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Rhizospheric biological weapons for growth enhancement and *Meloidogyne incognita* management in *Withania somnifera* cv. Poshita

Shilpi K. Saikia, Sudeep Tiwari, Rakesh Pandey *

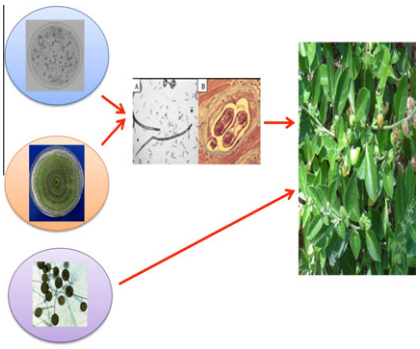
Microbial Technology and Nematology, CSIR-Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow 226015, India

HIGHLIGHTS

- *Withania somnifera* roots are economically important for its withanolide content.
- *Meloidogyne incognita* is a prime concern causing serious root damage.
- Rhizospheric microbes promote plant growth.
- Some microbes possess nematicidal potentials; helpful in *M. incognita* management.

GRAPHICAL ABSTRACT

Rhizospheric biological weapons for growth enhancement and *Meloidogyne incognita* management in *Withania somnifera* cv. Poshita.



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ABSTRACT

Withania somnifera L. (Family Solanaceae) is an angiospermic medicinal herb, well recognized for the immense therapeutic potentials of its roots containing several withanolides. *W. somnifera* is also a susceptible host for southern root knot nematode, *Meloidogyne incognita*. The nematode infestation in roots causes serious crop losses in terms of yield and chemo-pharmaceutical quality of this medicinal herb. In the present study, influence of five rhizospheric microbes, namely *Bacillus megaterium* (ATCC No. 14581), *Pseudomonas fluorescens* (ATCC No. 13525), *Trichoderma viride* (MTCC No. 167), *Paecilomyces lilacinus* (PDBC PL55) and *Glomus intraradices* was studied for the management of *M. incognita* in *W. somnifera* cv. Poshita under greenhouse conditions. All rhizospheric microbes, except *G. intraradices*, displayed nematicidal potentials via ovicidal and larvicidal actions *in vitro* and, resulted in significant improvement in plant growth parameters. The rate of nematode damage to *W. somnifera* was directly proportional to *M. incognita* (number of J2) population.

1. Introduction

Withania somnifera L. commonly known as Ashwagandha or Indian ginseng is a valuable medicinal herb from family Solanaceae. The plant has a long documented history of usage as "Rasayana" drug in Ayurveda with strong anti-inflammatory (Rasool et al., 2000), antioxidant (Panda and Kar, 1997), immunomodulatory (Rai et al., 2003; Archana and Namasivayam, 1999), anti-cancerous (Devi et al., 1992; Mohan et al., 2004), anti-stress and adaptogenic (Bhattacharya et al., 2002) properties. Besides, the plant is also known to possess rejuvenating action for treating several neurodegenerative (Bhattacharya et al., 1987; Naidu et al., 2003; Ahmad et al., 2005; Dhuley, 2001; Chaudhary et al., 2003; Jain et al.,

* Corresponding author.
E-mail addresses: shilpisaikia@yahoo.co.in (S.K. Saikia), sudeep_tiwari@ymail.com (S. Tiwari), r.pandey@cimap.res.in (R. Pandey).

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2001), endocrine (Schliebs et al., 1997; Panda and Kar, 1998), cardiovascular stimulatory activities (Mishra et al., 2000; Mohanty et al., 2004). Most of the therapeutic actions of *W. somnifera* are attributed to the presence of several steroidal lactones (withanolides, withaferins) and acyl steryl glycosides (sitoindosides VII–X) in its roots, leaves and berries (Bhattacharya et al., 1997; Kulkarni et al., 1998; Tohda et al., 2005).

W. somnifera is able to thrive in most of the soils under varied climatic conditions. In India, the plant is widely cultivated over an area of 10,780 ha in the sub-tropical and semi-temperate regions of Uttar Pradesh, Rajasthan and Andhra Pradesh (Kothari et al., 2003). However, against the annual requirement of nearly 7000 t for *W. somnifera* roots, India has an approximate production of 2000 t only (Patra et al., 2004). Due to increasing demand for its roots, growth promotion and resistance against diseases is a major obligation for *W. somnifera* cultivation. The infestation of root-knot nematode, *Meloidogyne incognita* (Kofoid & White) Chitwood is a major yield constraint and visible threat to *W. somnifera* cultivation (Sharma and Pandey, 2009; Gupta et al., 2004). *M. incognita* infection causes several biochemical changes such as alterations in the levels of amino acids (Sikora and Schuster, 2000), organic acids (Freire and Bridge, 1985) and reduced chlorophyll content (Ferraz et al., 1989) in the plants. Heavy infestation causes stunting, reduced tillering, yellowing, premature drying of leaf tips and margins, narrowing of leaf blades, delay in flowering, immature fruit drop, excessive root branching and reduction in the unit biomass yield of roots, leaves and their bioactive constituents (Kingland, 2001; Pandey et al., 2003).

India, the diversified land of crops and climate still relies mainly on synthetic nematicides viz. halogenated aliphatic hydrocarbons (e.g., 1,3-dichloropropene), methyl isothiocynate, oxamyl, thionazin and carbofuran for nematode management. These chemical nematicides besides being costly are not so ecofreindly and poses potential risk to non-target organisms as has been emphasized under 'Food and Environment Protection act, 1985, Part III. Generalizations on the dosage of nematicides are not possible due to their dependence upon the live standard. However, field application of aldicarb or carbofuran at 1.5 kg/ha has been found to reduce *M. incognita* populations effectively (Jagdale et al., 2000). Therefore, search for more benign and acceptable biological control measures is a global research priority today (Kalra et al., 1996; Akhtar and Malik, 2000; Jagdale et al., 2000; Atta and Saad, 2001; Sharon et al., 2001). Rhizospheric microbes are one of the most promising groups of microorganisms for plant growth promotion, playing a crucial role in soil health and plant growth by a plethora of mechanisms in variety of crop plants (Siddiqui et al., 2005; Lee et al., 2011). Besides, they also serve as ideal biocontrol agents since they share the rhizosphere with their host plants, providing frontline defence for roots against pathogen attack. Among the microorganisms that parasitize/reduce nematode populations by antagonistic behavior, rhizospheric microbes hold an important position where some of them have shown great potential as biocontrol agents (Akhtar et al., 2012).

Although encouraging success has been obtained in efforts to develop high yielding cultivars, no cultivar of *W. somnifera* resistant to *M. incognita* is yet available. Therefore, the management of plant parasitic nematodes on this crop relies mainly on application of high rates of the nematicides viz. carbofuran. However, the decreasing efficacies of the chemical nematicides as well as risks associated with them have highlighted the need for a more effective and safer alternative control measures. Thus, the present study was designed to determine the effect of the rhizospheric microbes viz. *Bacillus megaterium* ATCC No. 14581, *Pseudomonas fluorescens* ATCC No. 13525, *Paecilomyces lilacinus* PDBC PL55, *Trichoderma viride* MTCC No. 167 and *Glomus intraradices* on the growth of *W. somnifera* cv. Poshita under greenhouse conditions in order to find eco-friendly and cost-effective treatments for the management of *M. incognita*. The nematicide carbofuran was included as a positive control since it is a popular treatment in *W. somnifera* cultivation.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of a high yielding cultivar of *W. somnifera* cv. Poshita were obtained from the National Gene Bank for Medicinal and Aromatic Plants at the Central Institute of Medicinal and Aromatic Plants (CSIR-CIMAP), Lucknow, India. The seeds were surface sterilized by soaking in 10% (v/v) sodium hypochlorite solution for 5 min, washed with distilled water and soaked for 4 h. After sterilization and soaking, healthy looking uniform sized seeds were sown in content plug plates filled with sterilized soil. Twenty-one days after sowing, healthy four leafed stage seedlings were transplanted into 7.0 kg soil capacity clay pots containing a mixture of autoclaved soil (76% sand, 8% silt, 16% clay, pH 7.7) and composted farm manure in 5:1 ratio.

2.2. Culture and maintenance of biocontrol agents

B. megaterium (ATCC No. 14581), *P. fluorescens* (ATCC No. 13525), *T. viride* (MTCC No. 167), *P. lilacinus* (PDBC PL55) and *G. intraradices* (Arbuscular mycorrhizal fungi) are regularly maintained in the Microbial Technology and Nematology Division, CSIR-CIMAP, Lucknow. The fungal biocontrol agents (BCA) were cultured using sand maize media while the bacterial isolates were cultured in Luria broth. For multiplication, the fungal cultures were kept in a BOD incubator for 96 h at 30 ± 1 °C and the bacterial cultures were placed on a rotary shaker at 28 ± 1 °C for 48 h at 200 rpm orbital shaking conditions. The fungal inoculants were mass multiplied in the previously mentioned media and after incubation, the fungal mycelial mat with conidia was homogenized and suspended in 500 mL of 0.1 M phosphate buffer (K_2HPO_4; KH_2PO_4) at 1.2×10^6 colony forming units (CFU) mL^{-1}. The bacterial cultures, *B. megaterium* (ATCC No. 14581), *P. fluorescens* (ATCC No. 13525), as luria broth suspensions were centrifuged at 6000*g* for 10 min. The supernatant was discarded and the pellet containing bacterial cells was suspended in 500 mL of 0.9% saline to a final density of 2.5×10^8 (CFU) mL^{-1} for *B. megaterium* and 1.8×10^8 (CFU) mL^{-1} for *P. fluorescens*.

G. intraradices inoculum was propagated on maize roots (*Zea mays*) for 10 weeks in a 1:1 (v/v) mixture of sterilized sand and soil (5 kg) of low phosphorus content (7.5 kg ha^{-1}) and subsequently left to shade-dry for 2 weeks. The inoculum was based on root fragments colonized (70%) with *G. intraradices* and the sand–soil fraction with AM fungus propagules (spores and mycelium) from dry maize pot culture. The roots in the pot culture were extracted from the soil, cut into 1 cm segments and thoroughly mixed with the sand–soil mix from the pot culture and stored at 5 °C until use. The inoculums potential (potential of a specific amount of inoculum to cause root infection under a standard set of conditions) of *G. intraradices* used in this study was 8.9 ± 1.3 infecting propagules g^{-1} sand–soil mixture. Bacterial agents were inoculated as 10 mL of 10^8 CFU/pot and fungal isolates as 10 mL of 10^6 CFU/pot. Inoculums of arbuscular mycorrhizal fungi, *G. intraradices* used in the present experiment consisted of soil containing spores (8–10 spores g^{-1}) and colonized roots of maize (*Zea mays* L.).

2.3. Nematode isolation

The population of *M. incognita* was maintained on brinjal (*Solanum melongena* L.) grown in sterilized loamy-sand soil in

earthen pots under greenhouse conditions. Mature egg masses from the roots were handpicked and allowed to hatch in distilled water for 48 h at 28 °C and then observed for survival under Leica stereoscopic microscope (Leica Microsystems AG, Germany). Total nematode counts in 1 mL of J2 suspensions were recorded in three independent aliquots and 1000 J2/pot were inoculated for all the treatments.

2.4. Preparation of cell free extract from rhizospheric microbes

A single colony/1 mm plug of rhizobacterial strains was cultured in 10 ml of nutrient broth, incubated at 28 °C on a rotary shaker at 150 rpm for 48 h. The cells were harvested by centrifugation at 10,000 rpm for 10 min and the supernatant was sterilized through 0.22 μ filters. This was designated as undiluted standard cell free extract of 100% concentration that was diluted with sterilized water to 50 and 25% respectively to study their effect on *M. incognita* (Nicknam and Dhawan, 2001).

2.5. In vitro egg hatch (Ovicidal) test/assay

Ovicidal action of biocontrol treatments was assessed by slightly modified procedure of Su and Mulla (1998). A known number of eggs (50) were carefully transferred into each well of 24-well microliter plate. In test wells, 500 μL of cell free culture filtrate of rhizospheric strains of 100, 50 and 25% concentrations was added. Treatment with distilled water was used as negative control. Three replicates for each treatment were maintained for the accuracy of the results. The plates were incubated under humidified condition at ambient temperature (27 °C) for 120 h. A drop of Lugol's iodine solution was added to each well to stop further hatching, and all the unhatched eggs in each well were counted microscopically. The hatch rates were assessed by the following formula.Percentage of egg mortality = no. of hatched eggs/total no. of eggs × 100

2.6. In vitro mortality test of J2 (Larvicidal assay)

Freshly hatched J2's (50 J2/100 μL) in distilled water suspension were added into each well of 24-well microtitre plate containing 400 μL of each of the rhizospheric strains cell free filtrates of 25, 50 or 100% concentrations (three replications per treatment). The plates were then incubated under humidified conditions at ambient temperature for 48 h. J2's populations were monitored microscopically after 12, 24 and 48 h exposure period and percentage mortality was calculated.

2.7. Biocontrol Treatments

The experiments were carried out on a complete randomized design with three replications. The various biocontrol treatments are described in Table 1. Pot experiments were carried out in winter from January to April, 2012 (mean day length 10.42–12.67 h; mean daily maximum temperature 17.9–21.8 °C; mean daily minimum temperature 6.7–12.2 °C). The bio-control agents were applied three days before *M. incognita* inoculation. One week after transplantation, each plant was inoculated with 1000 freshly hatched J2 per pot. In addition, the following three treatments were maintained as controls: untreated-uninoculated, -inoculated, and carbofuran at 2 kg a.i. ha^{-1} (0.0020 g a.i./kg soil) treated-inoculated. Plants were maintained in a greenhouse at 30 ± 6 °C and 13 h day length until the experiment was terminated 90 days after inoculation. Plant growth was determined by measuring root length, shoot height, fresh and dry weight of root and shoot. Root and shoot dry weights were determined after drying in a hot air oven at 60 °C. Roots were rated for galling severity according to Krusberg and Nielson (1958) on a scale of 0–4.

2.8. Effect of M. incognita and rhizospheric strains on W. somnifera growth

For assessing *M. incognita* population in the infested roots, root samples were processed by a modified maceration method (McSorley, 1999). Briefly, the roots were washed free of soil and chopped in 1 cm pieces by scissors. The chopped root were immersed in sufficient amount of water and macerated by a waring blender for 1–2 min. Water was added to this suspension and 1 mL aliquot was taken by micropipette for *M. incognita* counting at 400× magnifications using a compound microscope. The number of *M. incognita* were determined from mean of three counts was taken in each case. Sieves used in nematode extraction were U.S. standard sieve series of 100, 200 and 325 meshes with openings of 149, 74 and 44 μ, respectively.

For getting the actual frequency and relative density of *M. incognita* in soil, 250 g soil sample from each of the replicative pots of all the treatments was collected. After mixing the soil properly, *M. incognita* from a composite subsample of 250 g soil were extracted by means of modified Cobb's sieving and decanting technique followed by Baermann funnel method (Southey, 1986). The suspension was passed through nested sieves of 149, 74 and 44 μ mesh sieves. Final volume of each of the samples was made up to 50 mL and nematode numbers were counted in a 1 mL capacity counting slide under stereoscopic binocular microscope and mean of three independent counts were taken in each case. Reproduction factor (RF) was calculated by the formula, Rf = Pf/Pi, where Pf = final *M. incognita* population and Pi = initial *M. incognita* population. The disease incidence was measured on the basis of nematode population per root system expressed as root knot index (RKI). The RKI was determined by counting the number of galls on the infested roots on a scale of 0–4: 0 = 0; 1 = 1–25; 2 = 26–50; 3 = 51–75; 4 = 76–100 (Krusberg and Nielsen, 1958).

2.9. Statistical analysis

Analysis of variance (ANOVA) techniques were used for the statistical analysis of data. To compare various treatments, Duncan multiple tests applicable to RCBD were performed by ASSISTAT software version 7.6 beta. Least Significant difference (LSD) was calculated at 5% probability level ($P = 0.05$) to compare treatment means for significance of difference between any two variables.

3. Results

3.1. Ovicidal and Larvicidal assay results

The nematicidal potentials of rhizospheric microbes as evaluated by *in vitro* egg hatch assay for *M. incognita* are shown in Fig. 1. It was observed that all the rhizospheric isolates were able to reduce the hatching of *M. incognita* eggs. The highest ovicidal potentials were shown by *P. lilacinus* allowing only less than 17 eggs to hatch at 100% cell extract concentration. It was observed that the isolates were able to control egg hatch at all the tested cell extract concentrations with 100% concentration showing the maximum activity.

The effect of rhizospheric microbes on percentage egg mortality is depicted in Fig. 2. The results clearly indicate a significant inhibition (mortality) as compared to distilled water control treatment.

Table 2 summarizes the larvicidal potentials of rhizospheric microbes on the J2's at different concentrations. Hatching of *M. incognita* juveniles decreased with an increase in concentration of microbial strains with a maximum percentage mortality observed for *P. lilacinus*.

Table 1
Experimental details (different treatments) for management of root-knot nematode *M. incognita* in *W. somnifera* cv. Poshita.

S. No.	Treatments	Details
1	Un-control	Without any microbial treatment and *M. incognita* involvement
2	Un-in. control*	Without any microbial treatment but having *M. incognita* association
3	Carbofuran + MI*	Chemical nematicide along with *M. incognita* inoculation
4	BM + MI*	*Bacillus megaterium* and *M. incognita* inoculation
5	PF + MI*	*Pseudomonas fluorescens* and *M. incognita* inoculation
6	TV + MI*	*Trichoderma viride* and *M. incognita* inoculation
7	PL + MI*	*Paecilomyces lilacinus* and *M. incognita* inoculation
8	GI + MI*	*Glomus intraradices* and *M. incognita* inoculation

* Each pot was inoculated with 1000 J2-juveniles of *M. incognita*.

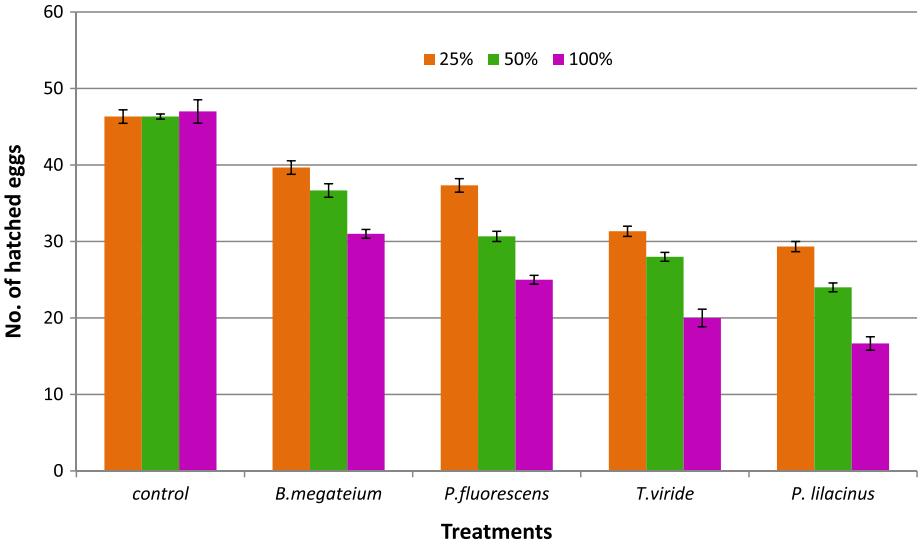

Fig. 1. Ovicidal effect of different cell extracts of rhizospheric microbes on *M. incognita* (*in vitro*).

3.2. Effect of M. incognita (J2) on W. somnifera growth

M. incognita being a sedentary plant parasitic nematode causes a severe loss to the host plants and the severity increases with an increase in inoculums density as is represented in Fig. 3. Dry weight parameters provide a quick glance to the damage ratio incurred due to increased inoculum density of the parasite. The results also suggest that reduction in plant dry biomass accumulation corroborated with damage ratio due to increased inoculum density of the parasite. The rate of growth inhibition

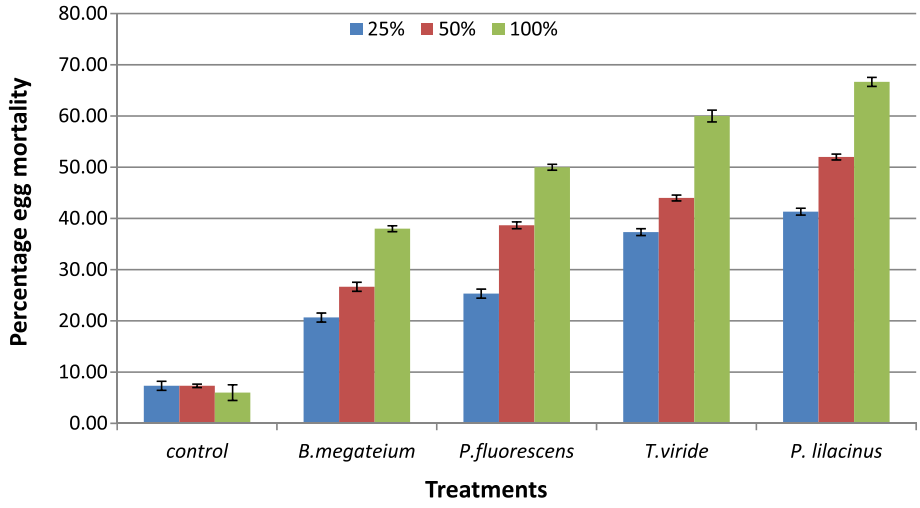

Fig. 2. Effect of different cell extracts rhizospheric microbes on percentage egg mortality of *M. incognita* (*in vitro*).

Table 2
Effect of different microbial cell extracts for their Larvicidal effects on J2's of *M. incognita*.

Treatments/ incubation time	25%			50%			100%		
	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h
Control	50.00 ± 0.0 (100)	49.00 ± 0.3 (100)	48.33 ± 0.3 (100)	50.00 ± 0.0 (100)	49.00 ± 0.6 (100)	48.33 ± 0.3 (100)	50.00 ± 0.0 (100)	49.00 ± 0.6 (100)	48.33 ± 0.3 (100)
B. megaterium	41.67 ± 0.33 (83.33)	39.00 ± 0.58 (79.59)	35.67 ± 0.33 (73.79)	37.00 ± 0.58 (74.00)	32.00 ± 0.58 (65.31)	25.67 ± 0.33 (53.10)	31.00 ± 0.58 (62.00)	25.33 ± 0.33 (51.70)	21.00 ± 0.58 (43.45)
P. fluorescens	39 ± 0.58 (78)	36.33 ± 0.88 (74.15)	31.33 ± 0.88 (64.83)	34.00 ± 0.58 (68.00)	30.00 ± 0.58 (61.22)	24.67 ± 0.33 (51.03)	31.00 ± 0.58 (62.00)	24.67 ± 0.88 (50.34)	18.67 ± 0.33 (38.62)
T. viride	35.67 ± 0.88 (71.33)	31.00 ± 0.58 (63.27)	26.67 ± 0.88 (55.17)	30.67 ± 0.33 (61.33)	26.33 ± 0.33 (53.74)	21.33 ± 0.33 (44.14)	24.67 ± 0.33 (49.33)	20.00 ± 0.58 (40.82)	15.00 ± 0.58 (31.03)
P. lilacinus	33.00 ± 0.58 (66)	26.33 ± 0.33 (53.74)	20.00 ± 0.58 (41.38)	24.00 ± 0.58 (48.00)	18.67 ± 0.33 (38.10)	13.00 ± 0.58 (26.90)	19.67 ± 0.33 (39.33)	15.00 ± 0.58 (30.61)	9.00 ± 0.58 (18.62)

J2-juveniles (mean values) with ± standard error (SE) are indicated.
Values in parentheses indicate the percentage mortality against distilled water control.

was also found to be in accordance with population density of J2's in the roots. The results emphasize that the rate of damage is directly proportional to the number of J2.

The multiplication of *M. incognita* inside the host roots as assessed by counting the number of galls, measured as RKI gave a good estimation of disease caused by *M. incognita*. The expression of *M. incognita* multiplication was based on the method given by Krusberg and Nielson (1958). RKI data also supports the damage due to *M. incognita* at higher densities. Treatments with *P. fluorescens* exhibited RKI of 1.7 (less than 50 galls), comparable to carbafuran treatment (Fig. 4). However, treatments consisting of *B. megaterium*, *T. viride* and *P. lilacinus* were more effective in controlling *M. incognita* population having an RKI of 1.3 (less than 25 galls). The plants having *G. intraradices* treatment showed a little higher RKI value (2.0; 50 galls/plant), yet it was much less than untreated- *M. incognita* inoculated control, RKI = 2.7 (more than 50 galls).

In terms of the reproduction factor (Rf) treatment involving *T. viride* as the biocontrol agent was found most effective resulting in least final population count of the parasite (Pf = 2620). *P. lilacinus* treatment, on other hand, resulted in a maximum Rf of 3.89 that was still much less than in the untreated-inoculated control (Fig. 5). Except for the treatment having *P. lilacinus*, all other microbial treatments resulted in a lower Rf value as compared to carbofuran treatment (Rf = 2.653) that clearly indicated their better efficacy in controlling *M. incognita* populations in *W. somnifera* roots.

The results clearly show that in the treatments of rhizospheric bacteria, Rf was similar to that of chemical nematicide, carbofuran but in combined treatments, Rf was drastically reduced proving the efficacy of microbial agents.

3.3. Growth parameters results

The overall plant growth inhibition data due to nematode population build up in infected plants is summarized in Fig. 6. The nematode significantly reduced plant growth (22.7 cm, shoot length and 11.3 cm, root length) in comparison to control plants (34 cm, shoot length and 12.3 cm, root length), leaf surface area (9.3 mm) against 12.3 mm of control plants besides other growth parameters like

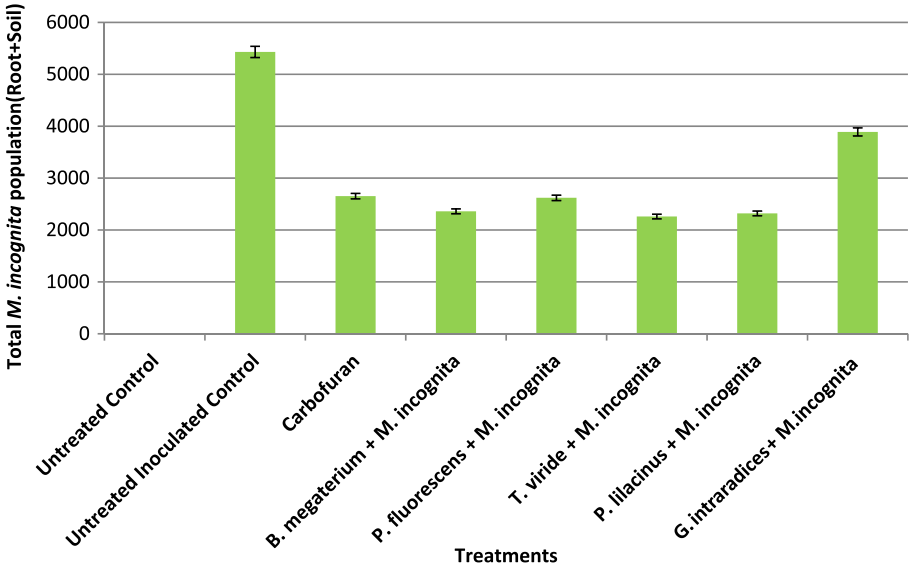

Fig. 3. Effect of different bioagents on total root-knot nematode (*M. incognita*) population in *W. somnifera*.

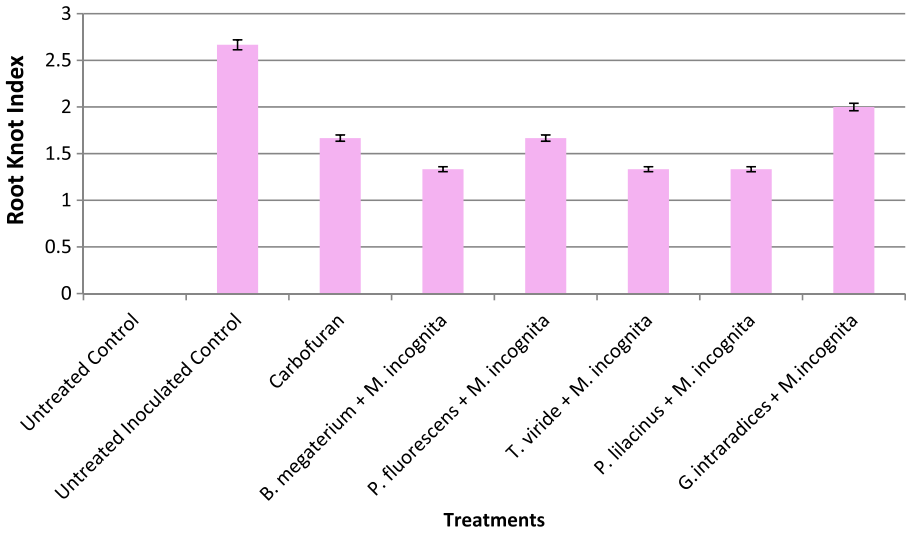

Fig. 4. Effect of different bioagents on root-knot index (RKI) of *M. incognita* in *W. somnifera*.

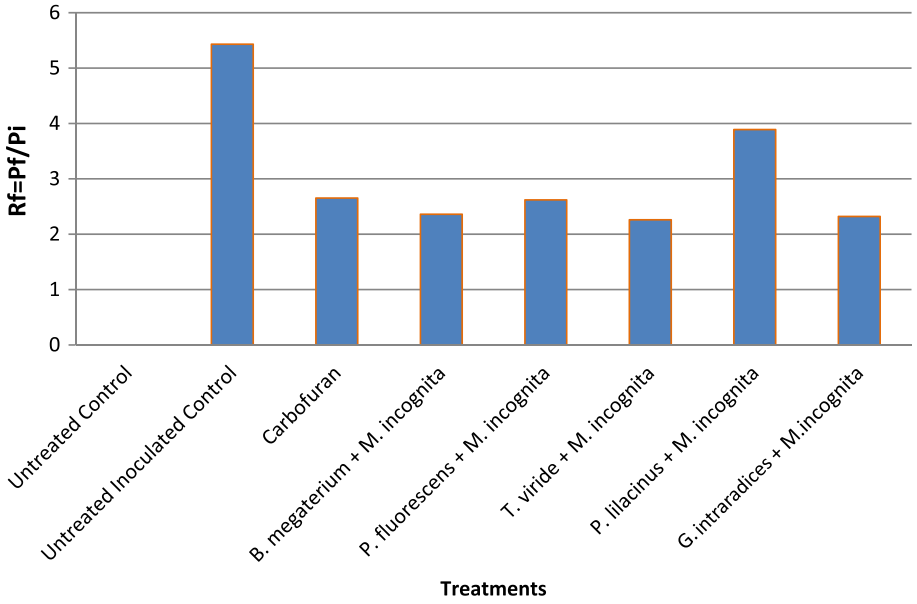

Fig. 5. Effect of microbes on Reproduction factor (Rf) of *M. incognita* in *W. somnifera*.

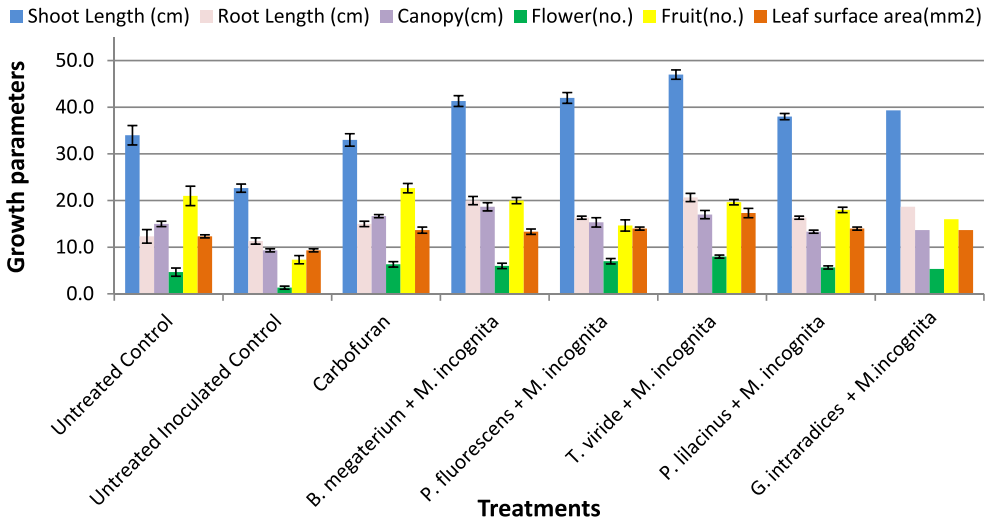

Fig. 6. Effect of rhizospheric microbes on plant growth parameters in *W. somnifera*.

Table 3
Statistical analysis for root and shoot fresh and dry weight in *W. somnifera* cv. Poshita.

Treatments	Shoot fresh wt. (g)	Root fresh wt. (g)	Shoot dry wt. (g)	Root dry wt. (g)
Un-control	31.33^{d}	5.87^{c}	5.37^{b}	2.17^{d}
Un-in. control*	18.33^{e}	4.23^{d}	3.67^{c}	1.13^{e}
Carbofuran*	34.6^{c}	7.03^{b}	5.72^{b}	3.01^{b}
BM + MI*	38.67^{b}	7.77^{b}	5.82^{b}	$2.71^{b,c}$
PF + MI*	37.67^{bc}	7.07^{b}	5.76^{b}	2.54^{c}
TV + MI*	42.67^{a}	9.93^{a}	8.50^{a}	3.90^{a}
PL + MI*	$37.33^{b,c}$	7.47^{b}	5.33^{b}	2.93^{b}
GI + MI*	35.33^{c}	7.67^{b}	5.47^{b}	$2.67^{b,c}$
CV%	4.77	7.46	5.17	7.62
LSD	2.85	0.92	0.51	0.35

[a–e] Mean in each column followed by same letters do not differ significantly ($P \leqslant 0.05$) according to Duncan's multiple range test.
* Each pot was inoculated with 1000 J2-juveniles of *M. incognita*.

plant canopy, number of flowers and fruits etc. (Fig. 6). ANOVA results for biomass depicted by root and shoot fresh and dry weight in *W. somnifera* cv. Poshita revealed significant mean differences against control (Table 3). The tests performed included Fisher's least significant difference (LSD) at $P = 0.05$ and Duncan's test for comparing control with treatment means (Zar, 1999). CV% value

for fresh weight (shoot) was 4.77 while it was 5.17 for shoot dry weight among various treatments. LSD value of 0.05 for percentage root weight, both fresh (0.92) and dry (0.35), indicated a highly significant result for various treatments. The Duncan test at 5% probability was applied to the treatments. Mean values for treatments followed by the same letter do not differ statistically between themselves. All the treatments were significantly effective in growth promotion The ANOVA results for fresh and dry shoot and root weight are represented in Table 4. The results significantly correlate the biocontrol agents, effective as growth promoting also.

The application of the various treatments significantly increased plant growth parameters of *W. somnifera* cv. Poshita and suppressed nematode soil population and root gall index when compared to non-treated inoculated plants. The treatments, *T. viride*, *B. megaterium* and *P. fluorescens* significantly ($P < 0.01$) increased plant growth, and were found to be effective for growth promotion. The results show that microbial agents completely counteract the negative impact of the nematode and reduce nematode population and root knot index, and were equally effective to carbofuran treatment.

4. Discussion

Buhrer (1938) first reported root knot nematodes (*Meloidogyne sp.*) on *Mentha arvensis*. The intricate association of plant parasitic nematodes causes significant damage to almost all the medicinal plants studied till date (Saikia et al., 2012). The magnitude of crop damage due to nematode–plant interactions has established *M. incognita* as the most important nematode parasites limiting production, with infestations reported on *Mentha arvensis*, *Ocimum sanctum*, *Papaver somniferum*, *Bacopa monnieri*, *Withania somnifera* and *Artemesia annua* (Pandey et al., 2009; Sikora et al., 2003). Gradual declines characterized by unthrifty growth and yellowing of leaves due to reduced the uptake of nutrients such as P, K, Zn, Mn and Cu are the prominent symptoms of *M. incognita* (Ferraz et al., 1988). The complete eradication of *M. incognita* from agricultural soils is nearly impossible due to the polyphagus nature of this pest. Inhibiting egg hatching in *M. incognita* is helpful in reducing its populations in soil and roots (Meyer et al., 2004). The root knot infested plants are stunted with smaller and chlorotic leaves, fewer rootlets and root-hairs and the roots bear small knots/galls. There are several earlier reports indicating an inverse relationship between nematode population and total foliage yield (Rhoades, 1984).

W. somnifera is economically important for its bioactive phytomolecules present in its roots, which are a major source of numerous alkaloids (sominiferine, somnine, withanine, tropine, isopelletierine, cuscohygrine, anaferine, anahygrine, visamine, etc.) and withanollides, a group of naturally occurring oxygenated ergostane-type steroids. *W. somnifera* is reported to be a highly susceptible host for *M. incognita* race 2 (Pandey et al., 2009). It has been demonstrated that application of carbofuran or aldicarb at 5 kg a.i./ha three times, every 3 months, results in increased growth and vigor of seedlings in both primary and secondary nurseries (Koshy, 1987; Ali, 1986, 1987; Eapen, 1995). Since these nematicides do not kill but only inactivate nematodes, repeated use is necessary to ensure good yield. However, the potential side effects associated with these synthetic treatments restricts their enormous usage. The use of antagonistic microorganisms can be considered suitable for nematode management programs, but still need advanced field testing (Pandey et al., 2003).

Rhizospheric microorganisms prove to be a potential source of novel natural products for exploitation in agriculture and are recognized as a promising group in terms of diversity and pharmaceutical potential (Masadeh et al., 2004). The reduction of inoculum density or disease producing activities of a pathogen by one or more microorganisms, termed as biocontrol is achieved by competition, hyperparasitism, induced resistance etc. Mycoparasitism and production of volatile and non-volatile antibiotics are important mechanisms operating in the case of *Trichoderma sp*. At CIMAP, earlier our group has identified a strain of *T. harzianum* that has been used to produce a disease-free, healthy nursery with a reduction in root-knot nematode infection and improving the growth and yields of *M. arvensis* (Kalra et al., 1996). With the growing public concern regarding environmental pollution and the increase in the costs of pesticides, efforts to develop non-chemical disease management strategies are indicated.

The pot experiment results demonstrated the nematicidal potential of the biocontrol agents tested, especially *P. fluorescens* and *B. megaterium*, which resulted in increased plant growth and yield of *W. somnifera*. Several mechanisms may be involved in the suppression of nematode soil populations and enhancement of plant growth and yield. Microbial extracts often contain phenolic compounds, organic acids and secondary metabolites (Spencer et al., 2003) which have been demonstrated to have nematicidal activity (Shaukat et al., 2004). This study revealed a drastic reduction in hatching of eggs by microbial inoculants with a significant reduction in RKI and increase in total weight of the plant. Bacteria destroy nematodes continuously in virtually all soils because of their constant association with nematodes in the rhizosphere. Although a large number of bacteria have shown antagonistic effects against nematodes but the most important genera include *Rhizobium* (*R. leguminosorum*), *Bradyrhizobium japonicum*, *Mesorhizobium sp*., *Azorhizobium sp*., *Pseudomonas* (*P. fluorescens* and *P. aeruginosa*) and *Bacillus* (*B. subtilis*). Similarly, Wescott and Kluepfel (1993) had reported that *Bacillus* and *Pseudomonas sp.* inhibit egg hatching by the production of exotoxic compounds as a result of cellular metabolism, which can also affect nematode juveniles. This antagonistic effect against *M. incognita* is due to the permeability changes of juveniles cuticle which is characterized by its selective permeability and this effect is more pronounced with molting inside eggs.

In this investigation, the addition of bio-control organisms improved plant growth and further suppressed *M. incognita* population as compared to the chemical nematicides, carbofuran. Reducing the hatching of *M. incognita* eggs will help in delaying the population build-up of *M. incognita* which will help in the efficient and harmless control of this pathogen. The possible mechanism for nematode population reduction might be related to the microbial ability to envelop or bind the root surface with

Table 4
ANOVA results for root and shoot fresh and dry weight in *W. somnifera* cv. Poshita.

	Degree of freedom (df)	Mean sum of squares (ss)			
		Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
Treatments	7	1122.67	55.70	37.29	12.95
Error	16	43.33	4.52	1.39	0.65
Total	23	1166.00	60.21	38.69	13.59
F-value		59.22**	28.21**	61.38**	45.89**
P-value		<0.001	<0.001	<0.001	<0.001

** Significative at a level of 1% probability.

carbohydrate lectin, thereby interfering with normal host recognition by the nematode. The use of rhizospheric microbes for the management of nematode populations is apparently effective and environmentally friendly compared to synthetic nematicides. The pot experiment was a preliminary test revealing the rhizospheric microbes as potential tools for nematode management in the near future.

5. Conclusions

Rhizospheric microbes are a poorly investigated group of microorganisms that represent an abundant and dependable source of bioactive and chemically novel compounds with potential for exploitation in a wide variety of medical, agricultural, and industrial arenas. The mechanisms through which these microorganisms exist and respond to their surroundings must be better understood. The results presented in this study show that rhizospheric microbes play an important role for protecting plants against diseases besides enhancing their growth parameters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2013.01.014.

References

Ahmad, M., Saleem, S., Ahmad, A.S., Ansari, M.A., Yousuf, S., Hoda, M.N., et al., 2005. Neuroprotective effects of *Withania somnifera* on 6-hydroxydopamine induced Parkinsonism in rats. Hum. Exp. Toxicol. 24, 137–147.

Akhtar, M., Malik, A., 2000. Roles of organic soil amendments and soil organisms in the biological control of plant-parasitic nematodes: a review. Bioresour. Technol. 74, 35–47.

Akhtar, A., Hisamuddin, Robab, M.I., et al., 2012. Plant growth promoting Rhizobacteria: an overview. J. Nat. Prod. Plant Resour. 2, 19–31.

Ali, S.S., 1986. Occurrence of root knot nematodes in cardamom plantations of Karnataka. Ind. J. Nematol. 16, 269–270.

Ali, S.S., 1987. Effect of three systemic nematicides against root knot nematodes in a cardamom nursery. Nematol. Mediterr. 15, 155–158.

Archana, R., Namasivayam, A., 1999. Antistressor effect of *Withania somnifera*. J. Ethnopharmacol. 64, 91–93.

Atta, F.A., Saad, O.A.O., 2001. Biofertilizers as potential alternative of chemical fertilizer for *Cathranthus roseus*. J. Agric. Sci. 26, 7193–7208.

Bhattacharya, S.K., Goel, R.K., Kaur, R., Ghoshal, S., 1987. Anti-stress activity of sitoindosides VII and VIII new acyl steryl glycosides from *Withania somnifera*. Phytother. Res. 1, 32–37.

Bhattacharya, S.K., Satyan, K.S., Ghosal, S., 1997. Antioxidant activity of glycol withanolides from *Withania somnifera*. Ind. J. Exp. Biol. 35, 236–239.

Bhattacharya, A., Muruganandam, A.V., Kumar, V., Bhattacharya, S.K., 2002. Effect of poly herbal formulation EuMil on neurochemical perturbations induced by chronic stress. Ind. J. Exp. Biol. 40, 1161–1163.

Buhrer, E.M., 1938. Addition to the list of plants attracted by root-knot nematode. Plant Dis. Rep. 22, 216–234.

Chaudhary, G., Jagannathan, N.R., Gupta, Y.K., 2003. Evaluation of *Withania somnifera* in a middle cerebral artery occlusion model of stroke in rats. Clin. Exp. Pharmacol. Physiol. 30, 399–404.

Devi, P.U., Sharada, A.C., Solomon, F.E., Kamath, M.S., 1992. In vivo growth inhibitory effect of *Withania somnifera* (Ashwagandha) on a transplantable mouse tumor Sarcoma 180. Ind. J. Exp. Biol. 30, 169–172.

Dhuley, J.N., 2001. Nootropic-like effect of Ashwagandha (*Withania somnifera* L) in mice. Phytother. Res. 15, 524–528.

Eapen, M., 1995. Rural non-agricultural employment in Kerala– inter-district variations. Econ. Polit. Weekly 30, 12.

Ferraz, H.B., Bertolucci, P.H.F., Pereira, J.S., Lima, J.G.C., Andrade, L.A.F., 1988. Chronic exposure to the fungicide maneb may produce symptoms and signs of CNS manganese intoxication. Neurology 38, 550–553.

Ferraz, E.C.A., Lordello, L.G.E., Gonzaga, E., 1989. Influence of *Meloidogyne incognita* (Kofoid & White 1919) Chitwood 1949 on chlorophyll content of black pepper (*Piper nigrum* L.). Agrotro 1, 57–62.

Freire, C.O.F., Bridge, T., 1985. Parasitism of eggs, females and juveniles of *Meloidogyne incognita* by *Paecilomyces lilacinus* and *Verticillium chlamydosporium*. Fitopatol. Brasil 10, 577–596.

Gupta, M.C., Misra, H.O., Kalra, A., Khanuja, S.P.S., 2004. Root-knot and wilt: a new disease of Ashwagandha (*Withania somnifera*) caused by *Fusarium solani*. J. Med. Arom. Plant Sci. 26, 285–287.

Jagdale, G.B., Ball, C.B., Potter, J., Brandle, J., Roy, R.C., 2000. Rotation crop effects on Pratylenchus penetrans and subsequent crop yields. Can. J. Plant Sci. 80, 543–549.

Jain, S., Shukla, S.D., Sharma, K., Bhatnagar, M., 2001. Neuroprotective effects of *Withania somnifera* Dunn in hippocampal sub-regions of female albino rat. Phytother. Res. 15, 544–588.

Kalra, A., Singh, H.B., Pandey, et al., 1996. Strain of *Trichoderma harzianum* useful as nematode inhibitor, fungicide and plant growth promoter and a process for the isolation there of. United States Patent No. 6475772.

Kingland, G.C., 2001. Diseases and insects of fruit and vegetable crops. Clemson Press, Victoria, Seychelles.

Koshy, P.K., 1987. Integratednematode management in plantation crops. Proceedings of the third Group Discussion on the Nematological Problems of Plantation Crops, 29–30 October. Sugarcane Breeding Institute, Coimbatore, Tamil Nadu, India, pp. 49–51.

Kothari, S.K., Singh, C.P., Kumar, Y.V., Singh, K., 2003. Morphology, yield and quality of Ashwagndha (*Withania somnifera* L. Dunal) roots and its cultivation economics as influenced by tillage depth and plant population density. J. Hortic. Sci. Biotech. 78, 422–425.

Krusberg, L.R., Nielsen, L.W., 1958. Pathogenesis of root-knot nematodes to Porto Rico variety of sweet potato. Phytopathology 48, 30–39.

Kulkarni, S.K., George, B., Mathur, R., 1998. Neuroprotection by *Withania somnifera* root extract against lithium-pilocarpine-induced seizures. Ind. Drugs 35, 208–215.

Lee, J.H., Kyung, C.M., Sug, J.K., Kang, B.R., Seon, K.I., Kim, Y.C., 2011. Nematicidal activity of a nonpathogenic biocontrol bacterium, Pseudomonas chlororaphis strain O6. Curr. Microbiol. 62, 746–751.

Masadeh, B., Alten, H.V., Gisela, G.S., Sikora, R.A., 2004. Biocontrol of root-knot nematodes using the arbuscular mycorrhizal fungus *Glomus intraradices* and the antagonist *Trichoderma viride* in two tomato cultivars differing in their suitability as hosts for the nematodes. J. Plant Dis. Prot. 111, 322–333.

McSorley, R., 1999. Host suitability of potential cover crops for root-knot nematodes. Suppl. J. Nematol. 31, 619–623.

Meyer, S.L.F., Huettel, R.N., Liu, X.Z., Humber, R.A., Juba, J., Nitao, K., 2004. Activity of fungal culture filtrates against soybean cyst nematode and root-knot nematode egg hatch and juvenile motility. Nematology 6, 23–32.

Mishra, L.C., Singh, B.B., Dagenais, S., 2000. Scientific basis for the therapeutic use of *Withania somnifera* (Ashwagandha) a review. Altern. Med. Rev. 5, 334–346.

Mohan, R., Hammers, H.J., Bargagna, M.P., Zhan, X.H., Herbstritt, C.J., Ruiz, A., et al., 2004. Withaferin A is a potent inhibitor of angiogenesis. Angiogenesis 7, 115–122.

Mohanty, I., Arya, D.S., Dinda, A., Talwar, K.K., Joshi, S., Gupta, S.K., 2004. Mechanisms of cardioprotective effect of *Withania somnifera* in experimentally induced myocardial infarction. Basic Clin. Pharmacol. Toxicol. 94, 184–190.

Naidu, P.S., Singh, A., Kulkarni, S.K., 2003. Effect of *Withania somnifera* root extract on haloperidol-induced orofacial dyskinesia possible mechanisms of action. J. Med. Food 6, 107–114.

Nicknam, G.R. and Dhawan, S.C., 2001. Effect of seed bacterization, soil drench and bare root dip application methods of Pseudomonas fluorescens isolate Pf1 on the suppression of Rotylenchulus reniformis infecting tomato (Abstract). In: Proceedings of the National Congress on Centenary of Nematology in India: Appraisal and Future Plans, IARI, New Delhi, India.

Panda, S., Kar, A., 1997. Evidence for free radical scavenging activity of Ashwagandha root powder in mice. Ind. J. Physiol. Pharmacol. 41, 424–426.

Panda, S., Kar, A., 1998. Changes in thyroid hormone concentrations after administration of Ashwagandha root extract to adult male mice. J. Pharm. Pharmacol. 50, 1065–1068.

Pandey, R., Kalra, A, Gupta, ML. and Sharma, P., 2003. Phytonematodes: Major pest of MAPs. In Proceedings of first National Interactive meet on medicinal and Aromatic Plants (Edited by Mathur et al. CIMAP, Lucknow, India), pp. 188–197.

Pandey, R., Gupta, A., Singh, H.N., and Kalra, A. 2009. Phytonematode management through Bacteria: An Underground Battle for existence In Recent Advances. Biopesticides Biotechnological Applications (Edited by J.K. Johri, NBRI, Lucknow), pp. 1–26.

Patra, D.D., Singh, K., Misra, H.O., et al., 2004. Agrotechnologies of Ashwagandha (*Withania somnifera*). J. Med. Arom. Plant Sci. 26, 332–335.

Rai, D., Bhatia, G., Sen, T., Palit, G., 2003. Anti-stress effects of *Ginkgo biloba* and *Panax ginseng* a comparative study. J. Pharmacol. Sci. 93, 458–464.

Rasool, M., Marylatha, L., Varalakshmi, P., 2000. Effect of *Withania somnifera* on lysosomal acid hydrolases in adjuvant-induced arthritis in rats. Pharm. Pharmacol. Commun. 6, 187–190.

Rhoades, H.I., 1984. Control of *Pratylenchus scribneri* on spearmint (*Mentha spicata*) with non-fumigant nematicides. Nematropica 14, 85–89.

Saikia, S.K., Tiwari, S., Pandey, R., 2012. Rhizospheric innovations for growth enhancement and *Meloidogyne incognita* management in *Mentha arvensis* cv. Kosi. Int. J. Environ. Sci. Eng. Res. 3, 26–34.

Schliebs, R., Liebmann, A., Bhattacharya, S.K., Kumar, A., Ghosal, S., Bigl, V., 1997. Systemic administration of defined extracts from *Withania somnifera* (Indian Ginseng) and Shilajit differentially affects cholinergic but not glutamatergic and GABAergic markers in rat brain. Neurochem. Int. 30, 181–190.

Sharma, P., Pandey, R., 2009. Biological control of root-knot nematode *Meloidogyne incognita* in the medicinal plant *Withania somnifera* and the effect of biocontrol agents on plant growth. Afr. J. Agric. Res. 4, 564–567.

Sharon, E., Eyal, M.B., Chet, I., Estrella, H.A., Kleifeld, O., Spiegel, Y., 2001. Biological control of root knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*. Phytopathology 91, 687–693.

Shaukat, S.S., Siddiqui, I.A., Zarina, B., 2004. Effect of some common weeds from Pakistan on plant-parasitic nematodes *in vitro* and population densities and survival of *Meloidogyne incognita* in okra and brinjal. Nematol. Medit. 532, 111–115.

Siddiqui, I.A., Haas, D., Heeb, S., 2005. Extracellular protease of *Pseudomonas fluorescens* CHA0, a biocontrol factor with activity against the root-knot nematode *Meloidogyne incognita*. Appl. Environ. Microbiol. 71, 5646–5649.

Sikora, R.A., Schuster, R.P., 2000. Handbuch der Phytonematologie. Shaker Verlag, Aachen, Germany.

Sikora, R.A., Niere, B., Kimenju, J., 2003. Endophytic Microbial Biodiversity and Plant Nematode Management in African Agriculture. In: Neuenschwander, P., Borgermeister, C., Langewald, J. (Eds.), Biological control in IPM systems in Africa. Cabi, U.K., p. 407.

Southey, J.F., 1986. (Ed.) Laboratory methods for work with plant and soil nematodes. Ministry of Agriculture, Fisheries and Food, Reference Book, London.

Spencer, M., Ryu, C.M., Yang, K.Y., Kim, Y.C., Kloepper, J.W., Anderson, A.J., 2003. Induced defense in tobacco by *Pseudomonas chlororaphis* strain O6 involves at least the ethylene pathway. Physiol. Mol. Plant Pathol. 67, 27–34.

Su, T., Mulla, M.S., 1998. Ovicidal activity of neem products (Azadirachtin) against Culex tarsalis and Culex quinquefasciatus (Diptera:Culicidae). J. Am. Mosq. Control Assoc. 14, 204–209.

Tohda, C., Kuboyama, T., Komatsu, K., 2005. Search for natural products related to regeneration of the neuronal network. Neurosignals 14, 34–45.