Bulletin of the National Research Centre

RESEARCH Open Access

Protective effect of *Bacillus subtilis*, *B. pumilus*, and *Pseudomonas fluorescens* isolates against root knot nematode *Meloidogyne incognita* on cowpea

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Abstract

Background: Due to the fact that chemical nematicides frequently cause environmental pollution and toxic hazards to human, plants, and domestic animals, certain biocontrol agents of environmentally and toxicologically safe properties and secure to human and animals were tested against root-knot nematode *Meloidogyne incognita* on cowpea.

Objective: The protective effect of two isolates of *Bacillus subtilis*, *B. pumilus*, or *Pseudomonas fluorescens* on *M. incognita* reproductive parameters infecting cowpea was examined, and consequently, plant growth and yield parameters were investigated. The nematicide, Furadan (10% G) was, also, included in this study for comparison.

Results: *Bacillus subtilis* (Bs_2) recorded the highest average total percentage reduction (82%) of *M. incognita* reproductive parameters followed by *B. pumilus* Bp_2 (81.8%). Also, Carbofuran 10% recorded the highest average total percentage reduction (76.5%) in terms of numbers of second-stage juveniles (J_2) in plant roots and soil as well as nematode galls and eggmasses compared to the untreated check. The highest total average percentage plant growth increase (99%) was obtained by *B. pumilus* (Bp1), but *B. subtilis* (Bs2) and medium recorded 26.3 and 12.8% only, respectively. *P. fluorescens* (Pf_1) scored the highest yield increase (97%) followed by Pf2 (63.8%). Number of bacterial nodules showed the highest percentage increase (78%) by *P. fluorescens* (Pf_2). *B. pumilus* (Bp_1) caused the highest increase of phenolic compound contents followed by *P. fluorescens* (Pf_1). The nematicide Carbofuran 10% G was the first in increasing soluble protein contents followed by *P. fluorescens* (Pf_1). *Bacillus pumilus* (Bp_1) caused the highest total contents of photosynthetic pigments followed by *P. fluorescens* (Pf_1) and (Pf_2).

Conclusions: The tested biocontrol agents could achieve various degrees of *M. incognita* control on cowpea under screen house conditions with consequent increase in cowpea growth and yield parameters. These bacterial isolates need to be studied under different field conditions for confirmation.

Keywords: Biocontrol agents, Bacterial isolates, Cowpea yield, Root knot nematode

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Background

The serious impact of phytonematodes on agriculture economy has recently been documented, especially for developing countries like Egypt (Abd-Elgawad and Askary 2015). Yet, due to the fact that chemical nematicides frequently cause environmental pollution, pest resistance, and toxic hazards to human, plants, and domestic animals, certain bioagents of environmentally and toxicologically safe properties and secure to humans and animals were tested. Osman et al. (2011) showed that *Pseudomonas fluorescens* reduced root knot parameters when treated as soil drench at S/2 dilution (10^8 CFU/ml/2). In another study, *Bacillus subtilis* and *B. thuringiensis* inhibited populations of root-knot nematode *Meloidogyne incognita* on tomato plants in the soil with 82.6 and 80.5% reduction, respectively (Khalil et al. 2012). Akhtar et al. (2012) showed that the rate of *M. incognita* on *Vigna mungo* was significantly reduced by *P. fluorescens* and *B. subtilis*. This reduction was reflected by increasing plant growth parameters as indicated by certain plant growth criteria and number of bacterial nodules per plant compared to the untreated control. Also, *P. fluorescens* and *B. subtilis* inhibited the number of galls by 42.79 galls per plant. Out of numerous plant growth-promoting rhizobacteria (PGPR) genera, *Bacillus* spp. have a considerable effect as biocontrol agents on plant-parasitic nematodes (PPNs) and on root colonization, multiple modes of action, and promising ability to sporulate under stressed conditions (Kavitha et al. 2012). Nematophagous bacteria as biocontrol agents of PPNs were recently reviewed (Eissa and Abd-Elgawad 2015). Abd-El-Khair et al. (2016) showed that eight selected isolates of *Bacillus* spp. reduced certain nematode criteria and improved plant growth compared to untreated check. Recently, Abd-Elgawad and Askary (2018) listed fungal and bacterial nematicides with their comprehensive references and relevant information, i.e., the active ingredient, product name, type of formulation, producer, targeted nematode species and crop, and country of origin. Sohrabi et al. (2018) showed that four species of PGPR of which *P. fluorescens* and *B. subtilis* inoculated in the presence or absence of root knot nematode significantly improved the tomato growth parameters. Also, the reproductive factor of *Meloidogyne javanica* was significantly affected by *P. fluorescens* and *B. subtilis* as it reduced from 112.15 to 24.94 and 24.96, respectively.

The purpose of this research is to investigate the nematicidal activity of two isolates of each of *Bacillus subtilis*, *B. pumilus*, and *Pseudomonas fluorescens*, on *Meloidogyne incognita* reproductive parameters and consequent plant growth and yield of cowpea under screen house conditions. Furadan (Carbofuran 10% G), a widely used chemical pesticide for its strong effect on PPNs and insect pests as a systemic nematicide and insecticide in Egypt (e.g., Abd-Elgawad and Kabeil 2010) and elsewhere (e.g., Faruk et al. 2012), was included for comparison with the bacteria tested herein.

Methods

Preparation of root-knot nematode inoculum

Pure culture of *M. incognita* was propagated on eggplant from stock of the nematode species. Newly hatched second-stage juveniles (J_2) from this culture were used as inoculum. Perineal patterns of adult females from eggplant roots were used to confirm the nematode species (Taylor and Sasser 1978).

Isolation of bacterial bioagents

Six bacterial bio-control isolates viz. *Bacillus subtilis* (Bs_1 and Bs_2), *B. pumilus* (Bp_1 and Bp_2), and *Pseudomonas fluorescens* (Pf_1 and Pf_2) were isolated from dry bean plant rhizospheres in Giza Governorate, Egypt. The bacteria were identified by morphological, cultural, and biochemical characters, using standard bacteriological methods, in the Department of Plant Pathology, National Research Centre, according to the methods described by Goszczynska et al. (2000).

Preparation of bacterial Inocula

For inoculum preparation of these bacteria, they were separately inoculated in nutrient sucrose (2%) broth medium (beef extract 3 g, peptone 5 g, glucose 10 g) in 1 l of distilled water, and pH was adjusted at 7.4 ± 0.2. The bacterial cultures were incubated at 28 °C for 48 h. Then, the bacterial inocula were adjusted to 10^7–10^9 colony forming unit (CFU)/ml by turbidity method (Baid et al. 2000). Bacterial inocula were used as a mixture of bacterial cells and cultural filtrate (Abd-El-Khair and Haggag 2007).

The procedures and experiment design

The experiment was conducted at screen house of Plant Pathology Department, National Research Centre (NRC), Giza, Egypt. Four of cowpea seeds were sown in plastic pots (20-cm diameter) containing 2 kg of solarized sandy-loam soil. Then, plants were thinned to two plants per pot. Each pot was inoculated with 1000 newly hatched J_2 of *M. incognita* (in four holes made around a plant). At the same time of nematode inoculation, cowpea plants were treated with each bio-agent inoculum via four holes around the plant. These treatments were compared with a nematicide, Furadan (Carbofuran 10% G), at the rate of 0.02 g/pot (equivalent to 10 kg/Feddan (Feddan = 4200 m^2) and medium. Four pots were used as replicates in each treatment. All pots were inoculated with Al-aukadin (containing nitrogen-fixing bacterium, namely, *Bradyrhizobium* spp. The pots were arranged in a completely randomized design on a bench under

screen house conditions maintained at 25 ± 5 °C; the plants were irrigated as needed. After 3 months of nematode inoculation (Harvest stage of cowpea), roots of cowpea were carefully uprooted and washed thoroughly with running tap water to avoid hanging soil particles and then hatched numbers of J_2 in eggmasses were extracted by an incubation method (Young 1954) in one half of roots. Parameters of *M. incognita* reproduction in terms of numbers of second-stage juveniles (J_2) in plant roots and soil per pot as well as nematode galls and eggmasses per plant in another half of roots were recorded. For soil extraction of nematodes, a sieving and decanting technique (Barker 1985) was used. The nematodes were counted using a light microscope.

Effects of the bacterial bioagents on vegetative parameters of cowpea, shoot length (cm), fresh and dry shoot weights, and fresh root weight (g) were recorded after 3 months of nematode inoculation. Also, the number as well as fresh and dry weights of pods and weight of 100 seeds were estimated. The method of Danil and George (1972) was used to determine total phenolic compounds from seeds colorimetrically by a Folin Ciocalteu phenol reagent. The determination of soluble protein was carried out by the method of Bradford (1976). Photosynthetic pigments (chlorophyll A, chlorophyll B, and carotenoids) in the fresh leaves were determined as described by Moran (1982).

Statistical analysis of data

Analysis of variance (ANOVA) procedures of the obtained data were performed through Computer Statistical Package (COSTAT) User Manual Version 3.03, Barkley Co., a computer-based program. To separate the means among treatments at 5% level of probability, Duncan's multiple range test (DMRT) was applied (Snedecor and Cochran 1999).

Results

Effect of the bacterial bioagents on nematode reproductive parameters

Results in Table 1 illustrate the significant ($P \leq 0.05$) effect of the tested bacterial isolates in controlling *M. incognita* on cowpea. Average total percentage nematode reduction was calculated to further determine the differences among treatments. It was observed that the highest average percentage total nematode reduction was achieved by using *B. subtilis* Bs_2 (82%) followed by *B. pumilus* Bp_2 (81.8%) compared to the untreated check. The two isolates of *P. fluorescens* Pf_1 and Pf_2 showed intermediate reduction (69.8 and 62.3%), respectively. Carbofuran 10% recorded higher reduction (76.5%). The least nematode reduction (61.5%) occurred by medium only.

Effect of the bacterial bioagents on plant growth parameters

Results in Table 2 indicate the significant ($P \leq 0.05$) effect of the tested bacterial isolates on cowpea growth criteria as influenced by *M. incognita* when compared to untreated check. It was observed that the highest percentage increase as a total average in plant growth criteria (99%) was achieved by using *B. pumilus* (Bp_1) followed by 86.8% achieved by *B. pumilus* (Bp_2) and 57.5% occurred by the nematicide. The lowest average total percentage increase (26.3%) occurred by *B. subtilis* (Bs2) and medium only (12.8%). Other isolates differed in their effect on plant growth criteria.

Effect of the tested bioagents on yield parameters and number of bacterial nodules

Table 3 illustrates the nematicidal significant ($P \leq 0.05$) effect of the tested bacterial species and isolates on the yield parameters and number of bacterial nodules of

Table 1 Effects of *Bacillus subtilis*, *B. pumilus*, and *Pseudomonas fluorescens* isolates on *Meloidogyne incognita* reproductive parameters on cowpea grown under screen house conditions

Treatments	*M. incognita* nematode parameters								Average total percentage nematode parameter reduction (%)
	J_2s in soil/pot		J_2s in roots/plant		Galls/plant		Eggmasses/plant		
	No.	Red. (%)	No.	Red. (%)	No.	Red (%)	No.	Red. (%)	
B. subtilis (Bs_1)	4270ef	86	223bcd	69	24def	73	19de	74	75.5
B. subtilis (Bs_2)	3050f	75	113de	84	16f	82	10f	87	82.0
B. pumilus (Bp_1)	7600de	88	260bc	64	27cde	70	21cde	72	73.5
B. pumilus (Bp_2)	3670de	90	155cde	79	32bcd	64	25bcd	66	81.8
P. fluorescens (Pf_1)	12,400c	60	210bcd	71	23ef	74	19de	74	69.8
P. fluorescens (Pf_2)	7370de	76	323b	56	38b	57	30b	60	62.3
Carbofuran 10%	9530cd	69	77e	89	26de	71	17ef	77	76.5
Medium only	17,000b	55	247bc	66	35bc	61	27bc	64	61.5
Nematode only (control)	30,800a	–	728a	–	89a	–	74a	–	–

Means are averages of four replicates. Means followed by different letter(s) are significantly different according to Duncan's multiple range test at $P \leq 0.05$.
Red. reduction

Table 2 Effects of *Bacillus subtilis*, *B. pumilus*, and *Pseudomonas fluorescens* isolates on vegetative growth parameters of cowpea grown under screen house conditions and infected with root knot nematode, *Meloidogyne incognita*

Treatments	Plant growth parameters/plant (g)								Average total percentage plant growth parameter increase (%)
	Shoot				Root				
	Fresh weight		Dry weight		Fresh weight		Dry weight		
	Weight	Inc. (%)	Weight	Inc. (%)	Weight	Inc. (%)	Weight	Inc. (%)	
B. subtilis (Bs_1)	63.9g	25	12.5ef	5	32.5abc	45	2.2ab	57	33.0
B. subtilis (Bs_2)	65.4f	28	12.8def	8	28.3bc	26	2.0abc	43	26.3
B. pumilus (Bp_1)	120.0a	135	22.7a	91	42.8a	91	2.5a	79	99.0
B. pumilus (Bp_2)	113.4b	122	19.2b	61	43.2a	93	2.4a	71	86.8
P. fluorescens (Pf_1)	68.8d	35	13.3d	12	26.6c	19	2.0abc	43	27.3
P. fluorescens (Pf_2)	66.7e	31	13.1de	10	41.6a	86	2.1ab	50	44.3
Carbofuran 10%	80.5c	58	16.2c	36	38.5ab	72	2.3a	64	57.5
Medium only	61.7h	21	12.3f	3	25.3c	13	1.6bc	14	12.8
Nematode only (control)	51.1i	–	11.9f	–	22.4c	–	1.4c	–	–

Means are averages of four replicates. Means followed by different letter(s) are significantly different according to Duncan's multiple range test at $P \leq 0.05$. *Inc.* increase

cowpea as influenced by root-knot nematode infection. It was observed that the highest average total percentage yield increase (97%) was achieved by using *P. fluorescens* (Pf_1) followed by *P. fluorescens* Pf_2 (63.8%). The least average total percentage yield increase was recorded by *B. subtilis* Bs_2 (41.3%) followed by medium only (14%). The number of bacterial nodules increased at the different treatments being the highest percentage by *P. fluorescens* Pf_2 (78%) followed by *B. pumilus* Bp1 (44%). The least increase (15%) occurred by using *B. subtilis* (Bs_1).

Effect of the bacterial bioagents on some biochemical compounds

It is clearly noticed that phenolic compounds and soluble proteins significantly ($P \leq 0.05$) increased as they are affected by the different treatments compared to those of the untreated check (Table 4). *B. pumilus* (Bp_1) was the first in increasing phenolic compound contents followed by *P. fluorescens* (Pf_1) followed by Pf_1 compared to the other treatments and untreated check. As for soluble proteins, the nematicide Carbofuran was the first in increasing soluble protein contents followed by *P. fluorescens* (Pf_1) comparable to the other treatments and untreated check.

Effect of the tested bacterial bioagents on photosynthetic pigments

Also, results in Table 4 illustrate the effect of the different treatments of the tested bioagents on chorophyll A, chlorophyll B, and carotenoid contents. It is well noticed

Table 3 Effects of *Bacillus subtilis*, *B. pumilus*, and *Pseudomonas fluorescens* isolates on yield parameters of cowpea grown under screen house conditions and infected with root knot nematode, *Meloidogyne incognita*

Treatments	Yield parameters/plant (g)									No. of bacterial nodules	
	Pod No.		Fresh weight		Dry weight		100 seeds weight		Average total percentage plant yield parameter increase (%)		
	No.	Inc. (%)	Weight	Inc. (%)	Weight	Inc. (%)	Weight	Inc. (%)		No.	Inc. (%)
B. subtilis (Bs_1)	3.5c	75	1.28d	28	0.69c	53	13.97b	54	52.5	31bc	15
B. subtilis (Bs_2)	3.1d	55	1.20e	20	0.61d	36	13.97b	54	41.3	36bc	33
B. pumilus (Bp_1)	3.7c	85	1.26d	26	0.68c	51	13.68b	51	53.3	39ab	44
B. pumilus (Bp_2)	3.6c	80	1.33c	33	0.70bc	58	13.71b	51	55.5	38ab	41
P. fluorescens (Pf_1)	5.1a	155	1.75a	75	0.80a	78	16.26a	80	97.0	37b	37
P. fluorescens (Pf_2)	4.7b	135	1.13f	13	0.73b	62	13.17bc	45	63.8	48a	78
Carbofuran 10%	3.7c	85	1.41b	41	0.68c	51	12.04c	33	52.5	39ab	44
Medium only	2.4e	20	1.03g	3	0.52e	16	10.63d	17	14.0	37bc	37
Nematode only (control)	2.0f	–	1.00h	–	0.45f	–	9.05e	–		27c	–

Means are averages of four replicates. Means followed by different letter(s) are significantly different according to Duncan's multiple range test at $P \leq 0.05$. *Inc.* increase

Table 4 Biochemical compounds in cowpea grown under screen house conditions and infected with root knot nematode, *Meloidogyne incognita*, as affected by *Bacillus subtilis*, *B. pumilus*, and *Pseudomonas fluorescens* isolates

Treatments	Biochemical compounds					
	Photosynthetic pigments (mg/g)				Phenolic contents (%)	Soluble proteins (%)
	Chlorophyll A	Chlorophyll B	Carotenoids	Total		
B. subtilis (Bs_1)	1.54ef	0.68d	0.12a	2.34d	2.94b	1.43ef
B. subtilis (Bs_2)	1.63de	0.62d	0.14a	2.39d	2.91b	1.45e
B. pumilus (Bp_1)	3.11a	1.20a	0.29a	4.60a	3.62a	1.42f
B. pumilus (Bp_2)	1.61de	0.65d	0.11a	2.37d	3.11b	1.51d
P. fluorescens (Pf_1)	2.23b	0.93b	0.18a	3.34b	3.15b	1.62b
P. fluorescens (Pf_2)	1.69de	0.66d	0.17a	2.52cd	3.01b	1.59c
Carbofuran 10%	1.88c	0.69cd	0.15a	2.72c	2.53c	1.67a
Medium only	1.72cd	0.66d	0.29a	2.66c	2.17d	1.41f
Nematode only (control)	1.40f	0.57d	0.14a	2.11e	1.87c	1.18g

Values are averages of four replicates. Values followed by different letter(s) are significantly different according to Duncan's multiple range test at $P \leq 0.05$

that the contents of photosynthetic pigments were significantly ($P \leq 0.05$) increased by different treatments compared to the untreated check. *B. pumilus* (Bp_1) recorded the highest total contents of photosynthetic pigments followed by *P. fluorescens* (Pf_1) and medium only. The least one occurred by untreated check.

Discussion

The present results showed that the two isolates from each of *B. subtilis*, *B. pumilus*, and *P. fluorescens* had nematicidal activities against *M. incognita* as well as enhancement of the growth, yield parameters and number of bacterial nodules of cowpea. *B. subtilis* Bs_2 followed by *B. pumilus* Bp_2 recorded 82% and 81.8% as average total percentage nematode reduction, respectively compared to the untreated check followed by *P. fluorescens*. The reduction in nematode numbers may be due to the tested rhizobacteria found in the plant rhizosphere which could possibly cause different modes of action against plant parasitic nematodes including producing antibiotics, enzymes, and toxins. They can parasitize and promote systemic resistance of plants against nematodes (Tian et al. 2007; Lugtenberg and Kamilova 2009; Osman et al. 2011; Norabadia et al. 2014).

The tested microorganisms can stimulate plant growth by using different mechanisms that include improvement of plant nutrition, production and regulation of phyto-hormones, and suppression of disease-causing organisms. In addition, these bacteria survive in soil around plants and can aggressively colonize plant roots and benefit plants by providing growth promotion materials (Tian et al. 2007; Lugtenberg and Kamilova 2009; Ali and Vidhale 2013). Also, Keneni et al. (2010) reported that *Bacillus* and *Pseudomonas* supply phosphate to plants. In the present study, although the highest average plant growth criteria increase was achieved by using *B. pumilus* (Bp_1) followed by *B. pumilus* (Bp_2), it is interesting to note that the moderate yield increases were recorded by these later isolates, respectively. On the other hand, the highest average percentage yield increase was achieved by using *P. fluorescens* (Pf_1) followed by *P. fluorescens* (Pf_2) in spite of their less average plant growth increases. *P. fluorescens* recorded intermediate nematode reduction in the present study, but the previous study by Khan and Haque (2011) proved that *P. fluorescens* achieved the greatest reduction in the studied nematode reproductive parameters and plant growth of tobacco which may be due to the differences in isolates.

Akhtar et al. (2012) reported that there was significant increase in chlorophyll content which was observed in T_7 which received a higher dose of bacteria (*P. fluorescens* (20 ml) + *B. subtilis* (20 ml) treatment compared to control. In the present study, *B. pumilus* (Bp_1) recorded the highest total contents of photosynthetic pigments followed by *P. fluorescens* (Pf_1) as single treatment for each. Formation of some phenolic compounds in seeds of resistant plants was involved in the mechanism of resistance against PPNs (Bajaj and Mahajan 1977; Giebel 1982). Resistance against nematodes has been affected by the performed phenol levels in roots of certain plant cultivars (Narayana and Reddy 1980).

It is well known that rhizobacteria are the most abundant microorganisms in plant rhizosphere exhibiting different modes of action against nematodes. Also, these microorganisms can stimulate plant growth by using a variety of mechanisms as mentioned previously. The present results clarified that inoculation of cowpea plants with two isolates from each of the abovementioned microorganisms at an early stage of plant development can reduce population of root-knot nematode and improve biomass production through direct effects

on root and shoot growth and nematode infection. Yet, before utilizing such bacteria for field application and commercialization as reported by Abd-Elgawad and Vagelas (2015), their experimentation under field conditions should be documented.

Conclusion

It can be concluded that using some antagonistic plant growth-promoting rhizobacteria achieved efficient control of the root-knot nematode problem. These treatments not only lowered the pathogenic effect of the nematodes, but also stimulated plant growth and yield, while avoiding toxicity and hazardous nature of chemical nematicides in the environment. However, it is necessary to further affirm these results and investigate various mechanisms involved caused by the studied bacterial isolates under field conditions.

Acknowledgements

This research work was supported in part by the In-house Project No. 11030133 entitled "Pesticide alternatives against soil borne pathogens attacking legume cultivation in Egypt" funded by National Research Centre, Egypt.

Funding

There is no funding.

Availability of data and materials

The tested bacterial isolates and nematodes are available in Egyptian environment and were identified in the laboratory.

Authors' contributions

All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Publisher's Note

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Received: 9 June 2018 Accepted: 1 April 2019
Published online: 17 April 2019

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