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Biocontrol of wilt-nematode complex infecting gerbera by *Bacillus subtilis* under protected cultivation

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Abstract

A strong antibiotic producer, *Bacillus subtilis* strain Bbv 57 (KF718836), has been utilized for the management of wilt-nematode complex (*Fusarium oxysporum* f. sp. *gerberae*, *Meloidogyne incognita*) in gerbera under greenhouse conditions in the Department of Floriculture, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India. The strain strongly inhibited *F. oxysporum* f. sp. *gerberae* (KM523669) mycelial growth to an extent of 44.33 and 63.33%, at 10 and 100 µl, of culture filtrate, respectively. Further, the culture filtrate at 100% concentration exerted lethal effect on nematode eggs (7.00 hatched) and juveniles (87% mortality) compared to control. The analysis of TLC revealed that Bbv 57 showed the cyclic antimicrobial peptides surfactin and iturin that were confirmed by PCR. Strain Bbv 57 was able to produce antifungal and anti-nematicidal activity with reduced wilt incidence (15.33%) and thus holds a great potential for use in the biocontrol of *Fusarium* wilt-root-knot nematode disease complex in gerbera under greenhouse conditions.

Keywords: *Bacillus subtilis* Bbv 57, Surfactin, Iturin, ß-Glucanase, *Fusarium* nematode complex, Greenhouse

Background

Gerbera (*Gerbera jamesonii* Bolus ex Hooker f.), commonly known as Barberton daisy or African daisy, is very attractive and the most important cut flower crop commercially grown for domestic and export purpose under hi-tech condition. In India, the gerbera production was 1470.90 lakh flowers from an area of about 680 ha whereas in Tamil Nadu, the production was 53 lakh flowers from an area of 25 ha (Sudhagar, 2013). The controlled environmental conditions favor the plant growth, but at the same time, poor sanitation enhances the pest attack (Rajendran et al., 2014). The wilt causing pathogen and nematode are soil borne, and yield loss due to *Meloidogyne incognita* (Kofoid & White) Chitwood. was estimated as 31% (Nagesh and Parvatha Reddy, 2000 and Kishore, 2007), which is a problematic factor for growing gerbera in commercial poly houses. In addition, in and around Bangalore, 40 to 60% yield loss was recorded in exotic variety from Europe (Nagesh and Parvatha Reddy, 1996). In gerbera, predisposition of *M. incognita* increased the severity of *Fusarium oxysporum* and their interactions result in synergistic flower yield losses (Parvatha Reddy, 2014 and Sankari Meena et al., 2015) along with measurable change in host physiology and morphology (Sankari Meena et al., 2016). To manage this complex infection, the ideal biocontrol agent should continue to exist in rhizosphere. Hence, the well-known Gram-positive *Bacillus subtilis* Cohn. known to live in both rhizosphere and phyllosphere possibly an alternative to chemical nematicide and fungicide has been exploited for its biocontrol activities (Ramyabharathi et al., 2016). Lipopeptide biosurfactant antibiotics, *viz.*, iturin and surfactin, are considered as antimicrobial peptide molecules that can induce systemic resistance as well as strongly exhibit biocidal activity against *F. oxysporum* (Ramyabharathi and Raguchander, 2014) and exert lethal effect on root-knot nematodes (Sankari Meena et al., 2016a).

With this background information, the present study was undertaken to detect the lipopeptide biosurfactant molecules in *B. subtilis* and their effect on wilt-root-knot nematode complex in gerbera.

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Materials and methods

This study was undertaken at the Department of Plant Pathology and Department of Plant Nematology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Bacillus DNA isolation

Authenticated *B. subtilis* strain Bbv 57 (KF718836) was obtained from the culture collection center of the abovementioned department. Bbv 57 was grown in nutrient broth at 28 °C. Total DNA that included chromosomal and plasmid DNA was extracted (Robertson et al., 1990). Cultures grown for 18 h in nutrient broth were centrifuged into a pellet, washed in TE buffer (10 mM Tris pH 7.5/1 mM EDTA pH 8.0), and suspended in 10% sucrose. Cells were incubated at 37 °C in lysozyme solution (5 mg/ml lysozyme, 50 mM Tris pH 7.5, 10 mM EDTA pH 8.0), followed by the addition of 20% SDS containing 0.3% beta-mercaptoethanol. DNA was purified and quantified.

Identification using genus-specific primers

For *Bacillus* sp., confirmation 16S rRNA intervening sequence-specific BCF1 (5′-CGGGAGGCAGCAGTAGGGAAT-3′) and BCR2 (5′-CTCCCCAGGCGGAGTGCTTAAT-3′) primers were used to get an amplicon size of 546 bp (Cano et al., 1994). A 20-μl reaction mixture containing 10× buffer (with 2.5 mM $MgCl_2$, 2 μl; 2 mM dNTP mixture, 2 μl; 2 M primer, 5 μl; *Taq* DNA polymerase, 3 U; H_2O 8 μl and 50 ng of template DNA samples) were amplified on DNA thermal cycler using the PCR conditions 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min. The total number of cycles was 40 with the final extension time of 10 min. The PCR products were resolved on 2% agarose and sequenced.

Lipopeptide antibiotic biosynthesis genes iturin (*ItuD* gene) and surfactin (*srfA* gene; *sfp* gene) amplification

Iturin

ITUD F (5′-GATGCGATCTCCTTGGATGT-3′) forward and ITUD R (5′-ATCGTCATGTGCTGCTTGAG-3′) reverse primers were used for amplification of *ItuD* gene (1203 bp) (Ramarathnam, 2007). The 20-μl mixture contained approximately 50 ng of total DNA, 5 mM each of dNTPs, 20 pmol each of forward and reverse primers, and 0.5 U of *Taq* DNA polymerase. PCR amplification was performed in a thermocycler (Eppendorf Master cycler, German), using the following conditions: initial denaturation at 94 °C for 3 min, 30 cycles consisting of 94 °C for 1 min (denaturation), 50 °C for 1 min (annealing), 72 °C for 1 min 30s (primer extension), and final extension 72 °C for 10 min.

Surfactin

The forward primer SRFA-F1 (5′-AGAGCACATTGAGCGTTACAAA-3′) and reverse primer SRFA-R1 (5′-CAGCATCTCGTTCAACTTTCAC-3′) were used for amplification of *srfA* gene (626 bp) (Hsieh et al., 2004). The 20 μl PCR reaction mixture contained DNA template 50 ng, 1× Taq buffer, 0.2 mM each of dNTP mixture, 1 μM of each primer, 1.5 mM $MgCl_2$, and 2 U of *Taq* DNA polymerase. The PCR conditions are as follows: an initial denaturation at 95 °C for 15 min; 40 - cycles of 95 °C for 1 min, annealing at 62 °C for 1 min, and 72 °C extension for 1.5 min; and a final extension at 72 °C for 7 min.

The forward primer SFP F (5′-ATGAAGATTTACGGAATTTA-3′) and reverse primer SFP R (5′-TTCCGCCACTTTTTCAGTTT-3′) were used for amplification of *sfp* gene (675 bp) (Hsieh et al., 2004). The 20-μl PCR reaction mixture contained DNA template 50 ng, 1× Taq buffer, 0.2 mM each of dNTP mixture, 1 μM of each primer, 1.5 mM $MgCl_2$, and 2 U of *Taq* DNA polymerase. The PCR conditions are as follows: an initial denaturation at 95 °C for 15 min; 40 cycles of 95 °C for 1 min, annealing at 55 °C for 1 min, and 72 °C extension for 1.5 min; and a final extension at 72 °C for 7 min.

Mycolytic enzyme β-1,3-glucanase detection

The forward primer β-GLU F (5′-AATGGCGGTGTATTCCTTGACC-3′) and reverse primer β-GLU R (5′-GCGCGTAGTCACAGTCAAAGTT-3′) were used for amplification of glucanase gene (400 bp) encoding PR2 protein (Baysal et al., 2008). The 20-μl PCR mixture contained approximately 50 ng of total DNA, 5 mM each of dNTPs, 20 pmol of each forward primer and reverse primer, and 0.5 U of *Taq* DNA polymerase. The PCR conditions are as follows: an initial denaturation at 94 °C for 5 min; 40 cycles consisting of 92 °C for 1 min (denaturation), 34 °C for 2 min (annealing), and 72 °C for 2 min (primer extension); and final extension 72 °C for 2 min.

Thin-layer chromatography (TLC)

Extraction of lipopeptide biosurfactants

Cultures of *B. subtilis* Bbv 57 were grown separately in 20 ml of pigment production broth (peptone, 20 g; glycerol, 20 ml; NaCl, 5 g; KNO_3, 1 g; distilled water, 1 l; pH 7.2) for 4 days on a rotary shaker at 30 °C. The fermentation broth was centrifuged at 15,000 rpm for 30 min in a tabletop centrifuge, and the supernatant was collected. It was acidified to pH 2.0 with 1 N HCl and then extracted with an equal volume of ethyl acetate. The ethyl acetate extract was reduced to dryness in vacuo. The residues were dissolved in methanol and kept at 4 °C until used for TLC (Rosales et al., 1995).

Surfactin and iturin identification

For the identification of surfactin and iturin, a volume of 4 μl of sample was spotted on to the aluminum-coated

sheets with silica gel TLC plates. Separation was performed with chloroform/methanol/distilled water (8:1:1) as a solvent system for surfactin and iturin. After separation, the spots were visualized under short wavelength (245 nm). For surfactin, the spots were visualized after spraying with 0.1% ninhydrin ethanol solution. *Rf* values for the spots confirming surfactin and iturin were calculated.

In vitro bioassay against F. oxysporum

A 9-mm mycelial disc of the wilt pathogen *F. oxysporum* f. sp. *gerberae* (KM523669) was placed in the center of the Petri plate. Sterile Whatman No. 40 filter paper discs with 6 mm dia were placed 1 cm away from the edge at four sides centering on the fungal disc. Different increments (10–100 μl) of crude extract of *B. subtilis* Bbv 57 were dropped over the sterile filter paper discs. The plates were incubated at room temperature (28 ± 1 °C) and were scored when the mycelium grew over the control disc. Control was maintained with the sterile distilled water instead of crude extract.

In vitro bioassay against M. incognita

Hatching test

Crude antibiotic extract of *B. subtilis* Bbv 57 was taken at different concentrations of 25, 50, and 100% in a 50-mm Petri dish, and five egg masses of *M. incognita* were placed in each dish and incubated at 28 ± 1 °C. Egg masses placed in distilled water without crude antibiotic extract served as control. The experiment was replicated four times. Observation on the number of hatched juveniles was made after 24, 48, and 72 h of exposure.

Mortality test

Crude antibiotic extract of *B. subtilis* Bbv 57 at concentrations of 25, 50, and 100% were poured into separate Petri dishes. The second stage juveniles of *M. incognita* were introduced at the rate of 100 juveniles in each dish and incubated at 28 ± 1 °C. Juveniles placed in dishes containing distilled water served as control. Each treatment was replicated four times. Observations were recorded on the mortality of juveniles after 24, 48, and 72 h of exposure period, and percent mortality was calculated. The inactive nematodes were transferred separately from each dilution into sterile distilled water and kept overnight to check whether mortality was permanent or temporary.

Greenhouse studies

The liquid formulation of *B. subtilis* Bbv 57 was assessed for its efficacy against wilt-nematode disease complex in Gerbera cv. Palm Beach under greenhouse conditions. A pot culture study was undertaken, using the completely randomized design (CRD), with three replications. The different treatments are listed below:

T1 = Seedling dip in liquid formulation of *B. subtilis* (Bbv 57) at 500 ml/ha (3.2×10^9 cfu/ml)
T2 = Soil drenching with *B. subtilis* (Bbv 57) 1000 ml/ha, at monthly interval (once in every month)
T3 = Soil drenching with *B. subtilis* (Bbv 57)1000 ml/ha at bimonthly interval (once in 2 months)
T4 = Seedling dip + soil drenching with *B. subtilis* (Bbv 57) at monthly interval
T5 = Seedling dip + soil drenching with *B. subtilis* (Bbv 57) at bimonthly interval
T6 = Soil application of carbendazim 0.05% + carbofuran (1 kg ai/ha)
T7 = Control

Observations on plant growth parameters like shoot length (cm), fresh shoot weight (g), root length (cm), fresh root weight (g), stalk length (cm), and flower stalk girth (cm) were recorded at 240 days after planting (DAP). For yield parameters, total number of flowers per plant, flower diameter (cm), disc diameter (cm), total number of normal flowers, total number of bent neck flowers, and vase life (days) were recorded till 240 DAP. Soil population of root-knot nematode was recorded. In roots, a number of females per gram of root, egg mass per gram of root, gall index, and percent wilt incidence were recorded at 240 DAP.

Statistical analysis

The data were analyzed statistically using the IRRISTAT version 92 (Gomez and Gomez, 1984). The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at two significant levels ($P < 0.05$ and $P < 0.01$), and means were compared by Duncan's multiple range test (DMRT) (Duncan, 1955).

Results and discussion

PCR amplification of lipopeptide and β-1,3-glucanase

The results of PCR amplification confirmed that Bbv 57 strain belongs to the group of *Bacillus* with the amplicon size of 546 bp (Fig. 1). This strain showed the presence of *ItuD* gene with an amplified gene product of size 1203 bp. It also showed 626 and 675-bp products indicating the presence of *srfA* and *sfp* gene of surfactin, respectively. The presence of β-1,3-glucanase gene in Bbv 57 was confirmed by PCR product size of 400 bp (Fig. 2). Antibiotic producer, *B. subtilis*, plays a major role in plant disease suppression (Kinsella et al., 2009). Similarly, *B. subtilis* Bbv 57 was positive for iturin (ItuD gene) and surfactin (*srfA* gene; *sfp* gene) lipopeptides. Among the lipopeptides, iturin has a strong broad

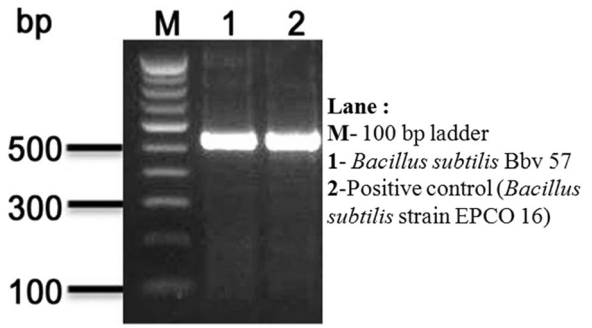

Fig. 1 PCR confirmation of *Bacillus* genus at 546 bp

spectrum antifungal activity. Several *Bacillus* strains have AMP biosynthetic genes like *ituC*, *srfAA*, and *srfAB* responsible for pathogen suppression (Gonzalez et al., 2010). The result is comparable with Mora et al. (2011) who reported the presence of AMP genes *srfA* (surfactin) and *ituC* (iturin) in 184 isolates of *Bacillus* spp. Cadena et al. (2008) reported that *B. amyloliquefaciens* strain FZB42 produced lipopeptide surfactin, bacillomycin D, and fengycins, which are secondary metabolites with mainly antifungal activity, also decreased gall formation, egg mass count, and juvenile counts of *M. incognita* extracted from roots of tomatoes. Similarly, in this study, the inhibition of *Fusarium oxysporum* f. sp. *gerberae* might be due to the production of AMP metabolites which are toxic to the pathogen. The presence of these diverse genes plays a crucial role in the biological control of root-knot nematode and *Fusarium* in gerbera both in greenhouse and in vitro through synergistic action of antimicrobial peptide genes. The presence of mycolytic enzyme, β-glucanase in *Bacillus*, is responsible for breaking down the fungal β-glucan that may be responsible for the rigidity of the fungal cell walls, thereby destroying cell wall integrity.

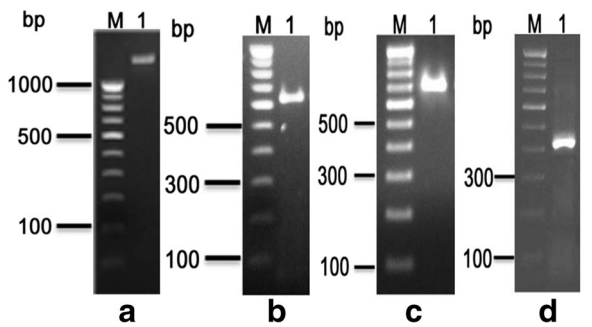

Fig. 2 PCR detection of lipopeptide biosurfactant and β-1,3-glucanase genes. **a** *ItuD* gene 1203 bp. **b** *srfA* gene 626 bp. **c** *sfp* gene 675 bp. **d** β-1,3-glucanase gene 400 bp. M 100 bp ladder. 1 Bbv 57

Identification of lipopeptide biosurfactants

Thin-layer chromatography results indicated that *B. subtilis* strain Bbv 57 produced surfactin and iturin, which was confirmed by the *Rf* values 0.3 for surfactin and 0.7 for iturin (Fig. 3) as that of standards used. In the TLC study, *B. subtilis* strain Bbv 57 produced both surfactin and iturin, which was confirmed by the *Rf* values as that of the standards, *viz*., iturin and surfactin, from Sigma-Aldrich. Mendizabl et al. (2012) identified iturin and surfactin lipopeptides from cell-free supernatants of *B. subtilis* strain CPA-8 by thin-layer chromatography, indicating that antibiosis could be a major factor involved in the biological control ability of CPA-8. Similarly, Ji et al. (2013) identified lipopeptide iturin in *B. amyloliquefaciens* strain CNU114001with *Rf* value of 0.41 using preparative silica gel TLC with chloroform/methanol (2:1 *v*/*v*) as mobile phase.

In vitro bioassay against *F. oxysporum*

B. subtilis strain Bbv 57 crude lipopeptide inhibited *Fusarium oxysporum* FOG 2 to an amount of 44.33 and 63.33% of control, at 10 and 100 μl of filtrate concentrations, respectively. There was no increase or decrease in the inhibition of mycelial growth of *Fusarium* with increase in the days of incubation either at lower (10 μl) or at higher (100 μl) concentrations of crude antibiotic (Fig. 4).

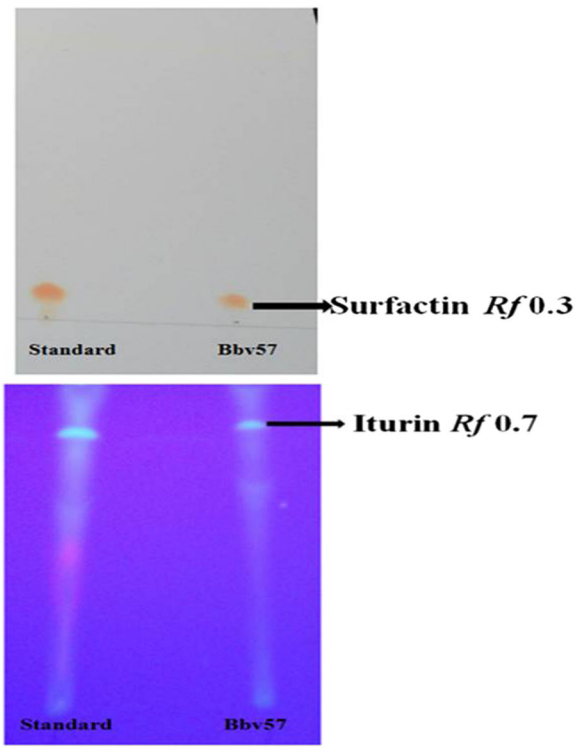

Fig. 3 Detection of iturin and surfactin in *B. subtilis* Bbv 57 through TLC

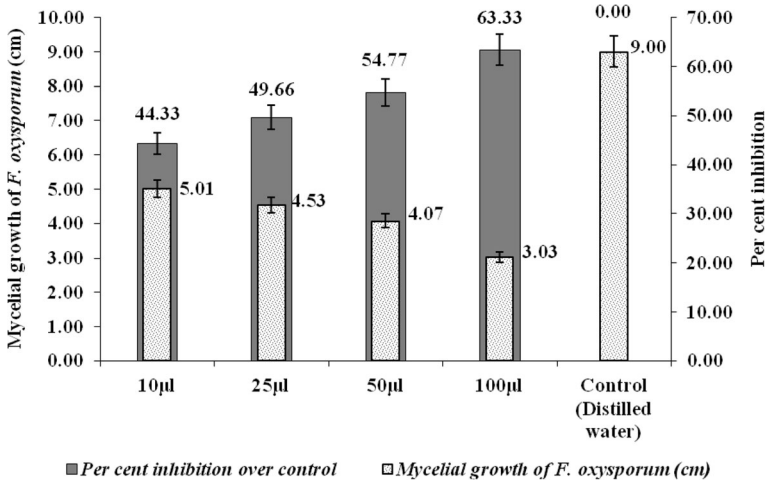

Fig. 4 Bioassay of crude antibiotic against *F. oxysporum* in vitro

Egg hatching and juvenile mortality of *M. incognita* in vitro

Eggs treated with *B. subtilis* strain Bbv 57 crude antibiotic showed poor egg hatching. A significant reduction in egg hatching (7.00 juveniles) was observed at 100% concentration after 72 h of exposure, compared to 98.33 juveniles in the control.

A gradual increase in juvenile mortality was observed with the increase in concentration of the crude antibiotic of Bbv 57 and with the increase in exposure time. The highest juvenile mortality (87%) was recorded in the 100% concentration of the crude antibiotic after 72 h of exposure (Fig. 5). *B. subtilis* association with the nematode galls and egg masses in root tissue was reported (Weller, 1988). Nematodes and their eggs also may have served as a food source for *Pochonia chlamydosporia*, *Pseudomonas fluorescens*, and *Bacillus subtilis.* The destructive effect of *Bacillus* species on *M. incognita* might be due to the production of lethal nematicidal antibiotic compounds and enzymes which directly affected the nematode reproduction and juvenile mortality (Gao et al., 2016).

The result is in accordance with Siddiqui et al. (2000) who reported the mortality and ovicidal activity of *M. javanica* using ethyl acetate and hexane fractions at different concentrations. Similarly, several authors have reported that cell-free culture filtrates from *Bacillus* species could be a major factor involved in the suppression of soil-borne plant pathogens in vitro (Salem et al., 2012). Hence, *B. subtilis* Bbv 57 crude antibiotic extracts proved a biocidal activity on *F. oxysporum* and *M. incognita* in a concentration-dependent manner.

Efficacy of biocontrol formulations on the reduction of fungal-nematode complex in gerbera under greenhouse condition

Growth parameters

The growth parameters were recorded at 8 months after planting. The treatment details are presented in Table 1.

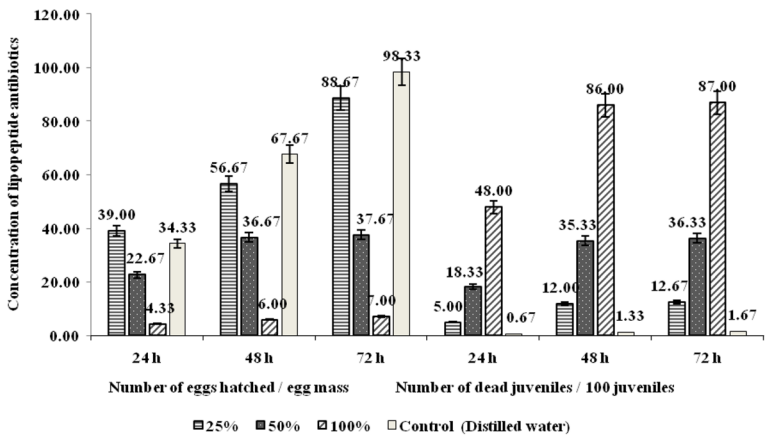

Fig. 5 Bioassay of crude antibiotic on egg hatching and juvenile mortality of *M. incognita*

Table 1 Effect of liquid formulation of *B. subtilis* (Bbv 57) on Gerbera cv. Palm Beach (yellow) under pot culture condition

Treatments	*Shoot length (cm)	*Root length (cm)	*Total no. of flowers/pl	*Stalk length (cm)	*Flower dia (cm)	*Disc dia (cm)	*Flower stalk girth (cm)	*Total no. of normal flowers/pl	*Total no. of bent neck flowers/pl	*Vase life (days)	Nematode population/250 cm^3 soil	Percent wilt incidence
T1	27.86^{c}	33.76^{d}	9.30^{e}	39.00^{d}	5.70^{e}	1.33^{d}	0.49^{b}	8.53^{e}	1.34^{a}	8.56^{c}	314.43^{e}	26.30^{e} (30.85)
T2	32.53^{b}	38.46^{c}	13.66^{d}	42.90^{c}	6.86^{d}	2.46^{b}	0.73^{a}	13.66^{d}	0.00^{b}	9.43^{ab}	285.01^{b}	16.73^{ab} (24.14)
T3	31.36^{b}	33.40^{d}	12.80^{d}	43.76^{bc}	6.53^{d}	1.73^{c}	0.76^{a}	12.80^{d}	0.00^{b}	9.06^{bc}	302.73^{d}	23.67^{d} (28.84)
T4	36.26^{a}	48.36^{a}	23.36^{a}	49.70^{a}	9.40^{a}	2.73^{a}	0.76^{a}	23.36^{a}	0.00^{b}	9.97^{a}	203.79^{a}	15.33^{a} (23.04)
T5	32.43^{b}	44.53^{b}	18.50^{b}	45.80^{bc}	8.00^{c}	2.73^{a}	0.49^{b}	18.50^{b}	1.32^{a}	8.86^{bc}	291.13^{c}	20.83^{c} (27.15)
T6	32.80^{b}	49.23^{a}	16.66^{c}	46.53^{b}	8.86^{b}	2.40^{b}	0.47^{b}	16.67^{c}	0.00^{b}	9.46^{ab}	281.73^{b}	18.36^{b} (25.36)
T7	25.23^{d}	34.43^{d}	7.90^{f}	36.33^{d}	6.73^{d}	1.76^{c}	0.37^{c}	7.90^{e}	0.00^{b}	6.73^{d}	715.01^{f}	56.63^{f} (48.81)
SE (d)	0.85	1.16	0.42	1.19	0.21	0.06	0.01	0.41	0.02	0.24	12.09	0.93

In a column, means followed by a common superscript letter are not significantly different at 5% level by DMRT. Values in the parentheses are arcsine transformed values
*Mean of three replications

T4 treatment gave maximum shoot length (36.26 cm) and root length (48.36 cm). This was followed by T6 treatment which recorded a shoot length of 32.80 cm and root length of 49.23 cm. T7 treatment recorded the minimum shoot length (25.23 cm) and root length (34.43 cm). In relation to the above statement, application of the liquid formulation of *B. subtilis* Bbv 57 at monthly interval (T4) increased the plant growth parameters in the greenhouse study.

Increase in the plant growth parameters in gerbera plants might be due to the root colonization of *Bacillus* species which improved mineral uptake and mineral release from the soil and organic matter and enhanced the production of plant growth hormones. Some bacteria make phosphorus as well as micronutrients more readily available for plant growth in some soils by solubilizing organic phosphate or inorganic phosphate in soil particles through the secretion of phosphatase or organic acids (Kloepper et al., 1989).

Yield parameters

The highest number of flowers (23.36 per plant) was recorded in T4 treatment, followed by T5 treatment which recorded 18.50 flowers per plant. The lowest number of flowers per plant (7.90) was recorded in control treatment (Table 1). Maximum flower diameter (9.40 cm) and disc diameter (2.73 cm) were observed in T4 treatment. The T6 treatment recorded flower diameter of 8.86 cm and disc diameter of 2.40 cm as against flower diameter of 6.73 cm and disc diameter of 1.76 cm in control treatment. The highest flower stalk length (49.70 cm) and stalk girth (0.76 cm) were recorded in T4 treatment. This was followed by T5 treatment which recorded stalk length of 45.80 cm and stalk girth of 0.49 cm. The lowest stalk length (36.33 cm) and stalk girth (0.37 cm) were recorded in control treatment.

Flower diameter and disc diameter in the gerbera treated with biocontrol agent at monthly interval were maximum than untreated control, indicating that the bacterial strain increased the flower size due to growth promotion and disease reduction. Production of surfactin is prevalent among *B. subtilis* and *B. amyloliquefaciens* which assists in cell attachment and detachment to surfaces during the formation of biofilm and in swarming motility (Raaijmakers et al., 2010). Similarly, in the present study, application of *Bacillus* species with antibiotic genes including surfactin might have helped in the attachment of all the strains of *Bacillus* and resulted in the multiplication of the bacteria in rhizosphere leading to the biofilm formation, thus suppressing the *F. oxysporum* and *M. incognita* in gerbera and increasing the plant growth promotion, flower yield, and soil health. Surfactin was also effective against *Pseudomonas syringae* pv. *tomato* and protects *Arabidopsis thaliana* against infection by the pathogen (Bais et al., 2004). The possible mechanism of action of *Bacillus* against nematode is not yet well defined.

Quality parameters

The highest number of normal flowers per plant (23.36) without any bent neck flowers per plant was observed in T4 treatment. In T5 treatment, 18.50 normal flowers per plant and 1.32 bent neck flowers per plant were observed. There were 16.67 normal flowers without bent neck flowers per plant in T6 treatment (Table 1).

The T4 treatment recorded the maximum vase life of 9.97 days, followed by T6 treatment with vase life of 9.46 days. The lowest vase life of 6.73 days for flowers was observed in control treatment.

Root population of nematode

All the treatments were found to reduce the female population in roots, and higher reduction was noticed in T4 treatment-treated root portion which recorded a reduced female population of 17.96 females per gram of root compared to control (Fig. 6). T6 treatment recorded 22.33 females per gram of root. The untreated root portion of the plants recorded significantly the highest

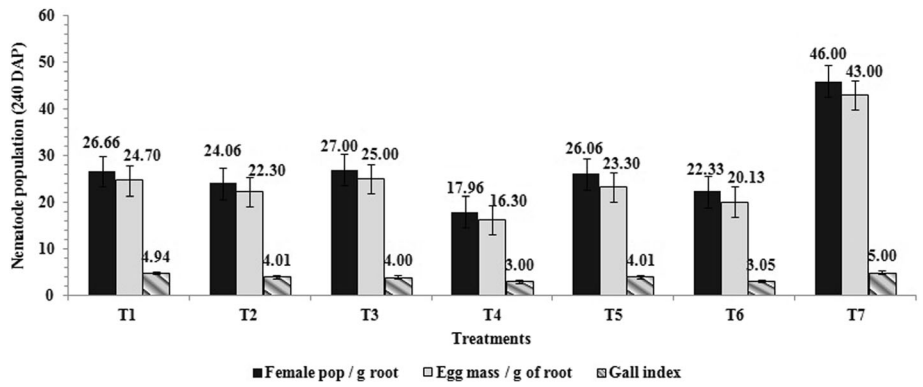

Fig. 6 Root population of root-knot nematode in gerbera under green house condition

nematode population of 46.00 females per gram of root at 240 DAP. Significant reduction in the number of egg mass (16.30 per gram of root) was observed in T4 treatment-treated root, followed by T6 treatment which recorded 20.13 egg masses per gram of root. The untreated root recorded significantly the highest number of egg masses of 43.00 per gram of root. The lowest gall index of 3.00 was observed in T4 treatment-treated root portion which was on a par with T6 treatment (3.05). Untreated plant root system showed significantly the highest gall index of 5.0.

Wilt incidence

Wilt incidence (15.33%) was lowest in T4 treatment. T6 treatment recorded 18.36%, whereas the control treatment recorded a maximum disease incidence of 56.63% (Table 1). The antibiotics and enzymes produced by *Bacillus* directly affected the nematode multiplication and juvenile mortality or made the roots less attractive and thus reduced nematode penetration which might have resulted in the reduction of nematode population. Bouizgarne (2013) attributed disease controlling efficacy of *Bacillus* spp. to their fast growing ability and a high rhizosphere colonization. This may reduce the feeding sites for root-knot nematodes.

Yedidia et al. (1999) reported that *Bacillus* spp. also induced systemic resistance mechanisms against pathogen and nematode. This is in confirmation with the present study that in gerbera plants also, *Bacillus* species provided the protection against *M. incognita* and *F. oxysporum*. Hence, identification for the presence of both iturin and surfactin in strain Bbv 57 in this study may help in the better management of gerbera *Fusarium* nematode complex under in vitro and in protected cultivation.

Conclusions

To conclude the *B. subtilis* strain Bbv 57 showed strong antibiotic production under in vitro and managed the Fusarium - root knot nematode complex in gerbera under protected cultivation.

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Authors' contributions
All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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