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Potential of *Pseudomonas putida*, *Bacillus subtilis*, and their mixture on the management of *Meloidogyne incognita*, *Pectobacterium betavasculorum*, and *Rhizoctonia solani* disease complex of beetroot (*Beta vulgaris* L.)

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Abstract

Effects of *Pseudomonas putida* and *Bacillus subtilis* alone, and in combinations for the management of *Meloidogyne incognita*, *Pectobacterium betavasculorum*, and *Rhizoctonia solani* disease complex of beetroot (*Beta vulgaris* L.), were studied. Application of *P. putida* or *B. subtilis* to plants with *M. incognita* or *P. betavasculorum* or *R. solani* singly or in combinations caused a significant increase in plant growth parameters and the activities of defense enzymes. A significant increase in chlorophyll fluorescence attributes, viz., Fv/Fm, ϕPSII, qP, NPQ, and ETR were recorded in plants treated with *P. putida* or *B. subtilis* over pathogen-inoculated plants. Inoculation of *P. putida* results in a higher reduction in galling and nematode multiplication than *B. subtilis*. Maximum reduction in nematode multiplication and galling occurred when a mixture of *P. putida* and *B. subtilis* was used. Soft rot and root rot indices were 3 when *Pectobacterium betavasculorum* and *Rhizoctonia solani* were inoculated alone. The disease indices were rated 5 when these pathogens and *M. incognita* were inoculated in combinations. Inoculation of *P. putida*/*B. subtilis* with *P. betavasculorum* or *R. solani* reduced soft rot and root rot indices to 2 out of 3, while the use of *P. putida* + *B. subtilis* reduced indices to 1. Disease indices were reduced to 2–3 out of 5, when *P. putida* + *B. subtilis* were used to plants inoculated with two or three pathogens. The principal component analysis showed significant correlations among the various studied attributes. Two principal components explained a total of 86.1 and 93.4% of the overall data variability. Therefore, the use of *P. putida* together with *B. subtilis* had the potential for successful management of disease complex of beetroot.

Keywords: *Beta vulgaris*, Bioagents, Disease complex, Defense enzymes, Chlorophyll fluorescence

Background

Beetroot, *Beta vulgaris* L., is a high nutrient vegetable crop distributed throughout the world and is being used commercially to produce red juice and natural pigments. It provides many health benefits, especially for disorders characterized by chronic inflammation (Clifford et al. 2015).

The root-knot nematode, *Meloidogyne incognita*, is a parasite of a wide range of vegetable crops including beetroot (Mashela 2016). It induces root galls, which severely limit the yield of beetroot (Arora and Saxena 2003). *Pectobacterium betavasculorum* causes vascular necrosis and soft rot disease of beetroot, as well; causes wilting and black streaks on the leaves and petioles; and in severe cases, may secrete various extracellular digestive enzymes (Nedaienia and Fassihiani 2011). *Rhizoctonia solani* is also an important pathogen of beetroot (Abawi et al. 1986), causing pre-and post-emergence death of seedling, crown rot, and root rot. These pathogens are often associated with the roots of beetroot under field conditions, causing a disease complex.

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Management of plant disease, using plant growth-promoting rhizobacteria (PGPR), is an eco-friendly and host-targeted (Biswas et al. 2012). PGPR is a diverse group of free-living soil bacteria that colonize the rhizosphere, promote plant growth, and increase the yield of agriculture crops (Kumar et al. 2016). The association of roots with PGPR can protect plants more specifically from soil-borne pathogens and can assist to acquire more nutrients and grow better by producing phytohormones (Lugtenberg and Kamilova 2009). PGPR can activate different plant defense mechanisms for the management of plant pathogens (Lee et al. 2015). *Pseudomonas* sp. and *Bacillus* sp. are known as the dominant antagonists of plant pathogens in the rhizosphere (Mhatre et al. 2018).

Fluorescent pseudomonads exhibit many traits that make them appropriate as biological control agents (Pastor et al. 2016). They may protect plants via induced systemic resistance (Kumudini et al. 2017), production of extracellular lytic enzymes, and antimicrobial secondary metabolites for biocontrol (Olorunleke et al. 2015). The rhizospheric strains of *Pseudomonas* have also shown a biocontrol potential against plant-parasitic nematodes (Tabatabaei and Saeedizadeh 2017). *Bacillus subtilis* produces a wide variety of antimicrobial substances and extracellular lytic enzymes, which inhibit the growth of plant pathogens (Sajitha and Dev 2016). This bacterium may promote plant growth and induce host systemic resistance (Sha et al. 2016).

Generally, a single biocontrol agent is used for the control of plant disease, which sometimes leads to its inconsistent performance. A single biocontrol agent is not active in all soil environments or against all pathogens that attack the host plant. The combinations of biocontrol agents may be useful for biocontrol of different plant pathogens via different mechanisms of disease suppression (Akhtar and Siddiqui 2008). Moreover, mixtures of biocontrol agents that require different optimum temperature, pH, and moisture conditions may colonize roots more aggressively and improve plant growth and the efficacy of biocontrol (Siddiqui 2006). Dual inoculation with biocontrol agents having different mechanisms of action is known to provide greater biocontrol against plant pathogens (Guetsky et al. 2002). Researchers around the world are striving to identify potential biocontrol agents that when mixed may result in a synergistic response in controlling plant diseases.

Therefore, the role of *P. putida* and *B. subtilis* individually, and their combinations on the management of *M. incognita*, *P. betavasculorum*, and *R. solani* disease complex of beetroot under greenhouse conditions was investigated.

Materials and methods

Preparation and sterilization of soil mixture

The sandy loam soil used in this study was collected from a field of Department of Botany, Aligarh Muslim University, Aligarh. The soil and river sand were mixed in a ratio of 3:1 (v/v). Soil samples were passed through a 2-mm sieve before analyses, and the following properties were determined: porosity and water holding capacity by hydrometry; pH, conductivity, and cation exchange capacity (CEC) using soil: distilled water in pH and conductivity meters. Nitrogen was determined by the Kjeldahl digestion (Nelson and Sommers 1972) and phosphorus by phosphomolybdic blue colorimetry (Jackson 1958), while flame photometer analyzed potassium. The soil mixture having pH 7.5, porosity 44%, water, holding capacity 40%, electrical conductance 0.62, available N 95.8 mg/kg soil, available P 8.8 mg/kg soil, and available K 157.0 mg/kg soil was added to jute bags, and small amount of water was poured into each bag. Sterilization of soil was done at 137.9 kPa for 20 min. The soil was allowed to cool before the filling of earthen pots with 1 kg of sterilized soil.

Experimental design

The experiment was performed in a completely randomized design in two sets. The first set had four experimental variables: (A) control, (B) *M. incognita*, (C) *P. betavasculorum*, and (D) *R. solani*, and this set received the following four treatments: (1) control, (2) *P. putida*, (3) *B. subtilis*, and (4) *P. putida* + *B. subtilis* ($4 \times 4 = 16$ treatments) (Tables 1, 2, and 3). The second set comprises of five variables: (a) Control, (b) *M. incognita* + *P. betavasculorum* (M + P), (c) *M. incognita* + *R. solani* (M + R), (d) *P. betavasculorum* + *R. solani* (P + R), and (e) *M. incognita* + *P. betavasculorum* + *R. solani* (M+P+R). This set also received the same four treatments: (1) control, (2) *P. putida*, (3) *B. subtilis*, and (4) *P. putida* + *B. subtilis* ($4 \times 5 = 20$ treatments). Each treatment including the control was replicated five times.

Growth and maintenance of test plants

Beetroot seeds (cv. New Ruby Queen) were surface-decontaminated with 0.1% sodium hypochlorite solution for 2 min, followed by three times washings in sterilized water. Five seeds were sown in each pot, and thinning was done after germination to maintain one plant per pot. Plants were placed in a glasshouse and water was provided whenever required. Two days after thinning, seedlings received the treatments, while un-inoculated plants served as a control.

Nematode inoculum

Meloidogyne incognita was collected from the beetroots and multiplied on the eggplant, *Solanum melongena* L., using a single egg mass. Egg masses were hand-picked from heavily infected eggplant roots with the help of sterilized forceps. These egg masses were washed by

Table 1 Influence of *Pseudomonas putida*, *Bacillus subtilis*, and their mixtures on the plant length, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight of beetroot infected with *Meloidogyne incognita*, *Pectobacterium betavasculorum*, and *Rhizoctonia solani*

Bioagents	Pathogens	Plant length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
Control	C	51.26^{d}	97.64^{d}	131.51^{d}	7.91^{d}	10.66^{d}
	M	35.34^{k}	60.32^{j}	76.32^{l}	4.89^{j}	6.19^{l}
	P	37.53^{j}	64.18^{i}	81.37^{k}	5.20^{i}	6.61^{k}
	R	33.21^{l}	57.54^{j}	73.43^{l}	4.66^{j}	5.96^{l}
B. subtilis	C	55.35^{c}	106.87^{c}	146.46^{c}	8.66^{c}	11.87^{c}
	M	41.42^{hi}	81.35^{gh}	102.31^{i}	6.59^{g}	8.31^{i}
	P	43.37^{gh}	83.44^{g}	105.43^{hi}	6.76^{g}	8.56^{h}
	R	39.61^{i}	78.57^{h}	98.67^{j}	6.20^{h}	8.01^{j}
P. putida	C	58.43^{b}	112.62^{b}	150.63^{b}	9.12^{b}	12.29^{b}
	M	43.41^{gh}	84.33^{g}	106.32^{gh}	6.83^{fg}	8.62^{gh}
	P	45.26^{g}	87.47^{f}	109.26^{g}	7.09^{e}	8.86^{g}
	R	42.81^{h}	82.52^{g}	103.52^{hi}	6.68^{g}	8.32^{i}
B. subtilis + P. putida	C	64.53^{a}	123.62^{a}	163.43^{a}	10.15^{a}	14.22^{a}
	M	48.43^{ef}	95.32^{de}	125.67^{e}	7.72^{de}	10.19^{e}
	P	50.47^{de}	97.21^{d}	128.43^{de}	7.87^{d}	10.41^{de}
	R	47.52^{f}	93.49^{e}	121.37^{f}	7.57^{e}	9.84^{f}
LSD $p = 0.05$	Bioagents (B)	1.02	1.47	1.79	0.13	0.14
	Pathogens (P)	1.02	1.47	1.79	0.13	0.14
	B × P	2.04	2.94	3.58	0.27	0.27
CV		3.50	2.70	3.57	2.96	2.29

Data are presented as treatments mean ($n = 5$). The mean values within a column followed by the different letters are significantly different at $p = 0.05$ by Duncan's multiple range test (DMRT)
CV coefficient of variation, *C* control, *M M. incognita*, *P P. betavasculorum*, and *R R. solani*

sterilized water and then placed in a small sieve (1-mm pore size) having a cross-layer of tissue paper. The sieve was then placed in a Petri plate containing distilled water deep enough to contact the egg masses. The assemblies were kept in an incubator running at 25 ± 1 °C to obtain the sufficient number of second-stage juveniles required for inoculation. The newly hatched second-stage juveniles were collected every 24 h and fresh water was added, and the process was repeated daily. For counting newly hatched juveniles, an average of five counts was made to determine the density of nematodes in the suspension. The volume of the nematode suspension was adjusted in such a way that each milliliter may contain 200 infective juveniles of *M. incognita*. Ten milliliters of this suspension (i.e., 2000 freshly hatched *M. incognita* juveniles) was used for inoculation.

Preparation of bacterial inoculum

The bacterium *P. betavasculorum* was isolated from infected beetroots exhibiting necrosis and rot symptoms, following surface sterilization with 0.1% sodium hypochlorite solution. Roots were cut into small pieces and placed aseptically in Petri dishes containing nutrient agar medium (Himedia Laboratories, India) at 30 ± 1 °C for 24 h. Bacteria developed on the medium were identified using Bergey's manual of determinative bacteriology (Garrity et al. 1984). For the confirmation of identity, nutrient agar plates were streaked separately with a pure colony of *P. betavasculorum* and incubated at 30 ± 1 °C for 24 h. For inoculum, single colonies from a 24-h old pure culture of *P. betavasculorum* were inoculated separately into nutrient broth (Himedia Laboratories, India) flasks and incubated at 30 ± 1 °C for 72 h. Cell density was determined and measured 1.2×10^5 colony-forming units (CFU)/ml.

Preparation of fungus inoculum

The fungus, *R. solani* was isolated from infected beetroots exhibiting root rot symptoms following surface sterilization with 0.1% sodium hypochlorite solution as described for a seed above. Roots were cut into small pieces and placed aseptically in Petri dishes containing potato dextrose agar (PDA) medium (Himedia Laboratories, India) at 25 °C for 15 days. For obtaining sufficient inoculum, *R. solani* was inoculated by a sterile inoculation needle into Richard's liquid medium (Riker

Table 2 Influence of *Pseudomonas putida*, *Bacillus subtilis*, and their mixtures on the chlorophyll content, and the activities SOD, CAT, PPO, and PAL of beetroot infected with *Meloidogyne incognita*, *Pectobacterium betavasculorum*, and *Rhizoctonia solani*

Bioagents	Pathogens	Chlorophyll (SPAD values)	SOD activity (U mg^{-1} FW)	CAT activity (U mg^{-1} FW)	PPO activity (U mg^{-1} FW)	PAL activity (U mg^{-1} FW)
Control	C	53.3^{d}	5.18^{i}	6.63^{h}	26.34^{m}	52.37^{m}
	M	33.4^{kl}	7.32^{h}	8.16^{g}	42.23^{k}	64.23^{k}
	P	35.2^{k}	7.06^{h}	7.92^{g}	44.34^{j}	66.46^{j}
	R	32.5^{l}	7.45^{h}	8.35^{g}	39.35^{l}	62.35^{l}
B. subtilis	C	58.3^{c}	10.13^{g}	9.31^{f}	49.53^{i}	70.37^{i}
	M	41.2^{ij}	12.34^{de}	14.53^{c}	60.24^{f}	83.58^{g}
	P	43.5^{gh}	12.15^{de}	14.31^{c}	62.33^{e}	85.34^{f}
	R	40.1^{j}	12.57^{de}	14.73^{c}	58.43^{g}	81.44^{h}
P. putida	C	60.2^{b}	11.21^{e}	10.43^{e}	51.47^{h}	71.43^{i}
	M	43.5^{gh}	12.45^{de}	14.62^{c}	62.27^{e}	86.31^{f}
	P	45.3^{g}	12.24^{e}	14.47^{c}	64.43^{d}	88.21^{e}
	R	42.1^{hi}	12.73^{d}	14.81^{c}	60.51^{f}	83.53^{g}
B. subtilis + *P. putida*	C	67.4^{a}	13.73^{c}	13.24^{d}	65.43^{d}	91.51^{d}
	M	50.5^{ef}	16.75^{a}	19.33^{a}	82.47^{b}	115.47^{b}
	P	52.3^{de}	16.13^{b}	18.71^{b}	85.64^{a}	118.41^{a}
	R	50.2^{f}	16.87^{a}	19.53^{a}	77.63^{c}	112.33^{c}
LSD $p = 0.05$	Bioagents (B)	0.96	0.21	0.22	0.82	0.69
	Pathogens (P)	0.96	0.21	0.22	0.82	0.69
	B × P	1.93	0.42	0.43	1.65	1.38
CV		3.06	2.89	2.97	2.24	1.31

Data are presented as treatments mean ($n = 5$). The mean values within a column followed by the different letter are significantly different at $p = 0.05$ by Duncan's multiple range test (DMRT)

CV coefficient of variation, *SOD* superoxide dismutase, *CAT* catalase, *PPO* polyphenol oxidase, *PAL* phenylalanine ammonia lyase, *C* control, *M M. incognita*, *P P. betavasculorum*, and *R R. solani*

and Riker 1936) containing 10 g potassium nitrate, 5 g potassium dihydrogen phosphate, 2.5 g magnesium sulfate, 0.02 g ferric chloride, 50 g sucrose, and 1000 ml distilled water. The Richards liquid medium was prepared and filtered through a muslin cloth and sterilized in an autoclave at 103.4 kPa for 15 min in 250 ml Erlenmeyer flasks containing 80 ml of liquid medium. The flasks were incubated at 25 ± 1 °C for 15 days. The liquid medium was filtered through Whatman filter paper No. 1. The fungal mycelia mat on the filter paper was washed in distilled water and excess water and nutrients removed with blotting paper. The inoculum was prepared by mixing 10 g of fungal mycelium in 100 ml of distilled water and blending it (10,000 rpm) for 30 s in a Waring blender. Ten milliliters of the suspension, containing 1 g fungus, was used for inoculation of beetroot seedlings.

Preparation of inoculum of biocontrol agents

The PGPR strains, viz., *Pseudomonas putida* (MTCC No. 3604) and *B. subtilis* (Acc. No. 2274), were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. These isolates were sub-cultured on nutrient agar, and the inoculum was produced on nutrient broth incubated at 30 ± 1 °C for 72 h. Ten milliliters of suspension (1.2×10^5 cells/ml) was used as inoculums.

Inoculation technique

Two-week-old, well-established seedlings were used for the inoculation of pathogens and biocontrol agents. For inoculation, the soil around roots was carefully removed, and suspensions of *M. incognita*, *P. betavasculorum*, *R. solani*, *P. putida*, and *B. subtilis* were uniformly poured around roots and the soil replaced. In control pots, a similar amount of water was poured in the same way around the roots.

Determination of growth parameters

The plants were harvested 90 days after inoculation. The length of the plant was recorded in centimeters from the top of the first leaf to the end of the root. Excess of

Table 3 Influence of *Pseudomonas putida*, *Bacillus subtilis*, and their mixtures on the chlorophyll fluorescence parameters of beetroot infected with *Meloidogyne incognita*, *Pectobacterium betavasculorum*, and *Rhizoctonia solani*

Bioagents	Pathogens	Fv/Fm	ϕPSII	qP	NPQ	ETR
Control	C	0.661^{c}	0.436^{de}	0.534^{c}	0.425^{d}	163.43^{c}
	M	0.472^{hi}	0.332^{ij}	0.363^{hi}	0.343^{ij}	137.44^{hi}
	P	0.493^{h}	0.345^{hi}	0.381^{h}	0.35^{hi}	141.31^{gh}
	R	0.462^{i}	0.314^{j}	0.354^{i}	0.332^{j}	134.32^{i}
B. subtilis	C	0.691^{b}	0.461^{c}	0.573^{b}	0.446^{bc}	171.54^{b}
	M	0.554^{fg}	0.364^{gh}	0.445^{fg}	0.362^{hi}	148.24^{ef}
	P	0.571^{ef}	0.372^{fg}	0.463^{ef}	0.384^{fg}	151.34^{def}
	R	0.543^{g}	0.354^{ghi}	0.435^{g}	0.352^{hi}	146.45^{fg}
P. putida	C	0.712^{b}	0.483^{b}	0.591^{b}	0.453^{b}	173.53^{b}
	M	0.561^{efg}	0.375^{fg}	0.462^{ef}	0.371^{gh}	150.42^{def}
	P	0.583^{e}	0.392^{f}	0.473^{e}	0.385^{fg}	153.35^{def}
	R	0.554^{fg}	0.364^{gh}	0.454^{fg}	0.361^{hi}	149.38^{ef}
B. subtilis + *P. putida*	C	0.782^{a}	0.534^{a}	0.651^{a}	0.472^{a}	187.26^{a}
	M	0.634^{cd}	0.425^{de}	0.514^{cd}	0.416^{de}	157.45^{cd}
	P	0.642^{c}	0.443^{cd}	0.536^{c}	0.431^{cd}	162.35^{c}
	R	0.616^{d}	0.416e	0.504^{d}	0.403^{ef}	154.26^{de}
LSD $p = 0.05$	Bioagents (B)	0.011	0.010	0.011	0.009	3.26
	Pathogens (P)	0.011	0.010	0.011	0.009	3.26
	B × P	0.023	0.021	0.022	0.018	6.52
CV		3.01	4.16	3.57	3.72	3.25

Data are presented as treatments mean ($n = 5$). The mean values within a column followed by the different letter are significantly different at $p = 0.05$ by Duncan's multiple range test (DMRT)

CV coefficient of variation, *Fv/Fm* maximal PSII efficiency, *ϕPSII* photochemical efficiency of PSII, *qP* photochemical quenching, *NPQ* non-photochemical quenching, *ETR* electron transport rate, *C* control, *M M. incognita*, *P P. betavasculorum*, and *R R. solani*

water was removed by blotting before weighing the plant for fresh weight. The plants were cut with a knife above the base of the root emergence zone to separate the shoot and root. The shoot and root were kept in an oven at 80 °C for 3 days for dry weights.

Enzyme assay

To determine the activities of superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), polyphenol oxidase (PPO, EC 1.10.3.1), and phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) samples were ground in liquid nitrogen and homogenized on ice bath with different buffers to assay different enzymes: 3 ml solution containing 50 mM potassium phosphate buffer (pH 6.8), 0.1 mM EDTA (ethylene diamine tetraacetate), and 1% polyvinyl pyrrolidone (PVP) for SOD, CAT, and PPO; 1.2 ml of 200 mM sodium borate buffer (pH 8.8) was used for PAL. The homogenate was centrifuged at 13, 000*g* for 20 min at 4 °C. The supernatants obtained were used as the crude enzyme source to assay enzymatic activities. Spectrophotometric determinations were performed using a UV visible spectrophotometer (UV-1700, Shimadzu, Japan).

Enzyme activities

The SOD activity was estimated by the method of Beyer and Fridovich (1987). CAT was assayed according to the method of Aebi (1984) by monitoring the disappearance of H_2O_2 at 240 nm. The activity of PAL was determined, based on the production of trans-cinnamic acid by employing the methodology of Tian et al. (2006). PPO activity was performed using catechol as a substrate following the methodology of Yu et al. (2014).

Estimation of chlorophyll content and chlorophyll fluorescence

Chlorophyll content in the intact leaves was measured with the help of SPAD chlorophyll meter (502 DL PLUS, Spectrum Technologies, USA). Chlorophyll fluorescence was measured using a saturation-pulse fluorometer (PAM-2000, Walz, Effeltrich, Germany). Before the measurement, leaf parts were dark adapted for at least 30 min in order to relax the reaction center. In the dark-adapted leaves the minimal fluorescence (F_0) and maximum fluorescence (Fm) were measured by applying a low measuring beam having a light intensity of 125 mmol m^{-2} s^{-1}; however, under light-adapted condition, minimal

fluorescence (F_0') and maximum fluorescence (Fm′) were measured in the same leaves with a saturating light intensity of 720 mmol m^{-2} s^{-1}, together with steady-state fluorescence (Fs). The variable fluorescence (Fv and Fv′) was determined using the values of Fm – F_0 and Fm – F_0', and actual PSII efficiency (ϕPSII) was calculated as Fm′ − Fs/Fm′, maximal efficiency of PSII by using Fv/Fm. The intrinsic efficiency of PSII was measured by using Fv′/Fm′, and photochemical quenching was calculated as (Fm′ − Fs)/Fv′ and NPQ as (Fm-Fm′)/Fm′.

Nematode population and number of galls per root system

To obtain nematode counts, a 250-g subsample of well-mixed soil from each treatment was processed by Cobb's sieving and decanting technique followed by Baermann funnel extraction. The suspension was collected after 24 h and numbers of nematodes counted in five aliquots of 1 ml of suspension from each sample. The means of five counts were used to calculate the population of nematodes·kg^{-1} soil. To estimate the numbers of juveniles, eggs, and females inside roots, a 1-g subsample of roots were macerated in a Waring blender and counts made from the suspension obtained. The numbers of juveniles and females of nematodes present in roots were calculated by multiplying the number of *M. incognita* juveniles and females present in 1 g of the root by the total weight of root. The numbers of galls per root system were also counted.

Soft rot and root rot indices

Soft rot and root rot symptoms on roots were observed. Soft rot and root rot indices were determined by scoring the severity of disease on visual observations of disease symptoms. Disease rating was on a scale from 0 to 5 where 0 = no disease (no soft rot/root rot symptoms observed), 1 = soft rot/root rot symptoms up to 12.5% on root, 2 = soft rot/root rot symptoms 12.6 to 25% on root, 3 = soft rot/root rot symptoms 25.1 to 37.5% on root, 4 = soft rot/root rot symptoms 37.6 to 50% on root, and 5 = more than 50% soft rot/root rot symptoms on roots.

Statistical analysis

Data were statistically analyzed through two-way analysis of variance (ANOVA), using R (2.14.0) statistical software (package library, agricolae), followed by Duncan's multiple range test (DMRT) to find out the significance ($p \leq 0.05$) of the data. The principal components analysis (PCA) was carried out to determine variability among various studied attributes using Origin (2018b) software.

Results and discussion

Beetroot infected with single pathogens

Influence on growth attributes

The inoculation of *P. putida*/*B. subtilis*, or their mixture to plants without pathogens, caused a significant increase in plant growth attributes over the un-inoculated control (Table 1). Plants inoculated with *M. incognita*/*P. betavasculorum* or *R. solani* showed a significant reduction in growth parameters than the un-inoculated control (Table 1). *R. solani* caused a maximum reduction in plant length (35.21%), in shoot fresh weight (41.07%), in root fresh weight (44.16%), in shoot dry weight (41.09%), and in root dry weight (44.09%), followed by *M. incognita* and *P. betavasculorum* (Table 1). The application of *P. putida*/*B. subtilis* or their mixture to plants with pathogens resulted in a significant increase of plant growth characters. A combined application of *P. putida* + *B. subtilis* caused the highest significant increase in plant length (37.04%), in shoot fresh weight (58.02%), in shoot dry weight (57.87%), in root fresh weight (57.83%), and in root dry weight (57.48%) over *M. incognita* inoculated plants. The use of *P. putida* with *B. subtilis* caused the highest significant increase in plant length (34.47%), in shoot fresh weight (51.48%), in shoot dry weight (51.35%), in root fresh weight (64.66%), and in root dry weight (51.35%) over *P. betavasculorum* inoculated plants. The application of *P. putida* with *B. subtilis* caused a highly significant increase in plant length (43.08%), in shoot fresh weight (62.48%), in shoot dry weight (62.45%), in root fresh weight (65.28%), and in root dry weight (65.10%) over *R. solani* inoculated plants (Table 1).

Influence on chlorophyll content and the activities of defense enzymes

Inoculation of *P. putida*, *B. subtilis*, and their mixture to plants without pathogens caused a significant increase in chlorophyll content and the activities of SOD, CAT, PPO, and PAL over un-inoculated control (Table 2). Plants inoculated with *M. incognita*/*P. betavasculorum* or *R. solani* showed a significant reduction in chlorophyll content and significant increase in the activities of SOD, CAT, PPO, and PAL (Table 2). *R. solani* caused a maximum reduction of 39.02% in chlorophyll content and a maximum increase in the activities of SOD and CAT, followed by *M. incognita* and *P. betavasculorum* (Table 2). However, the highest increase in the activities of PPO and PAL was recorded in plants inoculated with *P. betavasculorum*, followed by *R. solani* and *M. incognita* (Table 3). The application of *P. putida*, *B. subtilis*, and their mixture to plants with pathogens resulted in a significant increase in chlorophyll content and the activities of enzymes. The maximum increase in chlorophyll content and increased activities of defense enzymes was

recorded in treated plants with the mixture of *P. putida* plus *B. subtilis* followed by *P. putida*/*B. subtilis* (Table 2).

Influence on chlorophyll fluorescence

Application of *P. putida*, *B. subtilis*, and their mixture to plants without pathogens caused a significant increase in chlorophyll fluorescence parameters, viz., Fv/Fm, PSII, qP, NPQ, and ETR, over un-inoculated control (Table 3). The inoculation of *M. incognita*/*P. betavasculorum* or *R. solani* caused a significant reduction in chlorophyll fluorescence attributes over un-inoculated control (Table 3). *R. solani* caused a maximum significant reduction of 30.11% in Fv/Fm, 27.98% in PSII, 33.70% in qP, 21.88% in NPQ, and 17.81% in ETR, followed by *M. incognita* and *P. betavasculorum*, respectively. The application of *P. putida* or *B. subtilis* or their mixture to plants with pathogens caused a significant increase in chlorophyll fluorescence characters. The maximum increase in the attributes of chlorophyll fluorescence was recorded in treated plants with the mixture of *P. putida* plus *B. subtilis*, followed by *P. putida*/*B. subtilis* alone (Table 3).

Influence on soft rot and root rot indices

Soft rot and root rot indices recorded 3, when *P. betavasculorum* and *R. solani* was inoculated, respectively (Fig. 1a). The indices were reduced to 2, when *P. betavasculorum* and *R. solani* inoculated plants were treated by *P. putida* or *B. subtilis*. The application of *P. putida* + *B. subtilis* to plants with *P. betavasculorum* and *R. solani* reduced disease indices to 1 (Fig. 1a).

Influence on nematode multiplication and galling

The nematode population and galling were high, when *M. incognita* was inoculated alone (Fig. 1c, e). Application of *P. putida* or *B. subtilis* caused a significant reduction in the number of galls and nematode multiplication. The combined application of *P. putida* + *B. subtilis* resulted in higher reduction in galling and nematode multiplication than caused by either of them singly (Fig. 1c, e).

Principal component analysis

The results of the principal component analysis (PCA) from all the treatments and their effects on various studied attributes are shown in Fig. 2a. The PCA explained a total of 86.1% (PC1 = 66.9%; PC2 = 19.2%) of observed variability in the data (Fig. 2a). Significant positive correlations were found in plant growth parameters, viz., PL, SFW, RFW, SDW, and RDW, with chlorophyll fluorescence attributes, viz., Fv/Fm, PSII, qP, NPQ, and ETR, respectively. Plant growth parameters and chlorophyll fluorescence characters were also found positively correlated with different enzymatic antioxidants like SOD, CAT, and defense enzymes such as PPO and PAL. The SOD and CAT activity was also positively correlated with PPO and PAL activity. The attributes of plant growth and chlorophyll fluorescence were negatively correlated with disease indices, nematode population, and galling. The increase in the activity of SOD, CAT, PPO, and PAL showed a negative correlation with nematode multiplication, galling, and soft rot and root rot indices. The PCA also clearly identified the ameliorative influence of *P. putida*, *B. subtilis*, and their mixture on various studied attributes as judged from the segregation of different treatments in the biplot (Fig. 2a, b).

Beetroot infected with two or three pathogens

Influence on growth attributes The inoculation of *M. incognita* plus *P. betavasculorum* or *M. incognita* plus *R. solani* or *P. betavasculorum* + *R. solani* or all the three pathogens together caused a significant reduction in plant growth attributes over the un-inoculated control (Table 4). The maximum reduction in plant growth parameters was noticed, when all the three pathogens were inoculated together, followed by the inoculations of *M. incognita* + *R. solani*, *M. incognita* + *P. betavasculorum*, and *P. betavasculorum* + *R. solani* (Table 4). The application of *P. putida* or *B. subtilis* or their mixture to plants with two or three pathogens resulted in a significant increase in plant growth parameters. The maximum increase in plant growth parameters was recorded in treated plants with the mixture of *P. putida* + *B. subtilis*, followed by *P. putida* and *B. subtilis* (Table 4).

Influence on chlorophyll content and the activities of defense enzymes The inoculation of *M. incognita* + *P. betavasculorum* or *M. incognita* + *R. solani* or *P. betavasculorum* + *R. solani* or all the three pathogens together caused a significant reduction in chlorophyll content, while the inoculation of these pathogens resulted in a significant increase in the activities of defense enzymes, viz., SOD, CAT, PPO, and PAL (Table 5). The inoculation of three pathogens together caused maximum reduction (59.47%) in chlorophyll content and maximum increase (62.74%) in SOD activity and CAT activity (46.90%), followed by the inoculation of *M. incognita* + *R. solani*, *M. incognita* + *P. betavasculorum*, and *P. betavasculorum* + *R. solani*. However, the inoculation of *P. betavasculorum* + *R. solani* resulted in a high increase (30.06%) in PPO activity and PAL activity (17.51%), followed by *M. incognita* + *P. betavasculorum*, *M. incognita* + *R. solani*, and co-inoculation of three pathogens (Table 5). The application of *P. putida* or *B. subtilis* or their mixture to plants with two or three pathogens resulted in a significant increase in chlorophyll content and the activities of SOD, CAT, PPO, and PAL. The

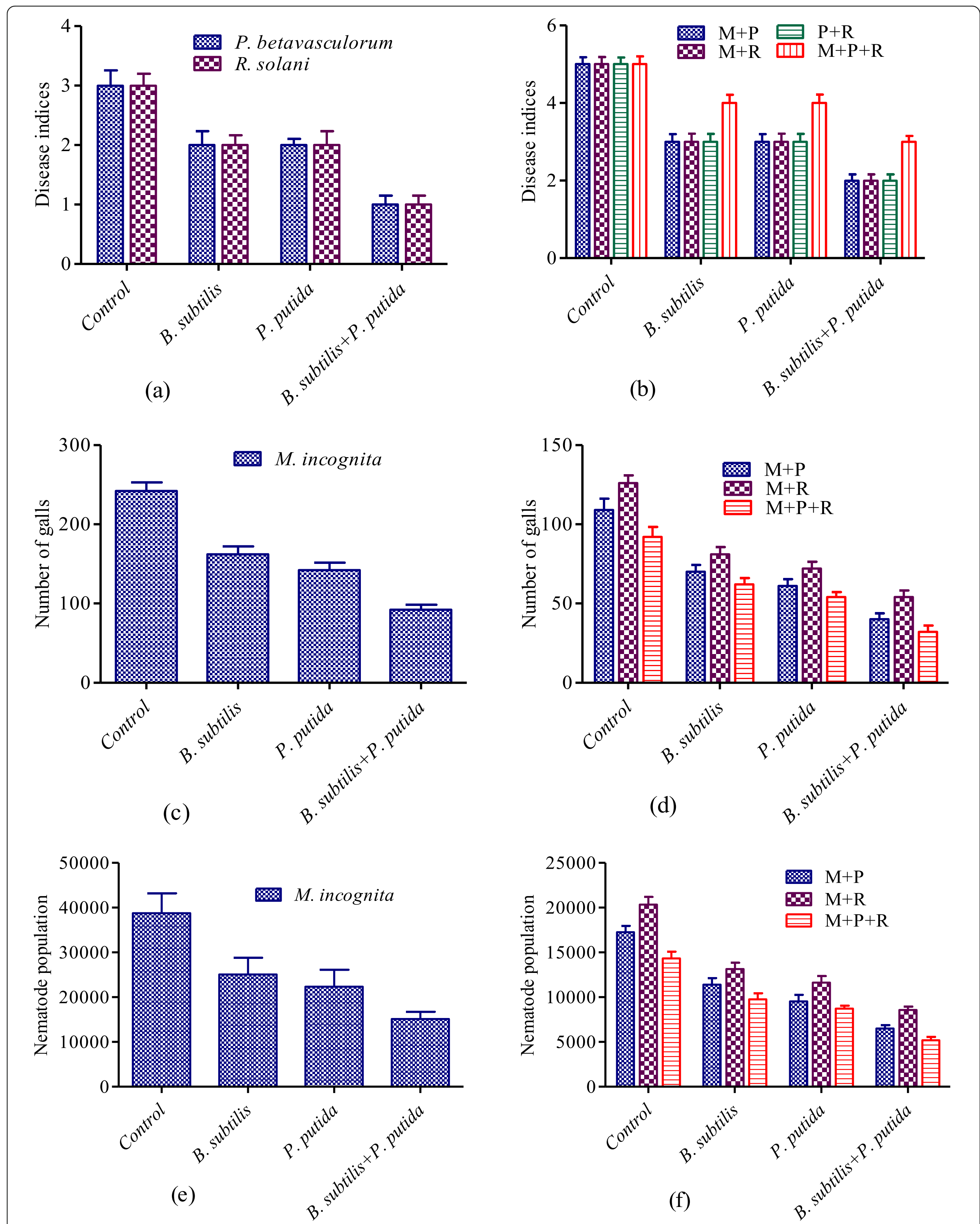

Fig 1 Effects of *Pseudomonas putida*, *Bacillus subtilis*, and their mixture on disease indices (**a**, **b**), root galling (**c**, **d**), and nematode multiplication (**e**, **f**) in beetroot infected with single, two or more pathogens. M *Meloidogyne incognita*, P *Pectobacterium betavasculorum*, and R *Rhizoctonia solani*

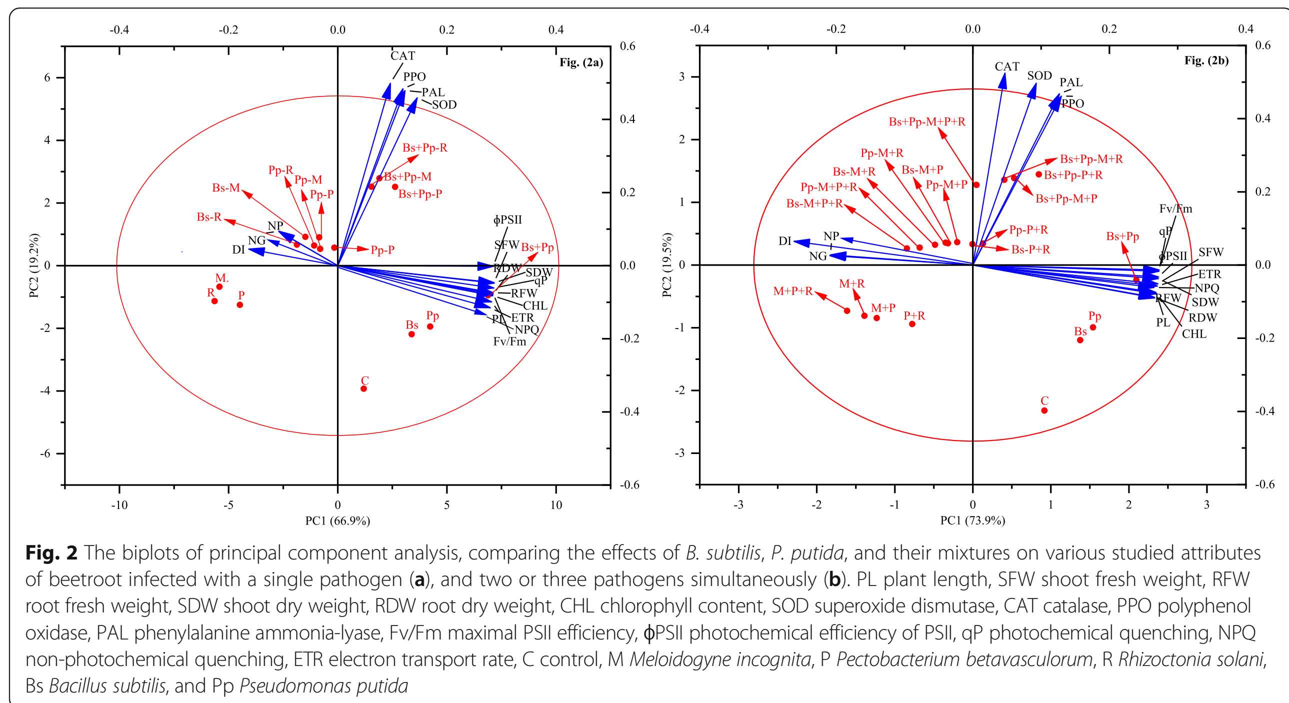

Fig. 2 The biplots of principal component analysis, comparing the effects of *B. subtilis*, *P. putida*, and their mixtures on various studied attributes of beetroot infected with a single pathogen (**a**), and two or three pathogens simultaneously (**b**). PL plant length, SFW shoot fresh weight, RFW root fresh weight, SDW shoot dry weight, RDW root dry weight, CHL chlorophyll content, SOD superoxide dismutase, CAT catalase, PPO polyphenol oxidase, PAL phenylalanine ammonia-lyase, Fv/Fm maximal PSII efficiency, ϕPSII photochemical efficiency of PSII, qP photochemical quenching, NPQ non-photochemical quenching, ETR electron transport rate, C control, M *Meloidogyne incognita*, P *Pectobacterium betavasculorum*, R *Rhizoctonia solani*, Bs *Bacillus subtilis*, and Pp *Pseudomonas putida*

maximum increase in chlorophyll content and the activities of SOD, CAT, PPO, and PAL were recorded in treated plants with the mixture of *P. putida* + *B. subtilis*, followed by *P. putida* and *B. subtilis* (Table 5).

Influence on chlorophyll fluorescence Inoculation of *M. incognita* plus *P. betavasculorum* or *M. incognita* + *R. solani* or *P. betavasculorum* + *R. solani* or all the three pathogens together caused a significant reduction in chlorophyll fluorescence parameters, viz., Fv/Fm, PSII, qP, NPQ, and ETR, over un-inoculated control (Table 6). The maximum reduction in chlorophyll fluorescence attributes was noticed, when all the three pathogens were inoculated together, followed by the inoculations of *M. incognita* + *R. solani*, *M. incognita* + *P. betavasculorum*, and *P. betavasculorum* + *R. solani* (Table 6). The application of *P. putida* or *B. subtilis* or their mixture to plants with two or three pathogens resulted in a significant increase in chlorophyll fluorescence parameters. The maximum increase in chlorophyll fluorescence was recorded in plants treated with the mixture of *P. putida* + *B. subtilis*, followed by *P. putida* and *B. subtilis* (Table 6).

Influence on soft rot and root rot indices Soft rot and root rot indices recorded 5, when *M. incognita*, *P. betavasculorum*, and *R. solani* were inoculated together (Fig. 1b). The indices were reduced to 3 when plants inoculated with two pathogens were treated with *P. putida* or *B. subtilis* and were reduced to 4 in plants with three pathogens. Indices were further reduced to 2 when plants inoculated with *M. incognita* plus *P. betavasculorum*/*R. solani* or *P. betavasculorum* + *R. solani* were treated with *P. putida* + *B. subtilis*. The index recorded 3 when plants with three pathogens were treated with *P. putida* + *B. subtilis* (Fig. 1b).

Influence on nematode multiplication and galling The application of *P. putida* caused a higher reduction in galling and nematode multiplication, followed by *B. subtilis* (Fig. 1d, f). The combined application of *P. putida* + *B. subtilis* caused greater reduction in galling and nematode multiplication than that caused by either of them alone. Inoculation of *P. betavasculorum* or *R. solani* also had an adverse effect on galling and nematode multiplication. *P. betavasculorum* had greater adverse effect on galling and nematode multiplication than *R. solani*. Co-inoculation of *P. betavasculorum* and *R. solani* had a more adverse effect than their individual inoculation (Fig. 1d, f).

Principal component analysis

The results of the principal component analysis (PCA) from all the treatments and their effects on various studied attributes are presented in Fig. 2b. The PCA obtained explained a total of (93.4%) (PC1 = 73.9%; PC2 = 19.5%) of variability in the data (Fig. 2b).

The application of *P. putida* and *B. subtilis* when applied singly and in combination acted as antagonists against *M. incognita*, *P. betavasculorum*, and *R. solani* in reducing nematode multiplication, galling, decreasing soft rot and root rot indices, and by enhancing growth of beetroot plants. Pseudomonads have been used to

Table 4 Influence of *Pseudomonas putida*, *Bacillus subtilis*, and their mixtures on the plant length, shoot fresh weight, root fresh weight, shoot dry weight, and root dry weight of beetroot infected with two/three pathogens simultaneously in a disease complex incited by *Meloidogyne incognita*, *Pectobacterium betavasculorum*, and *Rhizoctonia solani*

Bioagents	Pathogens	Plant length (cm)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
Control	C	51.26^{d}	97.64^{d}	131.51^{d}	7.91^{d}	10.66^{d}
	M + P	28.23^{k}	50.47^{l}	68.32^{no}	4.19^{no}	5.68^{o}
	M + R	26.53^{kl}	48.35^{l}	65.47^{o}	4.02^{o}	5.44^{p}
	P + R	30.31^{j}	54.23^{k}	71.52^{mn}	4.42^{mn}	5.94^{n}
	M + P + R	22.63^{m}	38.57^{m}	55.63^{p}	3.21^{p}	4.62^{q}
B. subtilis	C	55.35^{c}	106.87^{c}	146.46^{c}	8.66^{c}	11.87^{c}
	M + P	34.41^{h}	63.47^{ij}	79.31^{jk}	5.27^{jk}	6.59^{l}
	M + R	32.35^{i}	61.38^{j}	77.43^{kl}	5.11^{k}	6.42^{lm}
	P + R	36.61^{gh}	67.64^{h}	82.23^{ij}	5.62^{i}	6.83^{k}
	M + P + R	25.36^{l}	54.52^{k}	69.62^{n}	4.53^{lm}	5.78^{no}
P. putida	C	58.43^{b}	112.62^{b}	150.63^{b}	9.12^{b}	12.29^{b}
	M + P	35.45^{h}	67.34^{h}	85.45^{hi}	5.59^{i}	7.11^{j}
	M + R	34.57^{h}	65.26^{hi}	83.63^{i}	5.42^{ij}	6.95^{jk}
	P + R	37.87^{g}	71.43^{g}	88.47^{h}	5.93^{h}	7.35^{i}
	M + P + R	27.22^{kl}	57.54^{k}	74.68^{lm}	4.78^{l}	6.21^{m}
B. subtilis + *P. putida*	C	64.53^{a}	123.62^{a}	163.43^{a}	10.15^{a}	14.22^{a}
	M + P	44.33^{ef}	83.34^{f}	105.44^{f}	6.92f	8.76^{f}
	M + R	43.24^{f}	81.53^{f}	102.62^{f}	6.77^{f}	8.52^{g}
	P + R	46.03^{e}	87.65^{e}	119.51^{e}	7.28^{e}	9.32^{e}
	M + P + R	34.52^{h}	74.55^{g}	93.89^{g}	6.19^{g}	7.81^{h}
LSD $p = 0.05$	Bioagents (B)	1.16	1.41	1.51	0.11	0.10
	Pathogens (P)	1.30	1.58	1.69	0.13	0.12
	B × P	2.61	3.16	3.39	0.26	0.24
CV		4.14	3.41	2.80	3.37	2.40

Data are presented as treatments mean ($n = 5$). The mean values within a column followed by the different letter are significantly different at $p = 0.05$ by Duncan's multiple range test (DMRT)
CV coefficient of variation, *C* control, *M M. incognita*, *P P. betavasculorum*, and *R R. solani*

enhance plant growth and to protect a wide range of plants from several biotic stresses (Beneduzi et al. 2012). *P. putida* was used for the biocontrol of *Fusarium oxysporum* f. sp. *cucumerinum* (Simeoni et al. 1987). It also reduced the disease severity of bacterial spot in sweet pepper (Tsai et al. 2004). The rhizospheric strains of *Pseudomonas* have shown pathogenic mechanisms against plant-parasitic nematodes (Tabatabaei and Saeedizadeh 2017). Several strains of *Bacillus* are also known to suppress nematodes and promote plant growth (Tabatabaei and Saeedizadeh 2017).

The *P. putida* and *B. subtilis* exhibit many traits that make them appropriate as biological control agents against a wide range of pathogens (Pastor et al. 2016). They may protect plants via induced systemic resistance (Kumudini et al. 2017), production of extracellular lytic enzymes (Sajitha and Dev 2016), competition with pathogens for organic carbon and iron on the root surface (Höfte and Bakker 2007), and antimicrobial secondary metabolites (Olorunleke et al. 2015). The enhanced disease protection by using a mixture of *P. putida* and *B. subtilis* could be because of the combined mechanisms and the availability of more accessible nutrient sources and suppression of pathogens.

In the present investigation, *P. betavasculorum* and *R. solani* adversely affected the multiplication of *M. incognita*. These findings are in accordance to Khan and Siddiqui (2017) who found the inhibitory effect of bacterium and fungus on the multiplication of *M. incognita*. The establishment of bacterial or fungal pathogen induces certain modifications in root system which are not favorable for nematodes (Hussain and Bora 2009).

The application of *P. putida* and *B. subtilis* both alone and in combination significantly enhanced the activity of antioxidant enzymes, viz., SOD and CAT, in response to pathogenic infection. The antagonistic bacteria enhanced the activities of antioxidant enzymes as a defensive mechanism against multiple pathogens (Singh and Gaur

Table 5 Influence of *Pseudomonas putida*, *Bacillus subtilis*, and their mixtures on the chlorophyll content, and the activities of SOD, CAT, PPO, and PAL of beetroot infected with two/three pathogens simultaneously in a disease complex incited by *Meloidogyne incognita*, *Pectobacterium betavasculorum*, and *Rhizoctonia solani*

Bioagents	Pathogens	Chlorophyll (SPAD values)	SOD activity (U mg^{-1} FW)	CAT activity (U mg^{-1} FW)	PPO activity (U mg^{-1} FW)	PAL activity (U mg^{-1} FW)
Control	C	53.3^{d}	5.18^{k}	6.63^{l}	26.34^{o}	52.37^{q}
	M + P	25.4^{n}	7.87^{ij}	9.42^{jk}	31.63^{m}	59.43^{o}
	M + R	24.2^{n}	8.11^{hi}	9.51^{jk}	30.51^{m}	57.62^{o}
	P + R	27.3^{m}	7.64^{j}	9.16^{k}	34.26^{l}	61.54^{n}
	M + P + R	21.6^{o}	8.43^{h}	9.74^{j}	28.32^{n}	55.72^{p}
B. subtilis	C	58.3^{c}	10.13^{g}	9.31^{jk}	49.53^{k}	70.37^{m}
	M + P	33.4^{j}	12.89^{d}	13.83^{def}	57.47^{g}	77.42^{ij}
	M + R	31.5^{k}	12.73^{d}	13.64^{efg}	55.63^{h}	76.33^{jk}
	P + R	36.3^{h}	12.12^{e}	14.14^{de}	60.43^{f}	81.47^{fg}
	M + P + R	27.4^{m}	11.63^{f}	13.12^{h}	53.54^{i}	73.34^{l}
P. putida	C	60.2^{b}	11.21^{f}	10.43^{i}	51.47^{j}	71.43^{m}
	M + P	35.5^{hi}	12.95^{d}	13.94^{def}	58.53^{g}	79.61^{gh}
	M + R	34.2^{ij}	12.87^{d}	13.75^{defg}	57.82^{g}	78.32^{hi}
	P + R	38.4^{g}	12.21^{e}	14.22^{d}	62.31^{e}	82.64^{f}
	M + P + R	29.3^{l}	11.62^{f}	13.54^{fgh}	54.55^{hi}	74.57^{kl}
B. subtilis + *P. putida*	C	67.4^{a}	13.73^{c}	13.24^{gh}	65.43^{d}	91.51^{e}
	M + P	43.1^{f}	17.44^{ab}	17.56^{b}	70.63^{b}	105.31^{b}
	M + R	41.6^{f}	17.32^{ab}	17.42^{b}	68.54^{c}	103.28^{c}
	P + R	46.5^{e}	17.65^{a}	18.11^{a}	75.32^{a}	109.37^{a}
	M + P + R	35.2^{hij}	17.04^{b}	16.34^{c}	65.44^{d}	98.23^{d}
LSD $p = 0.05$	Bioagents (B)	0.80	0.19	0.22	0.70	0.83
	Pathogens (P)	0.90	0.21	0.24	0.78	0.93
	B × P	1.80	0.43	0.49	1.56	1.87
CV		3.55	2.88	3.13	2.35	1.90

Data are presented as treatments mean ($n = 5$). The mean values within a column followed by the different letter are significantly different at $p = 0.05$ by Duncan's multiple range test (DMRT)

CV coefficient of variation, *SOD* superoxide dismutase, *CAT* catalase, *PPO* polyphenol oxidase, *PAL* phenylalanine ammonialyase, *C* control, *M M. incognita*, *P P. betavasculorum*, and *R R. solani*

2017). The activation of antioxidant system in response to oxidative burst after pathogen infection has been proposed for conveying the signal transduction for downstream defense responses (Liu et al. 2017). SOD catalyzes the dismutation of $O_2{*}^-$ into H_2O_2, and O_2, and a change in the activity of SOD may indicate an increased concentration of intracellular $O_2{*}^-$ (Wang et al. 2004). In this way, the increased SOD activity may have contributed to an increase in H_2O_2 concentration, which results in the enhanced plant defense responses against pathogens (Shetty et al. 2008). However, excessive accumulation of H_2O_2 is potentially harmful to the host plant, and to prevent damage, it must be converted into substances, which are less dangerous to the cell. The catalase is the main enzymatic H_2O_2 scavenger in plant cells is frequently used to catalyze the decomposition of H_2O_2 into water rapidly and molecular oxygen (Scandalios et al. 1997). Therefore, the high activities of SOD and CAT in the present study suggested that the antioxidant defense system was maintained at a high level in plants colonized by *P. putida* and *B. subtilis* in response to pathogenic infection.

Beneficial microbes stimulate a variety of defense enzymes in host plants in response to the pathogenic attack (Raj et al. 2016). The highest activities of PAL and PPO reduced the disease incidence. PPO, a copper-containing bifunctional enzyme, is known to hydroxylate and oxidize phenolic compounds into highly reactive ortho-quinones, which possess antipathogenic properties (Li and Steffens 2002). The enhanced activity of PPO participates in plant defense against a broad spectrum of pathogens and pests (Kampatsikas et al. 2019).

Table 6 Influence of *Pseudomonas putida*, *Bacillus subtilis*, and their mixtures on the chlorophyll fluorescence parameters of beetroot infected with two/three pathogens simultaneously in a disease complex incited by *Meloidogyne incognita*, *Pectobacterium betavasculorum*, and *Rhizoctonia solani*

Bioagents	Pathogens	Fv/Fm	ϕPSII	qP	NPQ	ETR
Control	C	0.661^{d}	0.436^{d}	0.534^{d}	0.425^{c}	163.43^{c}
	M + P	0.404^{l}	0.252^{jk}	0.263^{m}	0.292^{j}	119.34^{gh}
	M + R	0.382^{m}	0.244^{kl}	0.254^{m}	0.271^{k}	116.31^{h}
	P + R	0.425^{k}	0.283^{hi}	0.291^{l}	0.314^{i}	123.43^{g}
	M + P + R	0.343^{n}	0.206^{m}	0.221^{n}	0.231^{l}	103.21^{i}
B. subtilis	C	0.691^{c}	0.461^{c}	0.571^{c}	0.446^{b}	171.54^{b}
	M + P	0.501^{h}	0.283^{hi}	0.384^{hij}	0.342^{gh}	135.37^{f}
	M + R	0.482^{hi}	0.271^{ij}	0.362^{j}	0.336^{h}	133.31^{f}
	P + R	0.525^{f}	0.304^{h}	0.403^{gh}	0.353^{efgh}	138.51^{f}
	M + P + R	0.446^{j}	0.225^{l}	0.321^{k}	0.264^{k}	121.42^{gh}
P. putida	C	0.712^{b}	0.483^{b}	0.591^{b}	0.453^{ab}	173.53^{b}
	M + P	0.523^{f}	0.294^{h}	0.386^{hi}	0.353^{efgh}	136.35^{f}
	M + R	0.503^{gh}	0.285^{hi}	0.374^{ij}	0.346^{fgh}	134.46^{f}
	P + R	0.535^{f}	0.326^{g}	0.412^{g}	0.362^{efg}	140.29^{ef}
	M + P + R	0.471^{i}	0.243^{kl}	0.341^{k}	0.281^{jk}	124.33^{g}
B. subtilis + *P. putida*	C	0.782^{a}	0.534^{a}	0.651^{a}	0.472^{a}	187.26^{a}
	M + P	0.593^{e}	0.352^{f}	0.463^{ef}	0.371^{e}	149.27^{d}
	M + R	0.582^{e}	0.346^{f}	0.451^{f}	0.364^{ef}	146.41^{de}
	P + R	0.601^{e}	0.381^{e}	0.482^{e}	0.395^{d}	151.38^{d}
	M + P + R	0.526^{f}	0.293^{h}	0.413^{g}	0.332^{hi}	134.63^{f}
LSD $p = 0.05$	Bioagents (B)	0.010	0.008	0.009	0.008	2.94
	Pathogens (P)	0.011	0.009	0.010	0.009	3.28
	B × P	0.023	0.019	0.021	0.019	6.57
CV		3.06	4.28	4.06	4.36	3.65

Data are presented as treatments mean ($n = 5$). The mean values within a column followed by the different letter are significantly different at $p = 0.05$ by Duncan's multiple range test (DMRT)

CV coefficient of variation, *Fv/Fm* maximal PSII efficiency, *ϕPSII* photochemical efficiency of PSII, *qP* photochemical quenching, *NPQ* nonphotochemical quenching, *ETR* electron transport rate, *C* control, *M M. incognita*, *P P. betavasculorum*, and *R R. solani*

Moreover, PAL catalyzes the first step of the phenylpropanoid pathway in the conversion of L-phenylalanine to trans-cinnamic acid, resulting in the biosynthesis of phytoalexins and phenolic compounds (Garcion et al. 2014). These compounds had a vital role in the protection of plants against a wide range of pathogens (Mierziak et al. 2014). Hence, the increase in the activity of PPO and PAL may prevent the pathogenic infection of beetroot due to the colonization by *P. putida* and *B. subtilis* and accumulation of the defense enzymes.

A significant reduction in chlorophyll content was observed in plants inoculated with *M. incognita*, *P. betavasculorum*, and *R. solani.* Chlorophyll pigments mainly capture light via photosystem II, with consequent electron transport. Plants suffer significant pigment loss when exposed to pathogen infection (Berova et al. 2007) also observed in the present study. The increase in chlorophyll content has been attributed to the increased photosynthetic leaf area of plants due to inoculation with plant growth-promoting bacteria (Ali et al. 2011).

Among the various attributes of chlorophyll a fluorescence, Fv/Fm, which reflects the quantum efficiency of PSII, is an excellent indicator of plant stress (Maxwell and Johnson 2000). In the stressed plants, a reduction in Fv/Fm values as observed in this study may indicate photoinhibition and damage to PSII (Araújo and Deminicis 2009). A decrease in NPQ values as recorded in the present study may indicate damage in the antennae pigments. This parameter is regarded as a very sensitive indicator of photosynthetic inhibition (Ricart et al. 2010). These results are supported by several previous findings in which pathogenic infection led to a decline in Fv/Fm, ØPSII, qP, NPQ, and ETR (Su et al. 2017), and a decrease in photosynthesis might be due to a downregulation of photosynthesis or damage of the photosynthetic apparatus.

In this investigation, *P. putida* and *B. subtilis*, as well as their mixture, were able to maintain the photosynthetic performance of pathogen-inoculated plants suggesting the protective role of these bioagents on photosynthetic apparatus and growth improvement in beetroot under biotic stress. The enhanced effects of biocontrol agents in combination against pathogens have been attributed to the potential synergistic effects of the different mechanisms (Manasfi et al. 2018). The mixture of microbes may enhance the genetic diversity of biocontrol systems, persist longer in the rhizosphere, and utilize a broader range of biocontrol mechanisms to suppress the pathogens.

Conclusion

The combined application of *P. putida* and *B. subtilis* improved plant growth, and chlorophyll fluorescence attributes and induced high levels of defense enzymes. The combined use of these biocontrol agents caused more reductions in galling, nematode multiplications, and disease indices. Therefore, *P. putida* together with *B. subtilis* may be used for the management of disease complex of beetroot.

Abbreviations

CAT: Catalase; ETR: Electron transport rate; Fv/Fm: Maximal PSII efficiency; NPQ: Non-photochemical quenching; PAL: Phenylalanine ammonia-lyase; PPO: Polyphenol oxidase; ϕPSII: Photochemical efficiency of PSII; qP: Photochemical quenching; SOD: Superoxide dismutase

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Authors' contributions

ZAS designed the experiment and drafted the manuscript. MRK carried out the experimental work and performed the statistical analysis. Both the authors read and approved the final manuscript.

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Availability of data and materials

All data of the study have been presented in the manuscript.

Ethics approval and consent to participate

This manuscript is in accordance with the guide for authors available on the journal's website. Also, this work has not been published previously and is approved by both authors.

Consent for publication

Both authors approve to the publication.

Competing interests

The authors declare that they have no competing interests.

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