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Effectiveness of *Bacillus subtilis*, *B. pumilus*, *Pseudomonas fluorescens* on *Meloidogyne incognita* infecting cowpea

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Abstract

Under screen house conditions, this experiment was conducted to investigate the effectiveness of single or combined application of bacterial inoculation of *Bacillus subtilis* (Bs), *B. pumilus* (Bp) and *Pseudomonas fluorescens* (Pf) on *M. incognita*, infecting cowpea cv. Baladi. Based on average percentages of nematode reduction, *P. fluorescens* (Pf) achieved (89.0%) followed by the combined treatments of Pf+Bs (88.5%) and Pf + Bs + Bp (86.3%) as compared to untreated positive and negative controls. As for cowpea vegetative growth, the highest plant growth increase (69.0%) was observed in combined treatment of Bs+Bp followed by 55.6% in single treatment of Pf and the least plant growth was obtained in combined treatment of Pf + Bs + Bp (54.8%). The highest percentage of yield increase (70.2%) was recorded in Bs+Bp treatment followed by 49.3% by *B. pumilus* (Bp). The highest percentage (116.7%) in the number of bacterial nodules on roots of cowpea was obtained by the combined treatment Pf + Bp, followed by the combined treatment Bs + Bp i.e. 88.9%. The combined treatments of Bs + Bp and Pf + Bp were responsible for increasing phenolic and soluble proteins, respectively. The effects of combined treatments of Pf+Bs and Pf +Bp were recorded for highest contents of photosynthetic pigments than the other treatments either as single or combined treatments.

Keywords: Biocontrol, *Bacillus subtilis*, *B. pumilus*, *Pseudomonas fluorescens*, cowpea, root-knot nematode

Root-knot nematodes (*Meloidogyne* spp.) are considered to be the most pathogenic plant parasitic nematodes. Application of nematicides for the control of these nematodes is a common practice. However, they cause hazardous effect to human and animals. Therefore, the use of biological control agents by plant growth promoting rhizobacteria (PGPR) is considered to be more effective and environmentally safe which can be a good replacement to chemical nematicides (Khan *et al*., 2011; Munshid *et al*., 2013). Out of several PGPR genera; *Bacillus* spp. and *Pseudomonas fluorescens* have considerable potential effect as biocontrol of plant parasitic nematodes through their root colonization, multiple modes of action and promising ability to sporulate under stressed conditions (Kavitha *et al*., 2012). It was studied that application of *Pseudomonas fluorescens* and *Bacillus subtilis* significantly reduced infectivity rate of *Meloidogyne incognita* on *Vigna mungo*. Plant growth parameters in terms of shoot length, root length, shoot fresh and dry weights, root fresh and dry weights and number of nodules per plant significantly increased in the plants treated with the two mentioned bacteria as compared to control. Maximum inhibition of root-knot (42.79 galls per plant) was observed in plants inoculated with *M. incognita* and at the same time treated with *P. fluorescens* and *B. subtilis* (Akhtar *et al*., 2012). The objective of this study was to investigate the potential nematicidal effect of *B. subtilis*, *B. pumilus* and *P. fluorescens* as simultaneous or

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single population of *M. incognita*, plant growth, bacterial nodulation and biochemical changes in cowpea cv. Baladi under screen house conditions.

Materials and Methods

Monoculture of root-knot nematode inoculum: The tested species of *M. incognita* was identified from adult females on the basis of the morphological characteristics of the female perineal pattern (Taylor & Sasser, 1978). Pure culture of *M. incognita* was reared on eggplant cv. Pusa Purple Long in a greenhouse at 30 ± 5°C by using a single egg-mass of this nematode. After getting pure culture, egg-masses were separated and by agitating in 0.05% NaOCl for 2-3 min. J_2 were obtained (Hussey & Barker, 1973).

Preparation of bacterial inocula: Bacterial isolates *viz*., *B. subtilis* (Bs), *B. pumilus* (Bp) and *P. fluorescens* (Pf) were isolated from Egyptian soil and identified according to standard microbiological soil. For preparation of bacterial inoculums they were separately inoculated in nutrient sucrose (2%) broth medium (Beef extract 3.0g ; Peptone 5.0g ; Glucose 10.0g in 1.0 liter of distilled water and adjusted pH at 7.4±0.2). The bacterial cultures were incubated at 28ºC for 48 h. Then, the bacterial inoculum was adjusted to 10^7-10^9 colony forming unit (CFU)/ml by turbidity method (Baid *et al*., 2000). Bacterial inoculum for each species was applied as mixture of bacterial cells and cultural filtrate (Abd-El-Khair & Haggag, 2007).

Pot experiment design: The experiment was carried out in pots at Plant Pathology Department, National Research Centre (NRC) Dokki, Egypt. Four seeds of cowpea were sown in each pot (30cm diameter) containing 6kg of solarized sandy loam soil. After that, plants were thinned to two plants per pot after 10 days. Each pot was inoculated with 1,000 newly hatched J_2 of *M. incognita* in April, 2017, in four holes made around the plant. At the same time of nematode inoculation, cowpea plants were treated with each bacterial species either as single or combined as mixture of cultural bacterial cells and filtrates at the tested rate (10^7-10^9 CFU/ml) in four holes around the plant. A nematicide, carbofuran 10g at the rate of 0.06 g/pot (equivalent to 10kg/Feddan=4200m^2) was applied as standard and plants without any treatment were used as positive and negative control. The treatments were as follows: *P. fluorescens* (Pf); *B. subtilis* (Bs); *B. pumilus* (Bp); Pf + Bs; Pf + Bp; Bs + Bp and Pf + Bs + Bp and carbofuran. Six pots were used as replicates for each treatment as well as for positive and negative controls i.e. nematode alone and untreated pots. All pots were inoculated with Al-aukadin (containing nitrogen fixing bacteria namely *Bradyrhizobium* spp). Pots were arranged in a completely randomized design on a bench under screen house conditions maintained at 25 ± 5 °C. Then, the plants were irrigated as needed. After 3 months of nematode inoculation at harvest stage of cowpea, reproduction parameters of *M. incognita* such as numbers of J_2 in soil, galls and egg-masses in cowpea roots were recorded. The number of J_2 in the soil was extracted using a sieving and decanting technique (Barker, 1985). Plants of cowpea were carefully uprooted and washed thoroughly under running tap water to get rid of debris. Then, they were incubated in tap water by incubation method (Young, 1954) to help hatching J_2 of egg-masses. All J_2 number of nematodes was counted under a stereoscopic microscope (Nikon ECLIPSE, E400, Japan). Effects of Bs, Bp and Pf were recorded on plant growth parameters of cowpea including shoot length, fresh and dry shoot weights and fresh root weight. Further, pod number, fresh and dry weights of pods and weight of 100 seeds were also recorded. Total phenolic compounds were extracted from dry seeds and determined colorimetrically using Folin Ciocalteu phenol reagent according to the method defined by Snell & Snell (1957). The amount of soluble protein was determined by Bradford, 1976

method. Photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) in the fresh leaf were determined as the method described by Moran (1982).

Statistical analysis of data: Statistical analysis of the obtained data was performed through Computer Statistical Package (COSTAT) User Manual Version 3.03, Barkley Co., a computer based program. Analyses of data were performed by using analysis of variance (one way ANOVA) procedures. Duncan's Multiple Range Test (DMRT) was applied to compare the means for each treatment at 5% level of probability (Snedecor & Cochran, 1999).

Results

Effect of the bioagents on population density of *M. incognita*: Significant efficacy ($P \leq 0.05$) of the tested bacterial isolates has been demonstrated in controlling *M. incognita* infecting cowpea (Table 1). It was observed that the highest nematode reduction (89.0%) was achieved by using *P. fluorescens* (Pf) followed by the combined treatment of Pf+Bs (88.5%) and Pf + Bs + Bp (86.3%) compared to other treatments and untreated controls. The least average nematode reduction (71.8%) occurred by single treatment of Bs.

Effect of the bioagents on plant growth: Table 2 indicates significant ($P \leq 0.05$) efficacy of the tested bacterial isolates on cowpea growth parameters as influenced by root-knot nematode infectivity. It was observed that the highest plant growth (69.0%) was achieved by using the combined treatment of Bs+Bp followed by 55.6% for single treatment of Pf than combined treatment of Pf + Bs + Bp (54.8%) and the least one (19.7%) occurred by using the tested nematicide. Other isolates differed in their effect on plant growth parameters.

Effect of the bioagents on yield parameters and number of bacterial nodules: Table 3 illustrates the nematicidal effect of the tested bacterial isolates on the yield parameters of cowpea as influenced by root-knot nematode infectivity. It was observed that the highest increase (70.2%) was achieved by using Bs+Bp followed by 49.3% by using *B. pumilus* (Bp). The least increase (18.2%) was occurred by using the tested nematicide carbofuran. Number of bacterial nodules differed in their response to the different treatments as follows: The highest increase (116.7%) in the number of nodules was achieved by using the combined treatment; Pf + Bp followed by the combined treatment Bs + Bp (88.9%). The least increase (27.8%) occurred by using single treatment of *P. fluorescens* (Pf) and the combined treatment of Pf + Bs.

Effect of the bioagents on photosynthetic pigments: Chorophyll A and Chlorophyl B and Carotenoid contents as affected by different treatments of the tested bioagents were recorded in Table 4. It is well noticed that the contents significantly (($P \leq 0.05$) decreased at untreated check as compared to different applied treatments. The single treatment of *B. pumilus* (Bp) recorded for maximum total contents of photosynthetic pigments followed by the other single treatments. The combined treatments of Pf+Bs found to be significantly increased the contents of photosynthetic pigments than the other treatments either as single or combined treatments.

Effect of the bioagents on biochemical compounds: Phenolic compounds and soluble proteins influenced by different treatments of the tested bioagents are presented in Table 4. It is clearly noticed that all contents significantly ($P \leq 0.05$) increased by applying different treatments as compared to those of the untreated check. *P. fluorescens* (Pf) had shown maximum increase of phenolic compound contents as compared to the other single treatments. However, the combined treatment, Bs + Bp had maximum effect that significantly increase phenolic contents i.e. 3.67% as compared to the other treatments either single or combined treatments. As for soluble proteins, *P. fluorescens* (Pf) has given significantly highest contents percentage as compared the other single treatments. However, Pf + Bp as combined

treatment was recorded for significantly highest increase of soluble proteins as compared to other combined treatments whereas Pf+Bs was found to be less effective than that.

Discussion

Application of *P. fluorescens*, *B. subtilis* and *B. pumilus* significantly reduced infectivity of *Meloidogyne incognita* on cowpea in the present study. Growth parameters in terms of shoot fresh and dry weights, root fresh and dry weights, yield parameters and number of nodules per plant were found to be significantly increased in the plants treated with the three bacteria than those of single application and control. The most obvious inhibition of root-knot nematode was noticed in plants treated with *P. fluorescens*.

These results agree with those obtained by Akhtar *et al.*, (2012). It is interesting to note that the highest plant growth (69.0%) was achieved by using the combined treatment of Bs+Bp and the same treatment has given the highest yield increase (70.3%). However, *P. fluorescens* (Pf) caused moderate increase in plant growth parameters (55.8%).

It is well known that the many tested microorganisms are found in plant rhizosphere may cause different modes of action against nematodes. The modes include producing antibiotics, enzymes and toxins production. When they colonize, induce systemic resistance in plants against pathogens and improve plant growth (Tian *et al*., 2007; Lugtenberg & Kamilova, 2009).

Numerous microbes are antagonistic to plant-parasitic nematodes but few of these organisms are commercially available for management of these pathogens. Varying performance of the applied biocontrol agents has proven to be a primary obstacle for the development of successful commercial products. Biocontrol preparation for overcoming these different performances can be obtained by combining the disease-suppressive activity of two or more beneficial microbes. Such combinations have more potential for colonization of the rhizosphere that might be more consistent under a wide range of soil conditions and antagonistic effect to a larger number of plant pests or pathogens than strains applied individually. These results agreed with those obtained by Hasan *et al*., 2014. Conversely, microbes applied in combination may have antagonistic interactions with each other (Jaizme-Vega *et al*., 2006). Unfortunately, the ecological basis for increased or decreased suppression has not been determined in many cases and needs further investigations.

Bacillus and *Pseudomonas* supply phosphate to plants (Keneni *et al*., 2010). *P. fluorescens, B. subtilis* and *Pantoea* sp. were tested for their nematicidal effects on root-knot nematode (*Meloidogyne javanica*) in cucumber. *P. fluorescens* was the most effective isolate on the studied nematode control. Combined application of *P. fluorescens*, *B. subtilis* and *Pantoea* sp. was more effective against root-knot nematode than that of single and increased the growth of plant (Majzoob *et al*., 2012).

Results obtained by Osman *et al*., (2012) revealed that Salicylic acid or *P. fluorescens* at dilution of S/2 (10^8 CFU/ml/2) as soil drench significantly ($p \leq 0.05$) reduced nematode reproduction and improved plant growth as compared to untreated plants. The activities of the enzymes, peroxidase, polyphenol oxidase and chitinase increased in the treated plants as indicator of inducing resistance against nematode.

Abd El-Khair *et al*., (2016) reported that primary bioassay test of the thirty rhizobacteria (RB) isolates (*Bacilllus* spp.) against *M. incognita* J_2 showed that the nematode mortality ranged from 81-97%. RB isolates of banana, bean and cucumber reduced *M. incognita* J_2 ranging from 81-97%. RB isolates of banana, bean and cucumber caused the mortality of *M. incognita* J_2 ranging from 81-97%, 85-96% and 84-95%, respectively. These results agree with those obtained by Muhae-ud Din *et al*., 2018.

Table 1. Effects of *Pseudomonas fluorescens*, *B. subtilis* and *Bacillus pumilus* singly or in combination on *Meloidogyne incognita* parameters in cowpea under screenhouse conditions.

Treatments	*M. incognita* parameters								General Average %Red.
	No. of J_2 /pot		No. of hatched J_2 in roots		No. of galls in roots		No. of egg-masses in roots		
	Count	Red. %	Count	Red. %	Count	Red. %	Count	Red. %	
B. subtilis (Bs)	4.35b	75	2.38c	87	1.45b	59	1.26b	66	71.8
B. pumilus (Bp)	4.21c	82	2.23cde	91	1.26c	74	1.12b	76	80-8
P. fluorescens (Pf)	3.90f	91	2.12de	93	1.06d	83	0.75d	89	89.0
Pf + Bs	4.00e	89	2.26cd	90	1.01d	86	0.74d	89	88.5
Pf + Bp	3.83g	93	2.39c	87	1.27c	73	0.96c	83	84.0
Bs + Bp	3.83g	93	2.10e	93	1.21c	75	0.93c	83	86.0
Pf + Bs + Bp	4.15d	84	2.29c	89	1.08d	83	0.76d	89	86.3
Nematicide	3.99e	89	2.57b	80	1.34bc	68	1.14b	74	77.8
Nematode alone	4.95a	-	3.26a	-	1.83a	-	1.72a	-	-

Values are averages of six replicates Means followed by different letter(s) are significantly different according to DMRT at p≤0.05.Red.=Reduction

Table 2. Effects of *Pseudomonas fluorescens*, *Bacillus subtilis* and *B. pumilus* singly or in combination on vegetative growth parameters of cowpea infected by *Meloidogyne incognita* under screenhouse conditions.

Treatment	Growth parameters								General Average %Inc.
	Shoot weight				Root weight				
	Fresh		dry		Fresh		dry		
	Weight (g)	Inc. %	Weight (g)	Inc. %	Weight (g)	Inc. %	Weight (g)	Inc. %	
B. subtilis (Bs)	43.64d	53.2	11.25d	73.1	28.35d	35.6	2.15c	10.3	43.1
B. pumilus (Bp)	43.24e	51.8	9.74f	50.0	24.10f	15.3	2.42b	24.1	35.3
P. fluorescens (Pf)	51.97c	82.4	11.49c	76.8	28.53c	36.5	2.47b	26.7	55.6
Pf + Bs	42.97f	50.8	10.24e	57.0	27.50e	31.6	2.60ab	33.3	43.2
Pf + Bp	39.95g	40.2	10.12e	55.7	23.60g	12.9	2.60ab	33.3	35.5
Bs + Bp	57.27a	101	12.23a	88.2	32.23a	54.2	2.60ab	33.3	69.0
Pf + Bs + Bp	52.49b	84.2	11.67b	80.3	29.70b	42.1	2.20c	12.8	54.8
Nematicide	30.60h	7.4	7.59g	16.8	23.97f	14.7	2.73c	40.0	19.7
Nematode only	28.49i	-	6.50h	-	20.90h	-	1.95d	-	-

Values are averages of six replicates. Means followed by different letter(s) are significantly different according to DMRT at p ≤0.05.Inc.=Increase

Table 3. Effects of *Pseudomonas fluorescens*, *Bacillus subtilis* and *B. pumilus* singly or in combination on yield parameters of cowpea infected by *Meloidogyne incognita* under screenhouse conditions.

Treatment	Yield parameters								No. of bacterial nodules	
	Pod						100 seeds weight		Count	Inc.%
	Number		fresh weight		dry weight					
	Count	Inc. %	weight (g)	Inc. %	weight (g)	Inc. %	weight (g)	Inc. %		
B. subtilis (Bs)	2.86e	43.0	1.06d	12.8	0.87b	70.6	12.56c	17.4	27	50.0
B.pumilus (Bp)	4.09b	104.0	1.09c	16.0	0.77c	51.0	13.41a	25.3	30	66.7
P. fluorescens (Pf)	4.10b	105.0	1.08cd	14.9	0.72e	41.2	11.96e	11.8	23	27.8
Pf + Bs	3.17d	58.5	1.18b	25.5	0.90a	76.5	11.55f	8.0	23	27.8
Pf + Bp	2.85e	42.5	1.42a	51.1	0.74d	45.1	11.45g	7.9	39	116.7
Bs + Bp	5.80a	190	1.20b	27.7	0.71e	39.2	13.24b	23.7	34	88.9
Pf + Bs + Bp	3.64c	82.0	1.20b	27.7	0.74d	45.1	12.39d	15.8	30	66.7
Nematicide	2.61f	30.5	0.97e	3.2	0.65f	27.5	11.92e	11.4	29	61.1
Nematode only	2.00**g**	-	0.94f	-	0.51g	-	10.70h	-	18	-

Values are averages of six replicates. Means followed by different letter(s)are significantly different according to DMRT at p≤0.05.

Table 4. Biochemical changes in cowpea plants infected by *Meloidogyne incognita* as affected by *Pseudomonas fluorescens*, *Bacillus subtilis* and *B. pumilus* singly or in combination.

Treatments	Biochemical compounds					
	Photosynthetic pigments(mg/g)				Phenolic content %	Soluble protein content %
	CA	CB	Car.	Total		
Bacillus subtilis (Bs)	1.52c	0.69bcd	0.32a	2.53b	2.98bcd	1.57d
Bacillus pumilus (Bp)	2.11b	0.73abcd	0.22ab	3.06b	2.79cd	1.86b
Psuedomonas fluorescens (Pf)	1.76bc	0.59cd	0.17b	2.52b	3.38ab	2.01a
Pf + Bs	2.03bc	0.84abc	0.20ab	3.07b	3.46ab	2.00a
Pf + Bp	2.78a	1.01a	0.31a	4.10a	3.27abc	2.06a
Bs + Bp	1.51c	0.64bcd	0.16b	2.31b	3.67a	1.74c
Pf + Bs + Bp	1.90bc	0.92ab	0.16b	2.98b	3.11abcd	1.72c
Nematicide	1.87bc	0.66bcd	0.20ab	2.73b	2.55de	1.40e
Nematode only	1.50c	0.51d	0.21ab	2.22b	2.20e	1.24f

Values are averages of six replicates. Means followed by different letter(s)are significantly different according to DMRT at p≤0.05.
CA= Cholorophyll A, CB= Chlorophyll B, Car. =Carotenoids

The complexity of interactions involved in the application of multiple organisms for biological control has shown slow progress toward development of successful formulations. However, an approach has potential for overcoming some of the efficacy problems by application of individual biocontrol agents (Meyer & Roberts, 2002). As for biochemical changes in cowpea seeds or roots, the studied biochemical compounds increased by applying different treatments, these findings are conformed with the results obtained by El-Nagdi *et al*., (2014) and Youssef *et al*., (2015).

Mechanism of resistance against plant parasitic nematodes included formation of some phenolic compounds in resistant plants (Bajaj & Mahajan, 1977; Giebel, 1982). Resistance against nematodes has been correlated with the levels of performed phenol in roots in certain plant cultivars (Narayana & Reddy, 1980). The results of chlorophyll in fresh leaves have shown consistency with the study carried out by Akhtar *et al*., (2012).

Conclusion

The present studies showed that efficient management of root-knot nematode problem may be carried out by using some antagonistic microorganisms either alone or combined with each other. These treatments not only annihilate the pathogenic effect of the nematodes, but also increased plant growth and yield, thus avoiding toxicity and hazardous nature of chemical nematicides in the environment. However, it is necessary to further affirm the results under field conditions and various synergistic mechanisms involved should be properly discussed.

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