality was calculated. Immobile second stage juveniles were transferred to petri-dish containing distilled water for confirmation of death. The whole experiment was repeated thrice.

2.7. Studies on inter-relationships between fungi and bacteria

Reciprocal effects between *B. subtilis*, *T. viride* and *F. oxysporum f. sp. melonis* between the strains were evaluated by dual culture test as described by Dennis and Webster (1971). In the first set, 9 mm dia mycelial disc of *F.oxysporum* f. sp. *melonis* was placed on one side of petri-dishes (90 mm) containing potato dextrose agar medium. It was then streaked with *B. subtilis* crisscrossed on the edge of a petri-dish and wrapped by parafilm. In the second set, 9 mm dia disc of *T. viride* grown on potato dextrose agar was placed in the petri dish adjacent to *F.oxysporum* f. sp. *melonis*. In the third set, 9 mm dia disc of *T. viride* was placed and *B. subtilis* was streaked opposite to *T. viride*. Three replicates were maintained for all treatments. Petri-dishes inoculated with single BCA were treated as control. The inoculated dishes were incubated for 6 days at 28 ± 2 °C. Consequently, the radial growth of the colonies was observed, and the rate of inhibition was calculated.

The per cent increase in the colony diameter was calculated using the following formula:

Percentage of Inhibition or Increase = T-C/C*100

T- Colony diameter in the treated

C- Colony diameter in control

Percentage of compatibility was calculated by using the formula: % compatibility = 100 - % 'I' where I is Inhibition.

2.8. Nematode fungus interaction studies in gherkin

The trials were carried out on a complete randomized design (CRD) with three replications during the month of January to March 2015 in the screen house at ICAR-IIHR, Bengaluru. Seeds of gherkin (cv. Ajax) were sowed in 5 kg pots filled with sterilized pot mixture (Red soil: Farmyard manure (FYM): Sand in 1:1:1). After 15 days, the plants were inoculated with *M. incognita* and *Fusarium oxysporum* f. sp.*melonis* as per treatment schedule (Table 1) at 2000 nematodes (J2) and or 20 ml of aqueous suspension with 2×10^6 cfu /ml (Ramamoorthy et al., 2002; Ramyabharathi and Raguchander, 2014: Mishra et al 2000) per pot near the root zone.Plants were uprooted 90 days after inoculation and observed for growth parameters *viz.,* root length (cm), shoot height (cm), root weight (g) and shoot weight (g). Roots were analysed for severity of root galling according to Heald et al. (1989) on a scale of 1–5 (Gall index: 1 = no galls; 2 = 1–25% galls; 3 = 26–50% galls; 4 = 51–75% galls; 5 = 76–100% galls per root system). Nematode population in soil was analysed as per Cobb's sieving and decanting method (Cobb, 1918) and Modified Baermann's funnel technique (Southey, 1986), and in roots by acid fuchsin staining (Bridge et al., 1982). Further, percent wilt incidence caused by the pathogen was calculated using number of plants affected by wilt/number of plants x 100 (Sowmya et al., 2012).

Table 1
Treatment details for the interaction between *M. incognita* and *F.oxysporum* f.sp.*melonis* in gherkin under screen house conditions.

S.No	Treatments	Details
1	MI + FO	Concomitant inoculation of *M. incognita* and *F. oxysporum*
2	MI alone	Inoculation of *M. incognita* alone
3	FO alone	Inoculation of *F. oxysporum* alone
4	MI-15 days	Inoculation of *M. incognita* 15 days earlier to fungus inoculation
5	FO-15 days	Inoculation of *F. oxysporum*15 days earlier to nematode inoculation
6	Un-inoculated	Pathogen free-healthy control

2.9. Evaluation of bioagents against nematode diseases complex under screen house conditions

Effect of liquid formulation enriched with vermicompost was evaluated against nematode-fungus diseases complex in Gherkin var. Ajax under screen house conditions at Division of Entomology and Nematology, ICAR-IIHR, Bengaluru. Bio-agent formulations were compared with standard chemicals *viz*., carbofuran (1 kg a.i/ha) and carbendazim (0.1%).

Uniform sized seeds of Gherkin var. Ajax were sown in 5 kg earthen pots filled with sterilized pot mixture (farm yard manure: sand: soil: 1:2:1) at two seeds/ pot. According to the treatment schedule (Table 2), seeds were treated with consortia formulation of BCA 1% A.S. @20 ml/ kg seeds, shade dried and sown in pots. For soil application, 200 g of BCA enriched vermicompost was applied per pot before sowing followed by monthly application of 100 g per pot for 3 months. For comparison with chemicals, carbofuran was applied @ 3.33 g per pot and carbendazim @ 0.1% (1 ml of carbendazim diluted in 1 L of water and drenched at 500 ml per pot) before sowing. After 10 days of sowing, *M. incognita* (2000 J2 per pot) and *F. oxysporum* (20 ml of spore suspension with 2×10^6 cfu/ml)) were inoculated in all the pots. The pots were arranged in a complete randomized design (CRD) with four replications. Experiments were carried out in autumn from September to December 2015 (mean day length 11.23 to 12.80 h); mean daily maximum temperature 26.1–28.2 °C; mean daily minimum temperature 15.1–19.2 °C).

Plants were uprooted after 90 days of inoculation and assessed for plant growth parameters *viz*., shoot height (cm), shoot weight (g), root weight (g) and root height (cm). Nematode population and wilt incidence were recorded as per the protocol in Section 2.8.

Root colonization by bioagents was assessed by serial dilution method with slight modifications. The roots were washed gently by running tap water and 1 g of root sample was grounded with mortar and pestle. Dilutions were prepared up to 10^{-5} and 1 ml of aliquots from 10^{-3} to 10^{-4} dilutions from each treatment were plated on nutrient agar (for bacteria) and potato dextrose agar (for fungi). Each treatment was replicated five times and plates were incubated at 27 ± 4 °C. The colonies were observed according to morphological structures for bacterial and fungal colonies (as per Section 2.3).

2.10. Evaluation of bioagent against nematode disease complex under field conditions

Two separate field experiments were conducted in pathogen sick plot located at Block VI, Division of Entomology and Nematology, ICAR-IIHR, Bengaluru during September to December 2015 and July to

Table 2
Treatment description for the effect of bio control agents on Nematode-fungus diseases complex in gherkin for screen house and field conditions.

Treatments	Details
ST	Seed treatment of Consortia formulation of *Bacillus subtilis* IIHR BS-21 and *Trichoderma viride* IIHR TV-2 (1% A.S)
ST + SA1	ST + Soil application of 2 tons ha^{-1} of vermicompost enriched with 5 l of *B. subtilis* IIHR BS-21, before planting followed by application at 1 t/ha at monthly interval.
ST + SA2	ST + Soil application of 2 tons $ha^{-1}$of vermicompost enriched with 5 l of *T.viride* IIHR TV-2, before planting followed by application at 1 t/ha at monthly interval.
ST + A3	ST + Soil application of 2 tons $ha^{-1}$of vermicompost enriched with 5 l Consortia of *Bacillus subtilis* IIHR BS-21 and *Trichoderma viride* IIHR TV-2 before planting followed by application at 1 t/ha at monthly interval.
ST + VM	ST + Soil application of 2 tons $ha^{-1}$of vermicompost, before planting followed by application at 1 t/ha at monthly interval.
CHEMICAL	Carbofuran (1 kg a.i/ha) + Carbendazim (0.1%)
CONTROL	Pathogen free-healthy control

September 2016 to evaluate the efficacy of consortia formulation of bioagents enriched with vermicompost against nematode-fungus diseases complex in Gherkin cv. Ajax. Initial nematode and wilt fungal population was estimated as 130 ± 30 J2 per 100cc of soil and 3.2×10^3 CFU/g soil, respectively for the first trial and 126 ± 15 J_2 per 100cc of soil and 2.0×10^3 CFU/g soil, respectively for the second season. Individual plot size of 10*2 m^2 was maintained for each treatment and randomized block design was followed with eight replicates of each treatment with 10 plants per plot. After 90 days, the experiment was terminated, and observations were recorded on root gall index, nematode population in soil and roots, wilt incidence and root colonization as per the procedures described in Section 2.9.

2.11. Statistical analysis

Statistical analysis was performed using the software SAS 9.3. For count data (juvenile mortality and hatching experiment) regression analysis was performed. The data was transformed into Y = ax + b for linear regression analysis, where Y = cumulative hatched eggs and x = number of days. Regression analysis was executed using 5 replicates for each treatment. The effect of different treatments on growth parameters, yield, gall index, nematode population and disease index were evaluated using ANOVA and the critical difference were determined using Duncan's Multiple Range Test. Since similar results were observed in the field trials for both years, the data were pooled and statistically analysed.

3. Results

3.1. Ovicidal and larvicidal assay

It was observed that the bioagents had the potential to inhibit egg hatching of *M. incognita* at all concentrations. Maximum ovicidal capability was revealed by *T. viride* IIHR TV-2 and *B. subtilis* IIHR BS-21 which recorded 94.56% and 92.15% inhibition in egg hatching, respectively at 100% concentration. The results clearly showed that increase in concentration of culture filtrates and duration (days) increased the suppression in egg hatching. It was followed by 92.48% and 90.49% inhibition in egg hatching at 75% concentration of *T. viride* IIHR TV-2 and *B. subtilis* IIHR BS-21, respectively. While in sterile broth, 22.56% was recorded and in distilled water, suppression in egg hatching was nil. All coefficients of determination were significant at $P < 0.001$. Regression analysis revealed the relationship between BCA concentration and hatching of nematode eggs. Suppression in egg hatch increased with increase in concentration (25–100%) and time (1–5 days) for both the BCAs (Figs. 1 and 3)

A similar trend was also observed for the larvicidal activity of *T. viride* IIHR TV-2 and *B. subtilis* IIHR BS-21 on *M. incognita* J2 at different concentrations. The maximum of 95.46% and 90.46% mortality was recorded at 100% concentration of *T. viride* IIHR TV-2 and *B. subtilis* IIHR BS-21, respectively. It was followed by 82.34% and 80.78% juvenile mortality at 75% concentration of *T. viride* IIHR TV-2 and *B. subtilis* IIHR BS-21, respectively. In sterile broth, 40.23% of nematode mortality was observed while in distilled water, mortality was nil. All coefficients of determination were significant at $P < 0.001$.Regression analysis showed the relationship of concentration *versus* nematode population. J_2 mortality increased with increasing concentration (25–100%) and time (1–5 days) for both the BCAs (Figs. 2 and 4).

3.2. Studies on inter-relationships of fungi and bacteria

In dual culture studies *in vitro*, significant inhibition in mycelial growth of *F. oxysporum f. sp. melonis* was detected in the presence of *T. viride* IIHR TV-2 (90.00%) and *B. subtilis* IIHR BS-21 (73.61%) (Table 9). *T. viride* IIHR TV-2 was observed to grow faster and inhibited the growth of *F. oxysporum* f. sp. *melonis*. Both the BCA strains were compatible to each other (91.84%) and it was observed that *T. viride* IIHR TV-2 overgrew on *B. subtilis* IIHR BS-21 without formation of inhibition zone.

3.3. Interactive effect of M. incognita and F. oxysporum on gherkin

In the sequential and concomitant inoculation of fungus and nematodes, the incidence of wilt was greater when compared to fungus alone. It was observed that presence of nematodes resulted in earlier onset of wilt symptoms and caused more stunting of plants. Sequential inoculation of nematodes 15 days earlier to fungus significantly increased the severity of the wilt incidence up to 96.81% followed by concomitant inoculation of nematode and fungus which resulted in 83.25% of wilt incidence (Table 3). Concomitant and sequential fungus and nematode inoculation caused a significant decrease in the nematode population and in root gall severity. Gall index was recorded 4.0 when the nematodes were inoculated 15 days early to the fungus which was significantly lesser compared to nematode alone (Gall Index - 5.0). Similarly, nematode population was found significantly lower in the sequential inoculation of fungus prior to nematode (Table 3).

3.4. Management of nematode disease complex under screen house conditions

In general, maximum plant growth promotion and minimum pathogen population were observed in plants treated with BCA and chemicals. Seed treatment along with SA3 (5 t ha^{-1}) showed the highest plant growth parameters and lowest nematode population in the soil, roots, gall index and wilt incidence (38.15%) (Table 4). It also recorded maximum plant growth parameters *viz.,* root length (24 cm), root weight (7 g), shoot length (174 cm) and shoot weight (79.4 g). It was followed by treatment with chemicals (Table 5). ANOVA results on plant growth promotion revealed significant mean differences among the treatments represented in Table 6.

In untreated control, maximum nematode population (216 per 100cc soil and 55 females g^{-1} of root) and wilt incidence (95.12%) were noticed followed by vermicompost alone (Table 4). Greater colonization of *T. viride* IIHR TV-2 and *B. subtilis* IIHR BS-21 in soil and

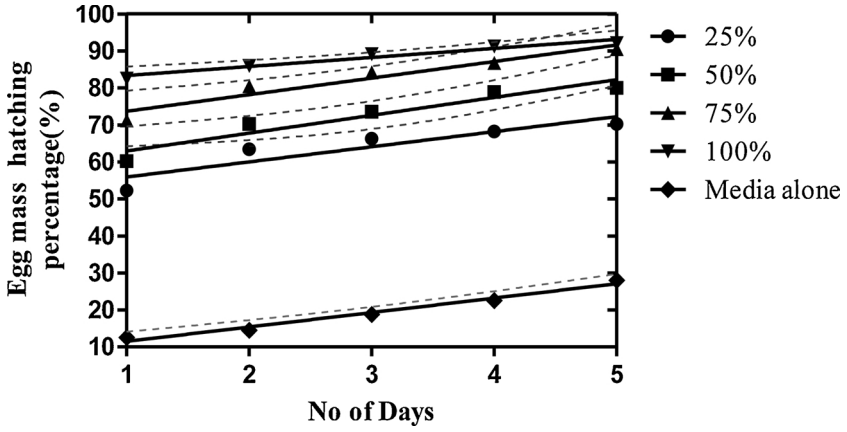

Fig. 1. Influence of *B.subtilis* concentrations on egg hatch(Y) of *Meloidgyne incognita* over the period of 5 days of incubation at various concentration. Mean values were derived from 5 replicates. Regression equations were $Y = 4.084x + 51.86$, $R^2 = 0.83$ (at 25%); $Y = 4.82x + 58.1$, $R^2 = 0.91$ (at 50%); $Y = 4.48x + 69.2$, $R^2 = 0.92$ (at 75%); $Y = 2.45x + 80.9$, $R^2 = 0.95$ (100%) and $Y = 3.90x + 7.60$, $R^2 = 0.57$ (Sterile broth).

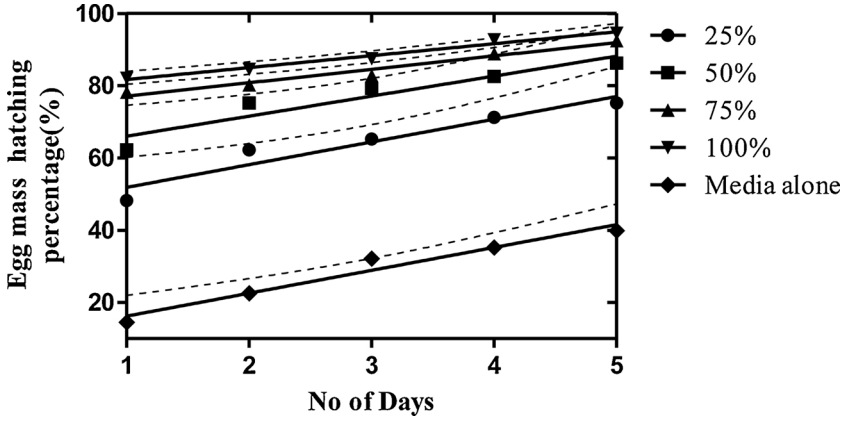

Fig. 2. Influence of *T.viride* concentrations on egg hatch(Y) of *Meloidgyne incognita* over the period of 5 days of incubation at various concentration. Mean values were derived from 5 replicates.. Regression equation were $Y = 6.29x + 45.55$, $R^2 = 0.92$ (at 25%); $Y = 5.53x + 60.49$, $R^2 = 0.89$ (at 50%); $Y = 3.71x + 73.38$, $R^2 = 0.96$ (at 75%); $Y = 3.3x + 78.88$, $R^2 = 0.97$ (at 100%) and $Y = 6.3 \times 3 + 9.88$, $R^2 = 0.46$ (for sterile broth).

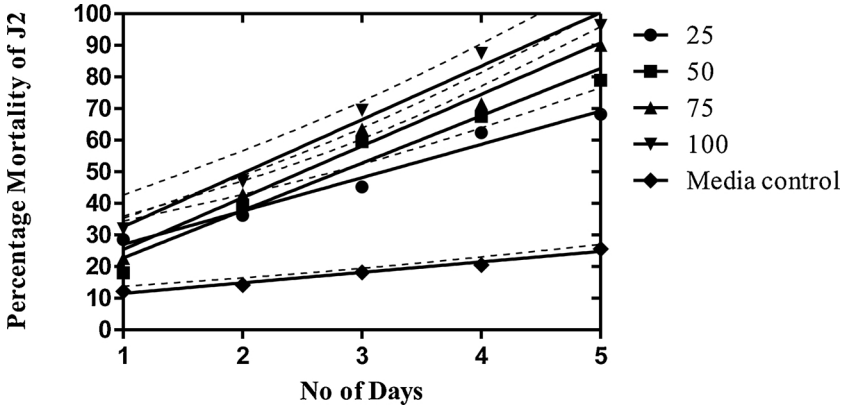

Fig. 3. Influence of *B.subtilis* concentrations on second stage juveniles(Y) of *Meloidgyne incognita* over the period of 5 days of incubation at various concentrations. Mean values were derived from 5 replicates. Regression equations were $Y = 9.05x + 13.31$, $R^2 = 0.94$ (25%); $Y = 6.86x + 29.44$, $R^2 = 0.95$ (50%); $Y = 9.04x + 29.56$, $R^2 = 0.97$ (75%); $Y = 10.39x + 42.04$, $R^2 = 0.97$ (100%) and $Y = 2.04x + 11.0$; R2 = 0.33 (Sterile broth).

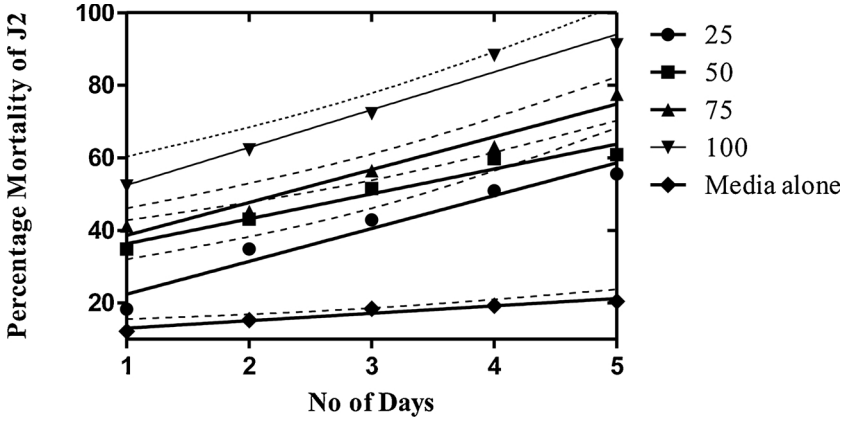

Fig. 4. Influence of *T.viride* concentrations on second stage juveniles(Y) of *Meloidgyne incognita* over the period of 5 days of incubation at various concentrations. Mean values were derived from 5 replicates. Regression equations were $Y = 10.455x + 16.58$, $R^2 = 0.97$ (25%); $Y = 14.99x + 7.74$, $R^2 = 0.96$ (50%); $Y = 16.35x + 9.04$, $R^2 = 0.98$ (75%); $Y = 16.93x + 15.68$, $R^2 = 0.99$ (100%) and $Y = 3.31x + 8.2$; R2 = 0.51 (Sterile broth).

Table 3
Interaction studies between *M. incognita* and *F.oxysporum* f sp *melonis* in gherkin.

Sno	Root length (cm)	Shoot length (cm)	Root weight (g)	Shoot weight (g)	No of egg mass/g of root	Root Gall index	Soil nematode population per 100 cc	Percent wilt incidence
Nematode alone	7.56 ± 0.32	78.5 ± 1.8	1.95 ± 0.14	11.23 ± 1.4	23.86 ± 1.6	5	347.88 ± 1.8	7.1 ± 0.51
Fungus alone	8.74 ± 0.19	90.48 ± 1.6	3.1 ± 0.39	14.56 ± 1.6	0	0	0	79.06 ± 3.5
Concomitant inoculation of nematode and fungus	9.1 ± 5.0	95.12 ± 3.3	2.35 ± 0.37	13.23 ± 1.5	21.54 ± 1.1	3.65	284.44 ± 1.2	85.29 ± 2.2
Nematode 15 days prior fungus prior to fungus inoculation	4.5 ± 0.29	65.23 ± 2.0	1.12 ± 0.34	8.5 ± 4.3	26.03 ± 1.2	4.1	323.10 ± 1.8	96.81 ± 1.1
Fungus 15 days prior fungus prior to Nematode inoculation	6.25 ± 0.48	86.25 ± 2.1	2 ± 0.15	16.12 ± 2.9	13.09 ± 1.5	3.05	258.99 ± 1.2	76.25 ± 1.7
Un-inoculated	10.23 ± 1.1	106 ± 2.2	4.2 ± 0.16	20.1 ± 5.4	0	0	0	0
CD 1%	1.01	4.00	0.51	6.36	3.24	0.60	3.19	2.94

Each value represents the mean and standard deviation of the mean from five replicates.

Table 4
Effect of bio control agents on *M. incognita* population and percent diseases incidence of *F.oxysporum* f sp *melonis* in gherkin under Screen house conditions.

Treatments	*M. incognita* population		Root gall index (1-5)	Per cent Disease incidence
	Soil (g)	Root (100 cc)		
ST	123.21^{C}	40.85^{D}	3.2^{C}	81.23^{C}
SA1	90.56^{B}	38.74^{CD}	2.1^{B}	60.48^{B}
SA2	87.00^{B}	36.41^{BC}	2.5^{B}	59.46^{B}
SA3	61.23^{A}	20.15^{A}	1.3^{A}	38.15^{A}
VC	160.89^{D}	40.85^{D}	3.8^{C}	88.98^{D}
CC	88.12^{B}	35.60^{B}	2.3^{B}	58.16^{B}
Control	216.14^{E}	55.00^{E}	5^{D}	95.12^{E}
CD1%	4.54	3.54	0.91	4.16
Sed	1.61	1.25	0.32	1.48

Each value represents the mean of the five replicates $p > 0.01$.

roots was documented in SA3 (Table 8). Also, treatment of gherkin seeds with *T. viride* IIHR TV-2 and *B. subtilis* IIHR BS-21 at 20 ml kg^{-1} seed resulted in significant colonization in soil and roots.

3.5. Field experiment

The pooled analysis for both the field experiments revealed that all the treatments with BCA and chemicals recorded significantly lower nematode population, and disease incidence compared to control. Soil application - SA3 (5 t ha^{-1}) recorded the maximum increase in plant growth and yield and minimum occurrence of nematode and disease incidence (Table 7). While in untreated control, maximum nematode population (195 per 100 cc soil and 59 females g^{-1} root) and higher wilt incidence (90.26%) was noticed.

Treating the seeds with BCA also recorded significantly higher yield and lower nematode and fungal population compared to untreated control. The effect of vermicompost application alone was on par with untreated control with respect to the yield and incidence of pathogens.

All BCA treatments showed significant colonization in both soil and roots under field conditions. Maximum population was observed in soil and root as 3.2×10^3 and 2.9×10^3, respectively for *B. subtilis* IIHR BS-21 followed by 5.2×10^3 and 4.9×10^3 respectively for *T.viride* IIHR TV-2 in SA3 followed by other treatments (Table 8).

4. Discussion

B. subtilis IIHR BS-21 and *T. viride* IIHR TV-2 exhibited nematicidal and fungicidal activities against *M. incognita* and *F. oxysporum* f. sp. *melonis*, both under *in vitro* and *in vivo* conditions. Previous reports by Killani et al. (2011); Huang et al. (2010) and Kavitha et al. (2012) revealed that *B. subtilis* produces several antibiotic compounds, nematicidal volatiles and lipopolypeptides which were antagonistic towards eggs and second stage juveniles of *Meloidogyne* spp. The antibiotic compound, fengycin produced by *B. subtilis* showed a strong fungi-toxic activity against several filamentous pathogenic fungi like F. *oxysporumf. sp. lycopersici, Pythium ultimum* and *Sclerotinia sclerotiorum* (Hofemeister et al., 2004; Koumoutsi et al., 2004; Seong et al., 2017).

T. viride is widely reported for its potential antagonism against *M. incognita* and *Fusarium* spp. on a range of horticultural crops such as tomato, mungbean, bell pepper and cauliflower (Meyer et al., 2001; Siddiqui et al., 1999; Siddique et al., 2001; Rajinikanth et al., 2013). *T. viride* produces enzymes like chitinases and glucanases which directly affect the egg membrane and cuticle layer of *M. incognita* (Brunner et al., 2003; Howell, 2003). It also produces β-1, 3-glucanases which shows strong antagonism, and undergoes the cell lysis against several phytopathogenic fungi (Matroudi and Zamani, 2009).

Also, in the present study, *B. subtilis* IIHR BS-21 and *T. viride* IIHR TV-2 were found compatible with each other. Compatibility of co-inoculated microorganisms is a pre-requisite for the success of effective

Table 5
Effect of bio control agents on plant growth parameters infected by nematode diseases complex in gherkin.

Treatments	Root				Shoot			
	Length (cm)		Weight(g)		Length (cm)		Weight (g)	
ST	11 ± 1.4	C	4.5 ± 0.6	E	130 ± 0.8	E	31.20 ± 1.8	EF
SA1	21 ± 1.6	AB	6.3 ± 1.0	C	142 ± 2.2	D	46.20 ± 0.18	D
SA2	22 ± 1.9	AB	6.8 ± 1.0	AB	151 ± 1.6	C	50.12 ± 1.6	BC
SA3	24 ± 4.3	A	7.0 ± 1.3	A	174 ± 0.7	A	79.4 ± 1.1	A
VC	10 ± 3.0	C	4.6 ± 1.2	E	124 ± 1.7	F	32.0 ± 1.5	E
Chemical	18.1 ± 5.4	B	5.9 ± 0.4	D	160 ± 1.6	B	52.0 ± 1.6	B
Control	9.2 ± 2.5	C	3.5 ± 0.5	F	105 ± 1.5	G	18.2 ± 0.24	G
CD 1%	2.00		2.76		0.98		2.46	

Each value represents the mean of the five replicates $p > 0.01$.

Table 6
ANOVA results for Shoot length, Root length, Shoot weight and Root weight in gherkin.

Sources	Degree of freedom	Sum of squares			
		Root length (cm)	Root weight (g)	Shoot length (cm)	Shoot weight(g)
Treatments	6	1175.47	14398.57	89.57	11274.56
Error	24	273.72	57.92	7.29	46.20
Total	34	1458.55	14464.20	114.69	11324.72
F-value		17.17	994.27	49.09	975.94
P-value		0.001*	0.001*	0.001*	0.001*

* Significative at a level of 1% probability. df- degree of freedom.

consortia formulations at field level. Similar combinations of BCAs *viz., T. harzianum* with *Pseudomonas fluorescens, Paecilomyces lilacinus* with *B.pumilus* and *P. chlamydosporia* with *P. fluorescens* have proved effective against nematode disease complexes in capsicum, eggplant and tomato (Rao et al., 2002; Naik and Rao, 2004; Rao et al., 2003).

The studies on interaction between the phytopathogens revealed a synergistic association between *M. incognita* and *F. oxysporum* f. sp. *melonis* and the resultant disease complex caused severe damage to gherkin. Inoculation of nematodes prior to fungus resulted in earlier onset of disease and caused maximum wilt incidence in gherkin compared to concomitant inoculation or individual inoculation of both the pathogens indicating that the nematodes allowed the fungus for earlier entry inside the hosts and aggravated the disease incidence. These results are in harmony with the experimental findings of Fritzsche et al. (1983) in cucumber wherein higher wilt incidence was observed with sequential inoculation of *M. incognita* followed by *F. oxysporum* f. sp. *cucumerinum,* than concomitant inoculation of the both the pathogens. In gerbera plants, severe wilt incidence due to fungus was observed due to pre-inoculation of nematodes (Sankarimeena et al., 2015). Nematode enters into the root system, alters the physiology and morphology of roots (Sankarimeena et al., 2011) and makes the plant more susceptible to the secondary pathogens (Mayol and Bergeson, 1970). However, the gall index in roots was rated significantly lesser with pre- inoculation of fungus 15 days before nematodes and concomitant inoculation of both pathogens compared to nematode alone. This might be due to the rotting of roots by *Fusarium* and reduction in the availability of feeding sites which deprived the nematodes for its nutrition and hence reduced its multiplication (Sankarimeena et al, 2015).

Integration of a cocktail of BCAs has gained importance in the recent years to reduce disease incidence level in economically important crops (Bharathi et al., 2004; Nandakumar et al., 2001; Senthilraja et al., 2010). In the present study, combination of *B. subtilis* and *T. viride* reduced nematode (69.78%), wilt incidence (59.26%), and promoted the plant growth and yield (25.6%) in gherkin, both under pot culture and field conditions. Different mixtures of microbial agents have been successfully used against several plant pathogens in different crops (Leibinger et al.,

Table 8
Soil rhizosphere density of BCA'S under green house and field conditions.

Treatments	Green house experiment				Field experiment			
	Soil density of *B.subtilis* IIHR BS -21		Soil density of *T.viride* IIHR TV-2		Soil density of *B.subtilis* IIHR BS-21		*Soil density of T.viride* IIHR TV-2	
	($X10^3$ CFU/g)		($X10^3$ CFU/g)		($X10^3$ CFU/g)		($X10^3$ CFU/g)	
	Soil	Root	Soil	Root	Soil	Root	Soil	Root
ST	3.1	2.1	4.1	3.9	2.1	1.1	3.8	3.1
SA1	6.5	5.2	0	0	5.2	4.1	0	0
SA2	0	0	8.1	7.2	0	0	7.2	7.3
SA3	4.9	4.2	6.1	5.1	3.1	2.9	5.2	4.9
VC	0	0	0	0	0	0	0	0
Chemical	0	0	0	0	0	0	0	0
Control	0	0	0	0	0	0	0	0
CD 5%	0.77	0.92	0.74	1.34	0.67	0.79	1.14	0.90

Table 7
Effect of BCAs on nematode induced disease complex in gherkin under field conditions.

Treatments	M. incognita population		Root gall index	Percent Diseases incidence	Marketable yield t/ha^{-1}	Percentage yield over the control (%)
	Soil(100cc)	Root(1 g)				
ST	116^{C}	39.84^{D}	3.5^{C}	72.56^{C}	6.8^{DC}	11.47
SA1	82^{B}	34.56^{CD}	2.4^{B}	62.16^{B}	7.2^{B}	18.03
SA2	81^{B}	35.26^{BC}	2.3^{B}	64.23^{B}	7.6^{AB}	24.59
SA3	62.56^{A}	22.15^{A}	1.8^{A}	42.56^{A}	8.2^{A}	34.42
VC	158^{D}	41.23^{D}	3.9^{C}	80.23^{D}	6.3^{E}	3.27
CC	75.23^{B}	60.07^{B}	2^{B}	48.23^{B}	7.1^{C}	16.39
Control	195^{E}	59^{E}	5^{D}	90.26^{E}	6.1^{D}	0
C.D 5%	3.33	2.59	0.67	3.05	0.56	0
S ed	1.61	1.25	0.32	1.48	0.72	0

S ed- Standard error of the mean. Each value represents the mean of the five replicates p.

Table 9
Interactive effect of *B.subtilis* IIHR BS-21, *T.viride* IIHR TV-2and *F. oxysporum* f. sp.*melonis* under dual culture technique.

Strains	*T.viride* IIHR TV-2		*F. oxysporum f. sp. melonis*		*B.subtilis* IIHR BS-21	
	Radial growth (7 days)	Per cent inhibition over control	Radial growth (7 days)	Per cent inhibition over control	Radial growth (7 days)	Per cent inhibition over control
B.subtilis	4.5	8.16	1.2	73.61	4.5*	0
T.viride	4.8*	0	1.0	90.00	4.2	6.66
F. oxysporum f. sp *melonis*	0.5	89.58	4.6*	0	1.2	73.33

* Respective Controls.

1997; Thilagavathi et al., 2007). Combined application of *T. harzianum* and *Pseudomonas* sp. reduced *Botrytis cinerea* in cucumber by 40% more than individual microbe application. It induced the systemic resistance (ISR) by activating different signalling pathways in cucumber against the pathogen (Alizadeh et al., 2013). Also, in pea plants, application of triple consortium consisting of compatible strains of *B. subtilis, P. aeruginosa* and *T. harzianum* increased the defence parameters by 1.4–2.3 folds. However, the increment was only 1.1–1.7 folds in individual microbial application compared to untreated control. The microbial consortia proved to induce the antioxidant enzyme activities and phenyl propanoid pathway leading to build-up of total phenols, proline and PR proteins upon pathogen infection (Jain et al., 2012).

The technology of enhancing BCA population in organic composts before field delivery has drawn much attention among the farmers as it reduces the cost of production and protection (Rao et al., 2015). Sharma (2002) reported that FYM and vermicompost were suitable substrates for growth of *P. fluorescens* and its soil application significantly decreased bacterial wilt in tomato. In the present study, *B. subtilis* IIHR BS-21 and *T. viride* IIHR TV 2 were enriched in such a way that 5 litres were enhanced in 5 tons of vermicompost. Combination of bio-agents with cow dung manure or farm yard manure decreases foot rot in lentil caused by *F. oxysporum* (Hannan et al., 2012).

Priti et al. (2016) reported that combination of *Bacillus pumilus* and *Pseudomonas* spp. enriched in vermicompost/neem cake reduced the nematode disease complex by 60–85% in onion. Likewise, Rao et al. (2017) had reported that *B. subtilis* enriched in vermicompost effectively reduced the *M. incognita* and *Pectobacterium carotovorum* disease complex in carrot. Also, (Cao et al., 2011) reported that organic fertilizer enriched with *B. subtilis* SQR 9 resulted in better plant growth, and significant reduction of *Fusarium* wilt in cucumber. Diverse mechanisms assessed by Oka 2010 showed that usage of manures decrease the nematode population by available nematicidal compounds through the process of decomposition, supports the growth of microorganisms and improves the soil physiology.

5. Conclusion

Experiments conducted in the present study established the first report on interaction between *M. incognita* and *Fusarium oxysporum* f. sp. *melonis* on gherkin and effective management of the nematode disease complex by combination of rhizospheric bioagents enriched in vermicompost. Such consortia formulations with combination of bioagents can thrive in different environments and act on diverse pathogens through multiple modes of action. Microbial consortia-based biocontrol agents have a huge potential for commercialization and such commercial products are of high demand in sustainable agriculture where priority is reducing the usage of chemical pesticides. Also, enrichment of biopesticides in organic composts is becoming popular among the farmers and commercial polyhouse growers as it is eco-friendly, economically feasible and environmentally sustainable. Thus, the standardisation of a suitable consortia formulation and mode of delivery of BCAs in gherkin makes it a potential IPM and IDM component and can be extended to other horticultural crops for successful nematode disease complex management.

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