Effect of integration of two bacterial bioagents and a plant residue extract for biocontrolling root-knot nematode, *Meloidogyne incognita* infesting potatoes

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Received: 28 August 2022
Revised: 5 October 2022
Accepted: 9 October 2022
Published: 9 March 2023

Egyptian Pharmaceutical Journal 2023, 22:67–77

Background
Recently, there has been an increasing attempt to explore nature-friendly compounds that could be substitutes for chemically synthesized products. It was found that some plant residues and certain microorganisms, including antagonistic bacterial species such as *Bacillus* spp. associated with plants, can act as biocontrol agents, achieving various degrees of control against *Meloidogyne incognita*, as well as increasing the plant growth and yield parameters.

Objectives
This research was designed to study the effect of *Bacillus subtilis* (Bs) and *B. pumilus* (Bp) alone or in combination with pomegranate peel aqueous extract (PP) on root-knot nematode, *M. incognita*, infesting potato cv. Spunta, as well as to examine the biochemical changes and total microbial counts under field conditions.

Materials and methods
Overall,100 g of crushed pomegranate fruit peel (PP) water extract and two bacterial biocontrol agents were applied in a field experiment for controlling root-knot nematode, *M. incognita.* These bacteria*, B. subtilis* (Bs) and *B. pumilus* (Bp), were isolated from rhizosphere soil and identified according to standard microbiological characteristics. In a field naturally infested with *M. incognita*, potato cv. Spunta tubers were planted during winter growing season. After planting, each of the bacteria was added in the soil at the tested rate (10^7–10^9 CFU/ml). Moreover, some tubers that were planted in the soil served as untreated control. The treatments included (a) *B. subtilis* (Bs)+pomegranate peel (PP) residue extract, (b) *B. pumilus* (Bp)+PP residue extract, (c) PP residue extract +medium (M), (d) Bs, (e) Bp, (f) PP residue extract, (g) medium (M), and (h) untreated control.

Results and conclusions
Based on the percentages of juvenile reduction in soil at the harvest time, Bs+PP recorded 84.0% juvenile reduction followed by Bp+PP (82.3%), revealing combined treatments to be more effective than single treatments (78.4% by Bs and 72.8% by Bp). The examination of co-toxicity of the two applied combined treatments at the harvest stage showed synergistic effects. All treatments significantly ($P \leq 0.05$) increased plant growth and yield criteria, especially individual treatments. Biochemical compounds and the total bacterial and fungal counts in potato rhizosphere varied with different treatments. It could be concluded from the present study that the combined treatments of *B. subtilis* or *B. pumilus* +PP inhibited *M. incognita* proliferation in potatoes more than single treatments. However, single treatments improved plant growth and yield more than the combined cases. Biochemical changes and microbial counts of potatoes were influenced by different treatments.

Keywords:
Bacillus pumilus, *Bacillus subtilis*, integration, *Meloidogyne incognita*, pomegranate residue extract, potato

Egypt Pharmaceut J 22:67–77

1687-4315

Introduction

Potato (*Solanum tuberosum* L.) is an important tuber crop grown in Egypt for either local consumption or export [1]. Potatoes are attacked by many pests and pathogens that cause significant losses in yield [2]. Among them, plant-parasitic nematodes (PPNs) represent the most important factors causing yield loss in quantity and quality [1,3]. Anatomical alterations in potato root tissues by root-knot

nematode, *Meloidogyne incognita*, in some potato cultivars, have been shown [4]. The symptoms depend upon their degree of susceptibility to this pest.

The application of chemical nematicides that are widely applied for controlling of PPNs, fungi, and bacteria is limited in many developing countries owing to non-availability and because they pollute environment and cause toxic hazards to human, plants, and domestic animals. Biological control of root-knot nematodes is essential for controlling phytoparasitic nematodes as an alternative strategy [5,6]. It is well known that certain beneficial rhizospheric microorganisms can act as biocontrol agents, achieving various degrees of control against root-knot nematodes as well as increasing plant growth and yield. One of them is the bacterium *Bacillus,* which can grow in the rhizosphere of most plants. Biological control of PPNs by using *Bacillus* spp. has been found to be a feasible option [7–11]. They have a great effect on PPNs owing to their ability to colonize roots and sporulation under stressed conditions [12].

Now, attempts have been carried out to substitute nature-friendly compounds instead of chemical products. Certain plants have been found to contain substances and products with the greatest therapeutic potential. Pomegranate contains antioxidants that have a vital role in various pharmacological activities, including anti-aging and anti-cancer activities [13]. An aqueous extract of pomegranate significantly reduced root-knot nematode, *Meloidogyne javanica*, infestation and increased plant growth, but its powder form exhibited phytotoxicity activities [14]. Pomegranate peel aqueous extract caused reduction in the number of nematodes in roots of date palm [15], sugar beet [16], and cucumber [17] plants infected by root-knot nematode, *M. incognita*.

The aim of this work was to clarify the effect of *Bacillus subtilis* (Bs) and *B*acillus *pumilus* (Bp) alone or in combination with pomegranate peel aqueous extract (PP) on root-knot nematode, *M. incognita*, as well as to examine the total microbial count and frequency % of common fungi on potato tubers under field conditions.

Materials and methods

Source and identification of potato tubers

Potato (*S. tuberosum* L.) cv. Spunta tubers were sourced and identified by vegetative Research Institute, Ministry of Agriculture and Land Reclamation, Egypt, and planted in this work.

Plant residue extract

Overall, 100 g of crushed pomegranate fruit peels (PP) was sourced from Horticultural Research Institute, Ministry of Agriculture and Land Reclamation, Egypt. They were soaked in 1 l of distilled water for 3 days and filtered through Whatman filter paper no.1. The filtrate (10%) was added to each plant at a rate of 200 ml.

Preparation of bacterial biocontrol agents

Two bacterial biocontrol agents were applied in a field experiment for controlling root-knot nematode, *M. incognita*. These isolates, *B. subtilis* (Bs) and *B. pumilus* (Bp), were isolated from rhizosphere soil and identified in the Plant Pathology Department according to standard microbiological characteristics. They were prepared separately and inoculated in nutrient sucrose (2%) broth medium (beef extract 3.0 g; peptone 5.0 g; glucose 10.0 g in 1.0 l of distilled water and adjusted pH 7.4±0.2). Incubation at 28°C for 48 h was carried out for the cultures. Then, an inoculum of 107–109 colony-forming unit (CFU)/ml by turbidity method was adjusted for inoculation [18]. A mixture of bacterial cells and cultural filtrate for each bacterial species was used as an inoculum [19].

Field experiment

An experiment in a field naturally infested with *M. incognita*, in a completely randomized block design, was conducted in Mansouryia village, Giza Governorate, Egypt, during the period from January 15 to May 17, 2020. The experiment was divided into rows, each of 3 m in length and 75 cm in width, and the distance among plants was 20 cm with one row for each biocontrol agent treatment and residue extract as well as untreated control. Recommended irrigation and fertilization were performed without adding any chemicals [2].

Potato cv. Spunta tubers were planted during winter growing season of 2020. After planting, each bacterial species was added into the soil at the tested rate (10^7 10^9 CFU/ml) in four holes around the plant. One tuber was planted per pit (Hill). There were five replicates (Hills) for each treatment. Equal replicates of tubers were planted in soil without any treatment as a control. The treatments included (a) *B. subtilis* (Bs) +pomegranate peel (PP) residue extract, (b) *B. pumilus* (Bp)+PP residue extract, (c) PP residue extract+medium (M), (d) Bs, (e) Bp, (f) PP residue extract, (g) medium (M), and (h) untreated control.

Meloidogyne adult females were sourced from galls found in potato roots and identified as *M. incognita*

based on their cuticular perineal pattern morphological characteristics [20].

The numbers of *M. incognita* nematode J_2s in the soil, 1 week before planting (initials) and after treatment at the mid-season after 2 months were determined in the soil. At the end of the growing season (at harvest), 4 months later, plants were carefully removed, and extraction of nematode J_2s in 250 g of soil was done. Soil was sieved and decanted [21]. Nematode parameters, including number of juveniles in the soil at mid-season and the number of juveniles in soil and roots and egg masses in roots and number of galls, were recorded at the harvest time in treated and untreated potato plants. The percentages of reduction for each nematode parameter were calculated according to the following formula:

Nematode reduction = (1–(PTA/PTB)–(PCB/PCA)×100 according toHenderson and Tilton formula [22].

Where PTA and PTB represent the number of each nematode parameter (P)in the treated plot (T) after (A) and before (B) application, respectively, and PCB and PCA represent the number of each nematode parameter (P) in the check plot (C) before (B) and after (A) application, respectively.

Co-toxicity of the tested materials was based on the percentages of reduction of juveniles in the soil in the combined treatments using the following formula:

E=X+Y-XY/100 according to Lempel's formula [23]

Where:

E=effect that expected for the mixture.

X and Y represent the effect due to each of single treatment A and Y alone, respectively.

The expected effect of mixture, calculated as additive or synergistic and antagonistic action, was based on the following equation [24]:

$$\text{Co-toxicity}=\frac{\text{Observed effect (\%)} - \text{Expected effect (\%)}}{\text{Expected effect (\%)}} \times 100$$

The obtained results can be classified as follows: +20 or more indicates potentiation, –20 or more is antagonistic, and immediate values ranging from –20 to+20 indicate additive or synergistic.

Some plant growth and yield parameters in potato plants were recorded. The percentages of increase in weight of branches and number and weight of tubers/plant were calculated.

Total soluble carbohydrates and total carbohydrates were determined in potato tubers using the colorimetric method [25]. Extraction of total phenolic compounds was performed from potato tubers and determined calorimetrically [26] using Folin-Ciocalteu phenol reagent. Photosynthetic pigments (chlorophyll a, chlorophyll b, and carotenoids) in the fresh leaves were determined [27].

Effect of Bs and Bp alone or combined with pomegranate peel extract (PP) on total counts of aerobic bacteria, spore-forming bacteria, and fungi in the rhizosphere of potato plants was carried out, 1 week before planting, after planting at mid-growing season, and at the end of growing season (at harvest time) by the dilution method using the plate count technique on suitable media [28,29]. Five soil samples (each 200 g soil) were taken from each plot at a depth of 15–30 cm, and then, the collected samples of each plot were used. Overall, 10 g of each collected soil sample was separately shaken in 90-ml of sterilized distilled water in a 250-ml flask to give a dilution of 10^{-1}. Then, serial dilutions of each fresh soil sample suspension were prepared up to 10^{-7}, by transferring 1 ml of sample suspension to 9 ml of sterilized distilled water in a test tube under sterile conditions. Four plates were prepared as replicates for each dilution of soil sample. Aliquots of 1.0 ml of 10^{-5}–10^{-7} dilution were transferred onto separated sterilized Petri plates filled with nutrient agar (NA) medium (peptone 5 g, beef extract 3 g, agar 15 g, distilled water 1L, pH 7) for determining the aerobic bacterial count. The resulted bacteria were recorded after 2 days of incubation at 30 ±2°C, as a number of CFU/10 g of soil. The sample dilution of 10^{-1} was pasteurized at 80°C for 20 min to determine the spore-forming bacteria count. Aliquots of 1.0 ml of each 10^{-3}–10^{-5} dilution were transferred onto separated sterilized petri plates filled with NA. The plates were incubated for 2 days at 28±2°C. The resulting spore-forming bacteria were recorded as CFU/10 g of soil. Aliquots of 1.0 ml of each 10^{-3} and 10^{-4} dilution were transferred onto separated sterilized petri plates filled with Martin medium (glucose 10 g, peptone 5 g, KH_2PO_4 1 g, $MgSO_4$ 0.5 g, Rose Bengal 30 µg, streptomycin 0.03 g, and agar 15 g). Distilled water (1 L) was used for counting the total fungi. Incubation at 30±2°C for 7 days was done to the inoculated plates, and then, the counts of the resulting fungi were recorded. Then, the total microbial counts were recorded as averages of counts for the aforementioned periods [30].

Effect of Bs and Bp alone or combined with PP extract on the frequency percent of common fungi in the potato rhizosphere was determined as CFU/10 g of soil on Martin medium as mentioned before [29]. There were five replicated plates per soil sample prepared for each dilution. The plates were incubated at 30±2°C for 7 days. Identification of the resulted fungi to the level of genus and species was based on the key of morphological and cultural characteristics [31,32]. Each isolated fungal genus or species was counted and its percentage frequency was calculated as averages of counts at three above periods according to the following formula:

Frequency of common mycoflora (%) = (fungus no./ total fungi no.) × 100

Data analysis

This experiment was laid out in a randomized block design. Analysis of variance test was performed for determining significance at *P* value less than or equal to 5% level of probability of the obtained data. Duncan's Multiple Range Test by Snedecor and Cochran [33] was used for mean separation. This was done by Computer Statistical (COSTAT) software.

Results

Effect on nematode parameters

From Table 1, the treatments of each of *B. subtilis* (Bs) and *B. pumilus* (Bp) significantly ($P \leq 0.05$) decreased nematode parameters as indicated by the number of the second-stage juveniles (J_2s) in soil at mid-season and number of J_2s in soil and roots and number of egg masses and galls on roots at the harvest stage. Based on the percentages of nematode reduction in soil, at mid-season, the different treatments decreased the number of J_2s at different degrees. At the harvest time, the combined treatment Bs+PP recorded 84.0% nematode reduction in soil followed by Bp+PP (82.3%). In single treatments, Bs registered less percentages of nematode reduction (78.4%) followed by percentage reduction (72.8%) by Bp compared with their combined treatments and untreated control. PP aqueous extract and medium (M) registered nematode reductions in soil by 52.2 and 45.4%, respectively. However, PP extract + M as combined treatment recorded nematode reduction of 56.3%, being higher than those recorded for single ones. Other nematode parameters differed in their reductions according to the tested materials.

Co-toxicity values were calculated for the two applied combined treatments of Bs or Bp with PP extract and PP+M in soil at the harvest stage. They exhibited additive or synergistic action for reduction of juveniles in potatoes. The combination between PP +M recorded antagonistic interaction (Table 2).

Effect on plant growth

As for plant growth of potatoes plants in Table 3, all treatments significantly ($P \leq 0.05$) increased plant growth criteria as indicated by length of branches and number of leaves per plant at mid-season and length and weight of branches and number of leaves per plant at the harvest stage compared with controls.

Table 1 Effect of *Bacillus subtilis*, *B*acillus *pumilus*, and pomegranate peel residue in single or combination on *Meloidogyne incognita* parameters in potatoes under field conditions

	Initial and mid-season nematode J_2 numbers in 250 g of soil		Number of nematode parameters in 5 g of roots and 250 g of soil at the harvest time			
Treatment	Initial	Mid-season	Final J_2s in soil	J_2s in roots	Galls	Egg masses
Bacillus subtilis+ pomegranate peel residue extract(Bs + PP)	440a	352b (82.2)	315h (84.0)	185f (54.1)	535b (24.4)	333d (40.3)
Bacillus pumilus +pomegranate peel residue extract (Bp + PP)	452a	245d (87.9)	359g (82.3)	138g (65.8)	166e (76.6)	91f (83.7)
Pomegranate peel residue extract + medium (PP + M)	436a	196e (90.0)	854d (56.3)	313c (22.3)	512b (27.7)	437b (21.7)
Bacillus subtilis (Bs)	428a	277c (85.6)	415f (78.4)	213e (47.1)	450c (36.4)	371c (33.5)
Bacillus pumilus (Bp)	430a	258d (86.6)	525e (72.8)	173f (57.1)	312d (55.9)	175e (68.6)
Pomegranate peel residue extract(PP)	438a	352b (82.1)	938c (52.2)	349b (13.4)	171e (75.8)	72g (87.1)
Medium (M)	437a	351b (82.1)	1070b (45.4)	249d (38.2)	316d (55.4)	172e (69.2)
Untreated Control	435a	1950a (0)	1950a (0)	403a (0)	708a (0)	558a (0)

Each value is average of five replicates (Hills). Means followed by the same letter in each column are not significantly different according to Duncan's new multiple range test ($P \leq 0.05$). Values of initial population were transformed to $\sqrt{x}$ before statistical analysis.
Figures between brackets indicate the percentages of nematode reduction.

Table 2 Type of interaction between pomegranate extract and bacterial isolates on potatoes infested with *Meloidogyne incognita*

Treatment	Effect based on percentages of juveniles reduction in soil at harvest stage		Co-toxicity	Type of interaction
	Expected	Observed		
Bacillus subtilis+Pomegranate residue extract(Bs + PP)	89.68	84.0	–5.7	Additive or synergistic
Bacillus pumilus + Pomegranate residue extract (Bp + PP)	87.0	82.3	–5.4	Additive or synergistic
Pomegranate residue + medium (PP+M)	73.90	56.3	–23.8	Antagonistic

Table 3 Effect of *Bacillus subtilis*, *B*acillus *pumilus,* and pomegranate extract in single or combination on growth parameters of potatoes infested by *Meloidogyne incognita* under field conditions

Treatments	Mid-season		Harvest stage			
	Branch length (cm)	Leaf No.	Branch length (cm)	Leaf No.	Branch weight (g)	%Increase
*B*acillus *subtilis*+ pomegranate residue (Bs + PP)	21ab	20b	45ab	23b	47b	135.0
*B*acillus *pumilus* + Pomegranate residue (Bp + PP)	25a	19b	46a	24b	45bc	125.0
Pomegranate residue + medium (PP + M)	18b	10cd	34bc	13de	43bc	115.0
*B*acillus *subtilis* (Bs)	18b	10cd	39ab	13de	48b	140.0
*B*acillus *pumilus* (Bp)	18b	28a	40ab	41a	59a	195.0
Pomegranate residue (PP)	16b	13c	34bc	16cd	35cd	75.0
Medium (M)	11c	12c	32c	18c	29de	45.0
Untreated control	11c	7d	29d	9e	20e	-

Each value is average of five replicates (Hills). Means followed by the same letter(s) in each column are not significantly different according to Duncan's new multiple range test ($P \leq 0.05$).

Table 4 Effect of *Bacillus subtilis*, *B*acillus *pumilus* and pomegranate extract in single or combination on yield parameters of potatoes infested by *Meloidogyne incognita* under field conditions

Treatments	Tuber no./ plant	% Inc.	Tuber weight/plant (g)	% Inc.	Average weight of tuber (stem)/plant(g)	% Inc.
Bacillus subtilis+ pomegranate residue extract (Bs +PP)	5a	150	600b	387.8	120b	95.1
Bacillus pumilus + pomegranate residue extract (Bp +PP)	5a	150	500c	306.5	100c	62.6
Pomegranate residue extract + medium (PP + M)	3a	50	310d	152.0	103.3c	68.0
Bacillus subtilis (Bs)	3a	50	530c	330.9	176.6a	187.5
Bacillus pumilus (Bp)	4a	100	710a	477.2	177.5a	188.6
Pomegranate residue extract (PP)	3a	50	290d	135.8	96.6c	57.1
Medium (M)	4a	100	170e	38.2	42.5e	–30.9
Untreated control	2a	0	123e	0	61.5d	0

Each value is average of five replicates (Hills). Means followed by the same letter in each column are not significantly different according to Duncan's new multiple range test ($P \leq 0.05$). Inc.=Increase.

On the basis of the percentages of increases of weight of branches, it was recorded that the highest percentages of increases (140 and 195%) were by using each of Bs and Bp as single treatments, respectively, at the harvest stage. The combined treatments of each of the two microorganisms + PP caused less percentages of branch weight increases (135 and 125%, respectively), compared with those of single treatments and control.

Effect on yield

As for yield of potato tubers in Table 4, individual treatments of each of Bs and Bp recorded the highest percentage increases of mean weights of tubers 187.5 and 188.6%, respectively, which were higher than those achieved by the combined treatments (Bs or Bp+PP extract), as they were 95.1 and 62.6%, with increases in only mean yield of tubers, respectively.

Effect on photosynthetic pigments

Chlorophyll A, chlorophyll B, and carotenoid contents were affected by different treatments, which were recorded in Table 5. It was well noticed that their total contents recorded the significant maximum by using the single treatment Bp followed by PP+M as compared with different applied treatments.

Effect on biochemical compounds

Total carbohydrates, polysaccharides, and phenolic compounds were influenced by different treatments of the tested materials and were presented in Table 5. It was clearly noticed that their contents increased by bacterial, PP, or M treatments compared with those of control. The combined treatment Bp+PP recorded significant maximum contents of the previous compounds followed by that of Bs+PP, regarding total carbohydrates and polysaccharides only. PP residue extract+M recorded the lowest contents of total carbohydrates and polysaccharides. As for soluble carbohydrates, Bs and Bs+PP recorded the highest contents followed by the combined treatment, Bp+PP and PP. The lowest content of soluble carbohydrates was recorded by M only.

Effect on microbial populations

The numbers of total counts of aerobic bacteria, spore-forming bacteria, and fungi in potatoes associated with its rhizosphere as affected by the different treatments at mid-season and at end of the growing season were listed in Table 6. The total aerobic bacteria count was increased through the growing season in the ranges of 7.07–7.47 and 7.21–7.73 $\log_{10}$ CFU/10 g soil with treatments of M, PP+M, and PP, PP+M at mid-season and end-season, compared with 6.88–7.15 and 6.70–7.13 CFU/10 g soil before planting, respectively. In untreated control, the aerobic bacterial counts were 6.62, 6.78, and 7.00 CFU/10 g soil before planting, at mid-season, and at end-season, respectively. At the end of season, the highest count of aerobic bacteria was recorded with PP+M (7.73), followed by Bp, M, Bs, Bs+PP, and PP, being higher than the untreated control at the end of season (7.00). The total spore-forming bacterial count also was increased through the growing season in the ranges of 4.95 to 5.41 and 5.14 to 5.53 $\log_{10}$ CFU/10 g soil with above treatments at mid-season and end-season, compared with 4.72–5.25 CFU/10 g soil before planting. In untreated control, the spore-forming counts were 4.78, 5.00, and 5.11 CFU/10 g soil before planting, mid-season and end-season, respectively. At the end of season, the highest spore-forming bacterial count was recorded with Bs (5.53) followed by PP+M, M, Bp, Bp+PP, PP, and Bs+PP, being higher than untreated control (5.11) at the same period.

The total fungal count also was increased through the growing season in the ranges of 4.95–5.22 and 5.12–5.35 $\log_{10}$ CFU/10 g soil with the above treatments at mid-season and end-season, respectively, compared with 4.83–5.11 $\log_{10}$ CFU/10 g soil before planting. At the end of season, the highest fungal count was recorded with Bs (5.35) followed by Bs+PP, PP+M, Bp+PP, PP, M, and Bp,

Table 5 Biochemical changes in potato tubers and photosynthetic pigments in potato leaves infested by *Meloidogyne incognita* influenced by bacteria, *Bacillus subtilis* and *Bacillus pumilus* and pomegranate peel extract singly or in combinations

Treatments	Photosynthetic pigments (mg/g fresh weight)				Fresh weight (mg/g)			Phenolic content %
	Chlorophyll a	Chlorophyll b	Carotenoids	Total	Total carbohydrates	Soluble carbohydrates	Polysaccharides	
Bacillus subtilis+ pomegranate peel residue extract (Bs + PP)	9.46e	2.87d	1.49e	13.82f	798.88b	40.20a	758.68b	3.10a
Bacillus pumillis + pomegranate peel residue extract (Bp + PP)	11.27d	3.50cd	1.92c	16.69e	806.79a	39.82b	766.97a	4.36a
Pomegranate peel residue extract + medium (PP+M)	14.49b	4.31ab	2.00b	20.80b	778.43de	38.24c	740.19e	3.78a
Bacillus subtilis (Bs)	11.72cd	3.47cd	1.93c	17.12de	785.97d	40.66a	745.31d	3.37a
Bacillus pumilus (Bp)	17.47a	4.79a	2.31a	24.57a	790.16c	38.79c	751.37c	3.31a
Pomegranate peel residue extract(PP)	12.87c	4.14bc	1.42e	18.93c	790.94c	39.19b	751.75c	3.73a
Medium (M)	12.72cd	3.61c	1.92c	18.25cd	787.00d	33.92d	753.08c	3.40a
Untreated control	12.28cd	3.66c	1.81d	17.75de	776.11e	39.86b	736.25e	3.07a

Each value is average of five replicates (Hills). Means followed by the same letter(s) in each column are not significant different according to Duncan's new multiple range test ($P \leq 0.05$). Inc.= Increase.

Table 6 Effect of *Bacillus subtilis* and *B*acillus *pumilus* alone or combined with pomegranate peels on total counts of aerobic and spore-forming bacteria and fungi in potatoes before planting, at mid-season and at harvest time under field conditions

	Log 10 CFU Total microbial counts[1]								
	Aerobic bacteria 10^{-4}			Spore-forming bacteria 10^{-4}			Fungi 10^{-4}		
Treatments	B	M	E	B	M	E	B	M	E
Bacillus subtilis+ Pomegranate peel extract (Bs + PP)	7.10ab	7.20bc	7.33d	4.72c	4.95d	5.14c	5.04ab	5.19a	5.31ab
Bacillus pumilus + Pomegranate peel extract (Bp + pp)	7.15a	7.28b	7.42c	5.06ab	5.13d	5.36b	4.98b	5.05b	5.24bc
Pomegranate peel extract+ medium (PP+ M)	7.13ab	7.47a	7.73a	4.96bc	5.18cd	5.52a	4.84cd	5.17a	5.31ab
Bacillus subtilis (Bs)	7.00bc	7.33b	7.41c	5.25a	5.41a	5.53a	5.11a	5.22a	5.35a
Bacillus pumilus (Bp)	7.00bc	7.47a	7.60b	5.11ab	5.32ab	5.45ab	4.83d	4.95bc	5.12de
Pomegranate peel extract (PP)	6.70bc	7.10c	7.21e	4.84cd	5.01e	5.18c	4.95bc	5.05b	5.19cd
Medium (M)	6.88c	7.07c	7.42c	4.96bc	5.25bc	5.48a	4.95bc	5.00bc	5.16cd
Untreated control	6.62d	6.78d	7.00f	4.78cd	5.00e	5.11c	4.59e	4.90c	5.04e

B=before planting, M=at mid-growing season, and E=at harvest. Means followed by the same letter(s) in each column are not significantly different according to Duncan's new multiple range test ($P \leq 0.05$).

being higher than untreated control (5.04) at the same period (Table 6).

Effect on common fungal frequency %

Results showed that *Aspergillus* spp., *Aspergillus niger*, *Penicillium* spp., *Penicillium chrysogenum, Penicillium citrinium, Rhizopus nigricans, Fusarium* spp., *Trichoderma* spp., *Rhizoctonia* spp., and others (unidentified fungi) were the most common fungi in the potatoes rhizosphere. Data of the percentages of fungal frequencies were illustrated in Table 7. The treatments of Bp+PP and Bp only increased the frequencies of *Aspergillus* spp. at mid (15.8 and 20.0%) or end of seasons (15.0 and 16.0%), being higher than those frequencies before planting (10.0 and 15.0%), and untreated control (11.1 and 13.0) at the same periods, respectively. The treatments of Bs +PP, PP+M, and M reduced the frequencies of *Aspergillus* spp. during the growing season, than before planting, especially at the end of season. However, Bs and PP increased the frequencies of the same fungal genus at mid-season (19.1 and 19.1%) and then, the treatments reduced it at the end of season (16.0 and 12.5%), compared with those recorded before planting (18.2 and 13.6%, respectively), but higher than untreated control at the same period. Results also revealed that the treatments of Bs+PP, Bp+PP, and Bp highly increased the frequencies of *Penicillium* spp. at two times of growing season, especially at mid-season, than before planting. The treatments of PP+M, Bs, and M only highly increased the frequency of this fungal genus at mid-season, being higher than those at end-season and before planting. The treatment of PP highly reduced fungal genus frequency during the growing season, especially at mid-season (14.3%), than that recorded at the end of season (16.7%) or before planting (18.2%), but higher than untreated control (11.1%) at the same period (Table 7). The treatments of Bp and PP increased the frequencies of fungi *Trichoderma* spp. during growing season, especially at the end of season (12.0 and 16.7%), than those before planting (10.5 and 13.6%, respectively). The treatments of Bs+PP, PP+M, and M only increased the frequencies of this fungal genus at end of season, than mid-season or before planting. The Bp+PP increased the frequency of that genus at mid-season (15.8%), than before planting or at the end season (15.0 and 15.0%), respectively, and untreated control (11.1%) at mid-season (Table 7).

The treatments of Bs+PP, Bp+PP, Bs, Bp, and PP highly reduced the frequencies % of *Fusarium* spp. at the end of season, than those recorded before planting or at mid-season. The treatment of PP+M highly reduced the fungal frequency at mid-season (11.1%), than at end of season (13.6%) or before planting (18.8%) at the same treatment. Most treatments highly reduced the frequency of *Rhizoctonia* spp. at mid-season compared with those at end-season and before planting. Bp and PP highly reduced the frequencies of that fungal genus at mid-season (5.0 and 4.8%), being less than those before planting (10.5 and 9.1%) and untreated control (11.1%) at mid-season (Table 7), but the frequencies of that genus increased at the end of season (8.0 and 8.3%).

Discussion

This study proved that each of *B. subtilis*, *B. pumilus*, and/or pomegranate peel extract had suppressive effect against *M. incognita* on potatoes with consecutive increase of the plant yield and growth parameters. Interestingly, the unusual quality of *M. incognita*-infected potato tubers, as illustrated [1], was much improved owing to the applied treatment. In the

Table 7 Effect of *Bacillus subtilis* and *B*acillus *pumilus* alone or combined with pomegranate peel extract on frequency % of common fungi in potatoes before planting, at mid-season, and at harvest time under field conditions

Treatments	Time	Frequency % of common fungi									
		Aspergillus Spp.	*Aspergillus* niger	*Penicillum* spp.	*Penicillum chrysogenum*	*Penicillum citrinum*	*Rhizopus nigricans*	*Fusarium* spp.	*Trichoderma* spp.	*Rhizoctonia* spp.	Others
Bacillus subtilis+ pomegranate peel extract (Bs + PP)	B	15.0a	5.0c	15.0a	5.0c	5.0c	5.0c	15.0a	15.0a	10.0b	10.0b
	M	14.6b	0.0e	33.8a	4.8d	4.8d	4.8d	14.3b	14.3b	9.5c	9.4c
	E	13.6c	4.6e	22.7a	9.1d	4.6e	4.6e	13.6c	18.1b	4.6e	4.5e
Bacillus pumilus+ Pomegranate peel extract (Bp + PP)	B	10.0b	5.0c	15.0a	10.0b	0.0d	10.0b	15.0a	15.0a	10.0b	10.0b
	M	15.8b	5.3d	21.1a	10.5c	0.0e	5.3d	10.5c	15.8b	5.3d	10.4c
	E	15.0b	5.0d	25.0a	10.0c	5.0d	5.0d	10.0c	15.0b	5.0d	5.0d
Pomegranate peel extract +medium (PP + M)	B	18.8a	6.3c	18.8a	6.3c	0.0d	6.3c	18.8a	12.4b	6.3c	6.1c
	M	16.7b	5.6d	22.2a	5.6d	0.0e	11.1c	11.1c	11.1c	5.6d	11.0c
	E	13.6b	9.1c	18.1a	4.6d	4.6d	9.1c	13.6b	13.6b	4.6d	9.1c
Bacillus subtilis (Bs)	B	18.2b	9.1d	22.7a	4.6e	4.6e	4.6e	13.6c	9.1d	9.1d	4.4e
	M	19.1b	9.5d	23.8a	9.5d	0.0f	4.8e	14.3c	9.5d	4.8e	4.7e
	E	16.0b	8.0d	20.0a	8.0d	4.0e	8.0d	12.0c	12.0c	4.0e	8.0d
Bacillus pumilus (Bp)	B	15.8a	5.3c	18.2a	10.5b	0.0d	5.3c	15.8a	10.5b	10.5b	10.5b
	M	20.0b	10.0d	25.0a	5.0e	0.0f	5.0e	15.0c	10.0d	5.0e	5.0e
	E	16.0b	8.0d	20.0a	8.0d	0.0e	8.0d	12.0c	12.0c	8.0d	8.0d
Pomegranate peel extract (PP)	B	13.6b	9.1c	18.2a	0.0e	9.1c	4.5d	13.6b	13.6b	9.1c	9.2c
	M	19.1a	9.5c	14.3b	9.5c	4.8d	4.8d	9.5c	14.3b	4.8d	9.4c
	E	12.5b	8.3c	16.7a	8.3c	4.2d	8.3c	8.3c	16.7a	8.3c	8.4c
Medium (M)	B	19.1a	4.8d	14.3b	9.5c	4.8d	4.8d	14.3b	14.3b	9.5c	4.6e
	M	12.5c	8.3d	20.8a	8.3d	8.3d	4.2e	16.7b	12.5c	4.2e	4.2e
	E	11.1c	7.4d	18.5a	7.4d	7.4d	7.4d	14.8b	14.8b	3.8e	7.4d
Untreated control	B	14.3b	4.8e	19.1a	4.8d	4.8e	4.8e	14.3b	9.5c	14.3b	9.3d
	M	11.1b	5.6c	11.1b	5.6c	5.6c	5.6c	22.2a	11.1b	11.1b	11.0b
	E	13.0b	8.7c	17.4a	4.4d	4.4d	8.7c	17.4a	8.7c	8.7c	8.6c

B=before planting, M=at mid-growing season and E=at harvest. Means followed by the same letter in each column are not significantly different according to Duncan's new multiple range test ($P \leq 0.05$).

present study, it was noticed that the combined treatments of the tested bacteria plus pomegranate extract recorded higher percentages of nematode reduction in soil at harvest stages than those achieved by their single ones, indicating that a synergistic action in reducing nematode parameters occurred. The effect of pomegranate extract was documented on root-knot nematode by several authors [15,16,34]. However, each solely applied microorganism achieved higher percentages of tuber and branch increases than those occurred by combination, which may be explained by that nutrients were faster absorbed via potato roots in the single case. This may be because pomegranate extract in combined treatments improved plant growth less than that in single treatments. As a result, fewer percentages of yield and branch increases occurred in combined case, which may be due to that plant growth was affected by pomegranate extract, as it has been reported that pomegranate powder amendment exhibited a phytotoxicity activity compared with the untreated plants [14].

Nematode reductions could be referred to that the tested rhizospheric bacteria possibly can act through different ways against PPNs by antibiotics, enzymes, and toxins produced by them. Systemic resistance of plants against nematodes can be induced by these substances [35–39]. When single treatment of *Bacillus* spp. was applied at different rates or three different times, it could control nematode parameters in eggplant under greenhouse conditions [40,41]. Moreover, *B. subtilis*, *B. megaterium*, *B. pumilus*, and *Pseudomonas fluorescens* reduced nematode parameters of *M. incognita* infecting sugar beet [8].

Co-toxicity for the applied bioagents+PP extract against *M. incognita* at harvest stage exhibited additive or synergistic effect on potato under field conditions which increased nematode reduction in the present study. As indicated in previous study [42], *P. fluorescens*+*B. megaterium* caused synergistic effect against green bean-infested *M. incognita.*

Plant growth and yield can be stimulated by using the tested bacteria through different mechanisms, and among them, reduction of pathogenic microorganisms occurred. In addition, these bacteria survive in rhizospheric soil colonizing plant roots and promoting plant growth [35,36,43]. Moreover, phosphate could be supplied to plants by *Bacillu*s [44]. Such bacterium was applied for field status and commercialization [39].

The photosynthetic compounds in the present study, chlorophyll (a and b), and carotenoid contents increased by applying different treatments, which were similar with the results obtained by different researchers [9,10,45]. Carotenoid levels contribute in photosynthesis, protect plants against oxidative damage, and are prospectors of volatiles that attract pollinators [46]. As for biochemical compounds, phenolic contents increased in the different treatments, which are essential for lignin formation, biosynthesis, and plant defense against pathogens resulting in resistant plants [47]. Seeds of resistant plants contain some phenolic compounds which were included as resistant mechanism against nematodes [48,49], confirming the present results. Moreover, the performed phenol levels in roots of certain plants affected resistance against nematodes [50].

The interactions between plant host and soil microbes, which naturally occur in soil, may play an important role in controlling soil-borne pathogen [51]. Our results cleared that the total counts of soil microbial community were variably increased as affected by the applied treatments in the potatoes rhizosphere during the growing season, than before planting, and untreated control. The antagonistic fungi, *Aspergillus* spp., *Penicillium* spp., and *Trichoderma* spp. differently occurred in rhizosphere of potatoes after planting and treatment. These obtained results agreed with those by certain scientists [52], as they indicated that *P. chrysogenum* (Snef1216) could cause the egg-hatching inhibition and increase mortality of *M. incognita*. It was concluded that fungus, *P. chrysogenum,* might serve as a novel nematicidal agent against root-knot nematode. Large diversity of microflora and fauna were found in soil horizons, and their populations were influenced by various factors, such as organic matter, oxygen and carbon dioxide concentration, and soil pH [53]. Rhizospheric microorganisms could decompose organic matter, detoxify the toxic substances, fix the nitrogen and its transformation, and provide phosphorous, potassium and other secondary micronutrients in soil to plants. Certain important natural enemies of nematode pests such as nematophagous bacteria can reduce nematodes by parasitizing; producing toxins, antibiotics, or enzymes; competing for nutrients or they can induce systemic resistance of plants against nematodes causing direct inhibition of nematodes, enhancing plant growth, and permitting to colonize rhizosphere and activate microbial antagonists [35]. A survey study revealed that *Alternaria* spp., *Aspergillus* spp., *A. niger*, *Fusarium* spp., *Penicillium* spp., *Trichoderma*

spp., and *Verticillium* spp. were the most frequently fungi in wheat plant rhizosphere in Egypt [54].

Conclusions

The combined treatments highly inhibited *M. incognita* in potatoes more than bacteria in the single treatments, which recorded less nematode reduction. However, single treatments increased the growth and yield parameters of potatoes more than the combinations. Moreover, the total populations of the associated aerobic and spore-forming bacteria and fungi were affected in the potatoes rhizosphere by using the tested treatments. The antagonistic microorganisms involved were *Aspergillus* spp., *Penicillium* spp., and *Trichoderma*, which may interact with nematodes, leading to their inhibited effects on host plants.

Acknowledgements

This research work was supported in part by the In-house Project No. 12050105 entitled 'Pesticide alternatives against soil-borne pathogens and pests attacking economically important solanaceous crops' carried out by National Research Center, Egypt.

Author contributions: W.M.A.EN. and M.M.A.Y. equally contributed in the design and execution of this experiment. M.M.A.Y. wrote the manuscript. H.AEK. isolated, reared, and identified the tested bacterial species. U.S.EK. carried out the experiment in the field. M.M.M.AE. provided the facilities during this work and reviewed the manuscript. M.G.D. carried out biochemical analysis. All authors have read and approved the final manuscript.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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