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RESEARCH

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Native bacteria for field biocontrol of black root rot in Egyptian strawberry

Farid Abd-El-Kareem, Ibrahim E. Elshahawy and Mahfouz M. M. Abd-Elgawad*

Abstract

Background: Increasing cultivation of strawberry in Egypt has boosted efforts to increase its yield. Biocontrol agents (BCAs) may avoid side effects and health risks caused by chemical fungicides used to control black root rot disease in strawberry. Some BCAs control the disease and augment strawberry yield, but additional research is needed to fit BCAs into emerging control strategies. The impact of six bacterial isolates of *Paenibacillus polymyxa* and *Bacillus brevis* on this disease and on berry yield is reported and compared to a common chemical fungicide.

Results: The bacterial isolates reduced the growth of the black root rot causal agents *Fusarium solani*, *Rhizoctonia solani*, and *Macrophomina phaseolina,* in dual culture bioassays. The greatest fungal inhibition was caused by *P. polymyxa* isolates 1 and 2 and *B. brevis* isolate 2. They suppressed the growth of *F. solani*, *R. solani*, and *M. phaseolina* by more than 74, 76, and 79%, respectively. Disease severity and incidence were significantly reduced on naturally infected strawberry plants in the field by the six isolates. The best results were obtained by the superior bioassay isolates which suppressed the disease incidence by 73, 77, and 71%, and its severity by 72, 78, and 70%, respectively. Disease suppression by bacteria was comparable to that by fungicide Actamyl. Bacteria surpassed Actamyl with regard to strawberry yield and enhancement of peroxidase and chitinase activities in the leaves.

Conclusions: These isolates are potential benign alternatives to fungicides used against black root rot in strawberry in Egypt. More studies are needed to examine their economic use on a wider scale.

Keywords: *Bacillus*, Black root rot, Enzyme activities, Strawberry yield

Background

Strawberry (*Fragaria ananassa* Duchesne) acreage has been expanding in Egypt due to several merits: the favorable Mediterranean climate, proper fertile soils, and geographic location offering closeness of export markets; all back high yield of a specialty crop. Abd-Elgawad (2019) emphasized that such factors can grant not only good strawberry fruit yield in size and quality with low produce prices but also first fruiting and lengthy reaping season. Admittedly, the ongoing economic crisis associated with corona pandemic enhanced the social and economic gains of the strawberry. The economic significance of the crop is quite apparent, for local consumption (e.g., fresh fruit and juice) and exportation to provide foreign exchange revenue for Egypt. However, the ongoing increase in Egyptian strawberry acreage is frequently accompanied with elevated plant infection by soilborne fungi (El-Shemy et al. 2013; Abd-El-Kareem et al. 2019a, b).

One of the serious and common diseases is the black root rot in Egyptian strawberry acreage. It is brought about by several fungal pathogens (singly or in combination); e.g., *Macrophomina phaseolina* (Hutton et al. 2013), *Fusarium oxysporum* (Juber et al. 2014), and *Rhizoctonia* spp. (Fang et al. 2013). This root disease ranks high among other strawberry rot diseases. A considerable incidence of the disease has been observed in Egypt in recent years (Abd-El-Kareem et al. 2019a). Our field monitoring indicated that *Macrophomina phaseolina*, *Pythium* sp. (fungal-like pathogen), *Rhizoctonia*

*Correspondence: mahfouzian2000@yahoo.com
Plant Pathology Department, National Research Centre, Dokki, Giza 12622, Egypt

solani, and *Fusarium solani* are the primary causal agents of the disease in Egypt. Its quite apparent symptoms are blackening of the main strawberry root resulting in gradual deterioration of the whole plant with decrease in its vitality and productivity. Ultimately, plant death usually occurs via killing and decaying of feeder rootlets (Abdel-Sattar et al. 2008; Fang et al. 2012; Ceja-Torres et al. 2014). Yield increase of 77.1 and 72.8%, was obtained with potassium silicate and calcium silicate, respectively, to control the disease (Abd-El-Kareem et al. 2019a).

Although a few chemical fungicides can sometimes result in reasonable management of fungal diseases, they generally bring about negative impact to the ecological settings and human health. This situation has been sparking researchers to earnestly work to avert the use of toxic fungicides against this serious disease (Abd-El-Kareem et al. 2019a, b, 2021). Biological control is one of the most promising and safe measure in this respect. Biocontrol agents (BCAs) can work through various mechanisms or modes of action (Abd-Elgawad and Askary 2018). Thus, BCAs may offer more durable and fungicidal effect without any toxic/unsafe residues in human food chain (Seddon et al. 2007; Choudhary and Johri 2009; Ahmed 2013). *Paenibacillus polymyxa* (Bacillales: Paenibacillaceae), the type species of genus, for instance, can be used to control several types of diseases in both horticultural and food crops. It has various benefits comprising promotion of plant growth, nitrogen fixation, solubilizing soil phosphorus, and producing exopolysaccharides, hydrolytic enzymes, antibiotics, and cytokinins (Raza et al. 2008; Lal and Tabacchioni 2009). Seddon et al. (2007) reported that the bacterium *Bacillus brevis* (Bacillales: Paenibacillaceae) operates against pathogenic fungi of plant via two mechanisms. Firstly, the gramicidin S as antifungal metabolite can directly suppress conidial evolution/growth of the pathogen. The second mechanism is through the bio-surfactant manufactured by *B. brevis*. It can lower surface wetness in events where saturation of relative humidity is not prevailed. Thus, the fungal conidia cannot germinate.

The objective of the present investigation was to evaluate the impacts of soil treatments with three isolates for each of *Paenibacillus polymyxa* and *Bacillus brevis* on strawberry black root rot disease and crop yield parameters. Putative enzymatic activities related to the presumed effects were measured.

Methods

Black root rot disease-causal fungi and their tested antagonists

Local isolates of the pathogenic fungi *Macrophomina phaseolina*, *F. solani*, and *R. solani,* the causal agents of this disease on strawberry roots and 3 isolates for each of *Bacillus brevis* and *Paenibacillus polymyxa* as antagonistic bacteria are maintained and supplied by Plant Pathology Department, National Research Centre (NRC) Giza, Egypt, for this study. These fungi and their antagonists were originally isolated from strawberry fields, Eldeer village, Qalioubia Governorate during previous experimentation (Abd-El-Kareem et al. 2019a, b; unpublished data). Among 67 isolates, we selected only six bacterial antagonists with the highest effectiveness against the pathogenic fungi. This research is a series of experiments on the use of biocontrol agents in controlling black root rot disease in strawberry plants. More details on the identification of these biologicals are given as a Additional file 1.

Bioassays of the bacterial isolates against the pathogens

The *P. polymyxa* isolates 1, 2, and 3 and *B. brevis* isolates 1, 2, and 3 were tested against the black root rot pathogens *M. phaseolina*, *R. solani*, and *F. solani* via the dual culturing as set out by Estrella et al. (2007). Each bacterial isolate was singly cultured at 1 cm from the edge of a Petri plate. It was streaked on fresh and sterilized potato dextrose agar (PDA) medium in the plate. Five 9-cm diameter Petri plates with the medium were used as replicates per treatment (a fungal species) and the untreated check. Suppressing the growth of the fungal mycelium was then estimated using Pandey et al. (2000)'s formula as follows:

$$R = (C - T/C) \times 100,$$

where R = growth reduction of the fungal mycelium (%), C = radius (cm) of growth of the fungal isolate in control dishes, and T = radius (cm) of growth of the fungal isolate in dual culture dishes.

Field trials

Field trials were conducted during 2019/20 and 2020/21 growing seasons (mid-October to end of April) at Qalioubia Governorate (Eldeer village, Toukh), Egypt, with light loamy soil and natural infestation. This field has a history of soil infested by the black root rot disease (Abd-El-Kareem et al. 2019a, b, 2021). Three plots (each of 1.2×5 m) were replicated for each treatment including each of the bacterial isolate and the fungicide Actamyl (3 g/l as recommended by manufacturer) in addition to the untreated check. A replicate contained 100 strawberry transplants. Seedlings of the cultivar Festival were dipped in bacterial cell suspension [1×10^8 colony forming units (CFU)/ml water per a bacterial isolate] for 30 min (Abd-El-Kareem et al. 2019b) then mingled with 5% Arabic gum to boost glutinous capacity and attain sound distribution of the bacteria on the surfaces of the addressed roots immediately pre-transplanting. Only water was used for soaking seedlings for the same term

to act as the control. The experimental layout was a completely randomized block model. All strawberry transplants had the same production practices of fertilizers, irrigation, and fruit collection plan (El-Shemy et al. 2013).

Rating disease incidence and severity

Disease incidence was rated 100 days after cultivation as follows:

$$\text{Disease incidence \%} = (\text{Number of infected plants}/\text{Total number of plants}) \times 100$$

Disease severity (DS) was computed at the season-end (6.5 months after transplanting) using the following 0–5 rate set out by Morocko (2006):

$$\text{Disease severity \%} = \frac{\sum (\text{Disease grade} \times \text{number of plants in each grade})}{\text{Total number of plants} \times \text{highest disease grade}} \times 100$$

Determination of plant growth parameters and yield

Strawberry plant weights (fresh and dry) in each treatment were recorded at season end. Also, assembled fruit yield (Ton/feddan) in each treatment was estimated at season-end.

Enzyme activities in strawberry leaves

Extraction of enzymes

Fresh strawberry leaves (g) were collected and homogenized according to Abd-El-Kareem et al. (2019a, b). A 0.1 M sodium phosphate buffer (pH 7.1) at the rate of 1/3 w/v (Goldschmidt et al. 1968) was used. The resultant material was subjected to centrifugation at 3000 rpm for 15 min, and the activities of the following enzymes were assessed in the supernatant. Three replicates for each treatment were used.

Peroxidase

Its activity was measured as set out by Abeles et al. (1971), and 0.1 ml of the enzyme extract was incubated with 4 ml of guaiacol solution for 1 min at 25 °C and absorbance at 470 nm. The solution of guaiacol comprised 0.5 ml of 2% guaiacol, 3 ml of 0.05 M potassium phosphate, pH 7, and 0.5 ml of 0.3% H_2O_2. The enzyme activity was shown as the increment in absorbance at 470 nm/gram fresh weight/1 min using spectrophotometer (Spectronic 20-D).

Chitinase

Chitin powder was used to prepare the substrate colloidal chitin (Ried and Ogryd-Ziak 1981). Monreal and Reese (1969)'s method was applied to determine the chitinase activity.

Reducing sugar was assessed in 1 ml of the supernatant by dinitrosalicylic acid. The optical density was determined at 540 nm using spectrophotometer (Spectronic 20-D). The enzyme activity was explained in terms of mM *N*-acetylglucosamine equivalent released/gram fresh weight tissue/60 min.

Statistical analysis

Analysis of variance (ANOVA) was applied on the bioassay of the growth areas for the tested fungi using dual culturing and on the combined treatments of the two seasons. Means were separated via Duncan's New Multiple Range Test (DNMRT).

Results

The antagonistic bacterial effect on the fungal growth

All the tested isolates of *B. brevis* and *P. polymyxa* differently decreased the growth area of *F. solani*, *R. solani*, and *M. phaseolina* (Table 1). The highest reduction was obtained with *P. polymyxa* isolates 1 and 2 and *B. brevis* isolate 2 which reduced the growth area by more than 74.4, 76.4 and 79.6% for *F. solani, R. solani*, and *M. phaseolina*, respectively. Moderate reduction was brought about by the other isolates. Examples are illustrated to demonstrate the reduction caused by *Bacillus brevis* isolate 2 (Fig. 1) and *P. polymyxa* isolate 1 (Fig. 2).

Field bacterial effects

The six isolates of *P. polymyxa* and *B. brevis* were tested for their impact on black root rot disease, strawberry vegetative characters, and yield as well as enzyme activities of strawberry plants under field conditions.

Their impacts on the strawberry disease

All the tested isolates of *P. polymyxa* and *B. brevis* considerably reduced the black root rot disease incidence and severity on strawberry plants (Table 2). Less plant intensity with more dead or unhealthy plants was found in the untreated control plots (Fig. 3). The best plant growth was found in plots treated with *P. polymyxa* isolates 1 and 2 and *B. brevis* isolate 2. The latter 3 isolates could reduce the disease incidence by 73.1, 76.9 and 71.2% and disease severity by 72, 78, and 70%. Meanwhile, the other isolates showed moderate bacterial impact. The reduction in disease incidence and severity obtained by *P. polymyxa*

Table 1 Reduction in the growth area of the strawberry black root rot-causal fungi as affected by six bacterial isolates

Bacterial isolates	Means of fungal growth area (cm^2)					
	Fusarium solani	**Reduction %**	***Rhizoctonia solani***	**Reduction %**	***Macrophomina phaseolina***	**Reduction %**
Paenibacillus polymyxa 1	15.0d	74.4	10.0c	84.2	12.0c	81.1
P. polymyxa 2	14.0d	78.0	12.0c	81.1	11.0c	82.7
P. polymyxa 3	22.0c	65.4	31.0b	51.3	30.0b	52.3
Bacillus brevis 1	22.0c	65.4	33.0b	48.1	32.0b	49.7
B. brevis 2	15.0d	74.4	15.0c	76.4	13.0c	79.6
B. brevis 3	31.0b	51.3	28.0b	56.0	30.0b	52.3
Control	63.6a	0.0	63.6a	0.0	63.6a	0.0

*Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to DNMRT

isolates 1 and 2 and *B. brevis* isolate 2 was not different ($P \leq 0.05$) from that scored by the fungicide Actamyl (Table 2).

Their impacts on strawberry growth

All the tested isolates of *P. polymyxa* and *B. brevis* considerably boosted fresh and dry weight of strawberry plants (Table 3). The highest increase was recorded by *P. polymyxa* isolates 1 and 2 which elevated fresh weight by 100 and 103% and dry weight by 112 and 106%, respectively. Meanwhile, the other isolates had less favorable effects.

Their impacts on strawberry yield

All the tested isolates of *B. brevis* and *P. polymyxa* significantly enhanced strawberry yield (Table 4). The highest increase was obtained by *P. polymyxa* isolates 1 and 2 and *B. brevis* isolate 2 which raised strawberry yield by 78.9, 84.2, and 68.4%, respectively. Meanwhile, other isolates showed moderate positive impacts. The superior isolates, *P. polymyxa* isolates 1 and 2 and *B. brevis* isolate 2, surpassed Actamyl in boosting the strawberry yield.

Their impacts on enzyme activities of strawberry plants

All the tested isolates of *P. polymyxa* and *B. brevis* significantly increased enzyme activity of strawberry plants (Table 5). The highest activities were caused by *P. polymyxa* isolates 1 and 2 and *B. brevis* isolate 2 which elevated peroxidase activity by 130, 145, and 140% and chitinase activity by 133.3, 140, and 68.4%, respectively. The other isolates showed moderate impact on the activities of the two enzymes (Table 5).

Discussion

The averages of strawberry plant weight and fruit yield of the two combined growing seasons were variably enhanced due to application of each bacterial isolate tested. Such increases were probably resulting from suppression of the fungal pathogens causing the root rot disease. This was evidenced by the big reduction in the growth area of the causal fungi, and the severity and incidence of the disease. Some strains of *P. polymyxa* are known to produce antibiotics as polymyxins and fusaricidin (Mahajan and Balachandran 2017; Shaheen et al. 2011) while those of *B. brevis* produce antibiotics as tyrocidine and gramicidin (Mohamed 2020). Superior isolates that brought about the best reduction and greatest yield were *P. polymyxa* 1 and 2 as well as *B. brevis* 2. Interestingly, these three isolates demonstrated notable reduction in disease incidence and severity comparable to the chemical fungicide Actamyl. This fungicide is effective and of common use (Abd-Elsayed et al. 2019; Abd El-Razek et al. 2021) as well as recommended by the Egyptian Ministry of Agriculture (Agricultural Pesticides Committee 2020). However, stakeholders should be aware of the development of the chemical fungicide resistance as it is impacted by multiple factors, e.g., its mode of action and use pattern, the biology of the targeted pathogen, and the cropping system.

Hence, these benign isolates can be tried in earnest as safe alternatives to the relevant chemicals that are commonly used against this disease. The use of chemical fungicides can result in numerous undesirable effects, comprising (1) side-effects on beneficial and non-targeted fungi; (2) outbreaks/resurgence of secondary pathogens pests; (3) development of fungicide resistance; (4) contamination/pollution of the ecological settings/wildlife ecosystem; (5) gradual gathering of fungicide residues in food stuffs/materials; (6) lack of harmony in beneficial processes, e.g., pollination as pollinators are adversely affected; and (7) human diseases such as carcinogenic impacts (Kumar et al. 2021; Liu et al. 2021).

Additionally, BCAs related to species of *Bacillus* work through different modes of action. This helps them to be both more effective and durable without any toxic residues in the human food chain (Choudhary and Johri 2009; Ahmed 2013). The three superior isolates were

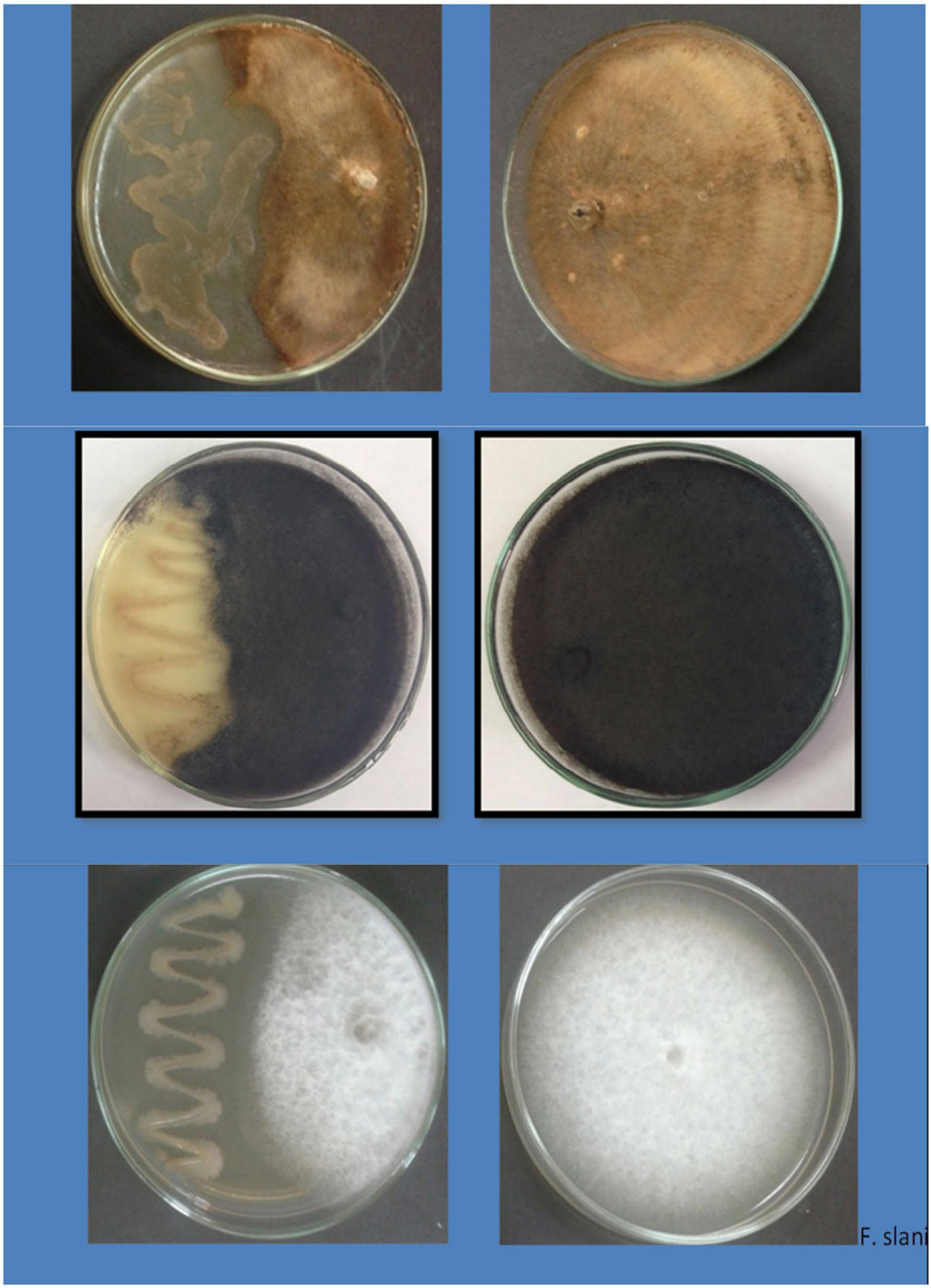

Fig. 1 Effect of *Bacillus brevis* isolate 2 on growth area of the pathogenic fungi *Rhizoctonia solani* (upper dishes), *Macrophomina phaseolina* (Medium dishes), and *Fusarium solani* (lower dishes)

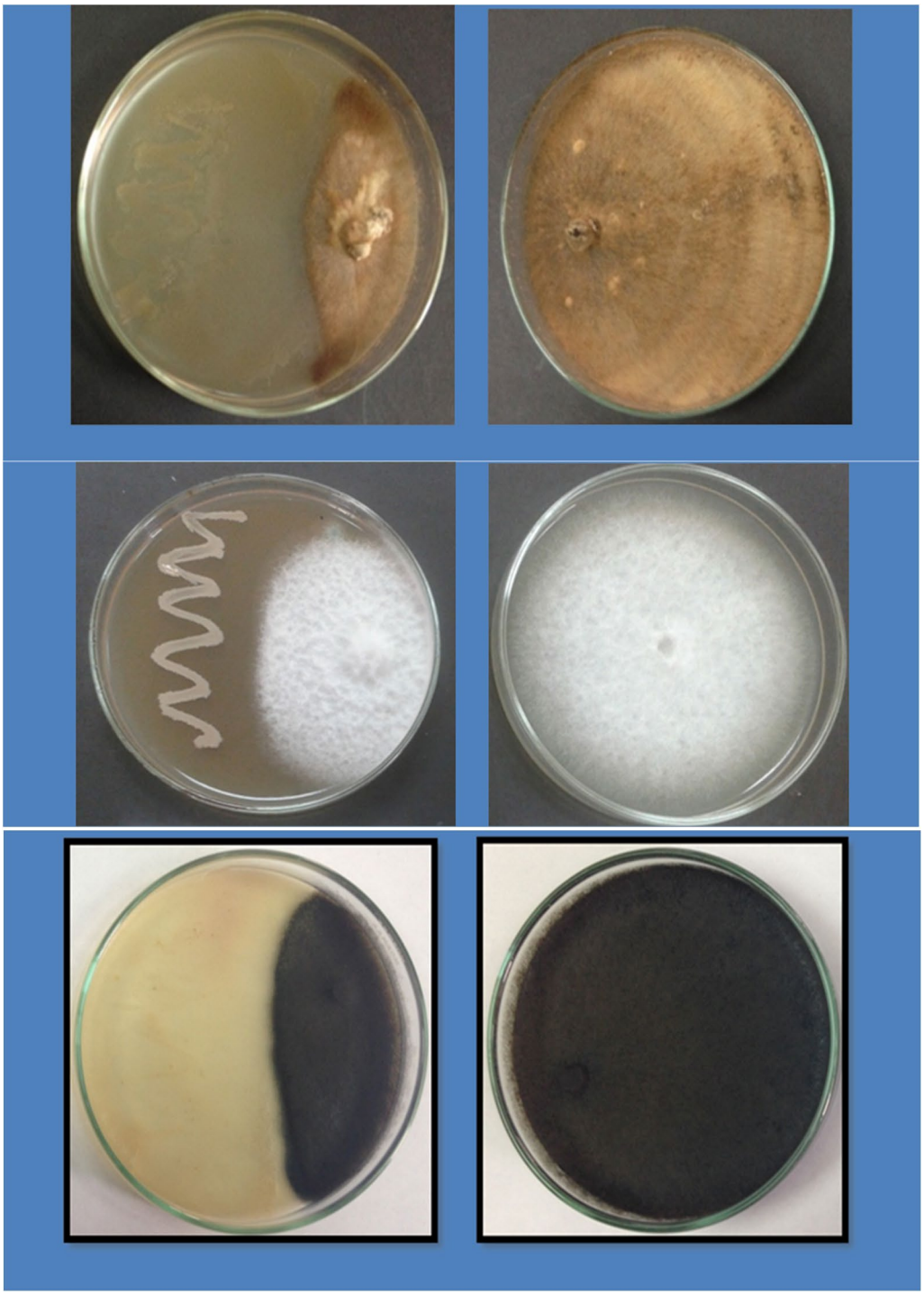

Fig. 2 Effect of *Paenibacillus polymyxa* isolate 1 on growth area of the pathogenic fungi *Macrophomina phaseolina* (upper dishes), *Rhizoctonia solani* (Medium dishes), and *Fusarium solani* (lower dishes)

also the best in increasing peroxidase and chitinase activities which imply plant resistance against diseases as these enzymes are pathogenesis-related proteins in strawberry plants. Although plant resistance is activated by the BCAs (increased chitinase and peroxidase plant activities), the same was fairly demonstrated in the case

Table 2 Impact of six bacterial isolates and chemical fungicide on strawberry black root rot disease in the field

Antagonistic bacterial isolates	Strawberry black root rot disease			
	Disease incidence	Reduction %	Disease severity	Reduction %
Paenibacillus polymyxa 1	14.0d	73.1	14.0e	72.0
P. polymyxa 2	12.0d	76.9	11.0e	78.0
P. polymyxa 3	24.0c	53.8	22.0d	56.0
Bacillus brevis 1	28.0b	46.2	27.0c	46.0
B. brevis 2	15.0d	71.2	15.0e	70.0
B. brevis 3	30.0b	42.3	32.0b	36.0
Actamyl 3 g/l (fungicide)	13.0d	75.0	12.0e	76.0
Control	52.0a	0.0	50.0a	0.0

*Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to DNMRT

Fig. 3 Effect of antagonistic bacteria on black root rot disease of strawberry plants, relatively unhealthy and less plant densities are seen in the untreated check

Table 3 Impact of antagonistic bacteria and chemical fungicide on the weight of strawberry plants in the field

Antagonistic bacterial isolates	Weight (g)/plant			
	Fresh	Increase %	Dry	Increase %
Paenibacillus polymyxa 1	267.0a	100.0	36.0a	111.8
P. polymyxa 2	264.0a	103.1	35.0a	105.9
P. polymyxa 3	252.0b	93.8	32.0b	88.2
Bacillus brevis 1	250.0b	92.3	31.0bc	82.4
B. brevis 2	250.0b	92.3	32.0bc	88.2
B. brevis 3	250.0b	92.3	32.0b	88.2
Actamyl 3 g/l (fungicide)	220.0c	69.2	30.0c	76.5
Control	130.0d	0.0	17.0b	0.0

*Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to DNMRT

Table 4 Effect of antagonistic bacteria and chemical fungicide on the strawberry yield in the field

Antagonistic bacterial isolates	Strawberry yield	
	Tons/feddan	Increase %
Paenibacillus polymyxa 1	17.0a	78.9
Paenibacillus polymyxa 2	17.5.0a	84.2
Paenibacillus polymyxa 3	13.0c	36.8
Bacillus brevis 1	13.0c	36.8
Bacillus brevis 2	16.0a	68.4
Bacillus brevis 3	14.0bc	47.4
Actamyl 3 g/l (fungicide)	14.0bc	47.4
Control	9.5d	0.0

*Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to DNMRT

of Actamyl which also elevated those enzymes to a lesser degree. That is possibly because plant defense system has different levels of specialization. A first or basic level induces a general innate defense. This basic level might slightly increase those enzymes but usually specialized pathogens overcome this defense level (Molinari 2020).

Moreover, *Paenibacillus polymyxa* has various advantages comprising nitrogen fixation, promotion of plant growth, solubilization of soil phosphorus, and production of exopolysaccharides, hydrolytic enzymes, antibiotics,

Table 5 Effect of antagonistic bacteria and chemical fungicide on peroxidase and chitinase activities of strawberry plants in the field

Antagonistic bacterial isolates	Enzyme activities			
	Peroxidase		Chitinase	
	Activity	Increase %	Activity	Increase %
Paenibacillus polymyxa 1	4.6a	130.0	7.0a	133.3
P. polymyxa 2	4.9a	145.0	7.2a	140.0
P. polymyxa 3	3.8d	90.0	6.4b	113.3
Bacillus brevis 1	4.2bc	110.0	6.0b	100.0
B. brevis 2	4.8a	140.0	7.1a	136.7
B. brevis 3	4.0cd	100.0	6.1b	103.0
Actamyl 3 g/l (fungicide)	3.8d	90.0	5.0c	66.7
Control	2.0e	0.0	3.0d	0.0

*Means in a column followed by the same letter are not significantly ($P \leq 0.05$) different according to DNMRT

and cytokinins (Lal and Tabacchioni 2009). *Paenibacillus polymyxa* can produce antibiotics that are so effective in controlling plant pathogens (Raza et al. 2008).

Many researchers have focused on biocontrol as a promising alternative for managing soil-borne diseases in sustainable and organic agriculture including integrated pest management (IPM). Their results revealed that a number of bacteria such as *Pseudomonas* spp. (Oktay and Kemal 2010), *Bacillus* spp. (Mansoori et al. 2013) and *Streptomyces* spp. (Xue et al. 2016) were able to outstandingly reduce plant diseases. More recently, studies have indicated that the plant growth-promoting rhizobacteria (PGPR) may also suppress different plant diseases (Remans et al. 2008; Rocheli et al. 2015). Among them, endospore-forming PGPRs, especially gram-positive *Paenibacillus polymyxa* and *Streptomycetes*, have better results than non-PGPRs due to being resistant to heat, radiation, drying, and toxic chemicals (Comasriu and Vivesrego 2002). These PGPR can produce a variety of antibiotics often associated with the ability of the bacterium to prevent proliferation of plant pathogens. Also, a number of PGPRs can generate enzymes such as proteases, chitinases, glucanases, and lipases that can lyse a portion of the cell walls of many pathogenic fungi (Majeed et al. 2015). The mechanisms by which these BCAs suppress many types of pathogens and pests differ among species/strains and therefore can be used in IPM (Dey et al. 2004; Lucy et al. 2004; Remans et al. 2008; Abd-Elgawad 2021a, b).

The bacterium *Bacillus brevis* operates as BCA against fungal plant pathogens via the two aforementioned modes of action. These mechanisms and their contribution to disease control were established and confirmed using wild-type and a gramicidin S-negative mutant of *B. brevis* (Seddon et al. 2007). Target crops were tomato, lettuce and cucumber. Cultures of *B. brevis*, fractionated into spore and supernatant fractions showed that disease control was due to gramicidin S and/or biosurfactant. Hence, disease control may vary due to the plant species and the prevailing ecological conditions.

Conclusions

Although six isolates of *Paenibacillus polymyxa* and *Bacillus brevis* tested herein proved to be safe and effective on strawberry black root rot disease, three isolates (*P. polymyxa* isolates 1 and 2 as well as *B. brevis* isolate 2) caused the best control. They inhibited the pathogen growth by more than 74, 76, and 79% for *F. solani*, *R. solani*, and *M. phaseolina*, respectively. Future research to use either of them singly or in combinations with a complementary disease control material(s) such as a plant extract should be attempted in earnest, especially in IPM programs.

Abbreviations

ANOVA: Analysis of variance; BCAs: Biological control agents; DNMRT: Duncan's new multiple range test; IPM: Integrated pest management; PDA: Potato dextrose agar; PGPR: Plant growth-promoting rhizobacteria.

Supplementary Information

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Additional file 1. Identification of the examined organisms.

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Authors' contributions
All authors equally participated in the development and implementation of the reviewing plan. Subsequently, they worked it out wrote the manuscript; the first author FA wrote and discussed the different parts of the article with IE and MA and all together finalized the manuscript. All authors have read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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