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# Potential activities of *Bacillus simplex* as a biocontrol agent against root rot of *Nigella sativa* caused by *Fusarium camptoceras*

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## Abstract

This study aimed to investigate the impact of two isolates of *Bacillus simplex* (PHYB1 and PHYB9) for controlling the root rot disease of black cumin (*Nigella sativa*) caused by *Fusarium camptoceras* (PHYF1) under greenhouse and field conditions at Assiut Governorate, Egypt. The highest reduction percentage of infection (22.5%) was recorded by the treatment of PHYB1 as suspension than the control (60%). Both isolates of *Bacillus* spp. provided the root and foliar dry weight under greenhouse and seed production in the field. The results showed that the isolate PHYB1 as a formulation gave the highest impact in the root dry weight (0.28 g/plant), followed by PHYB9 (0.22 g/plant), with insignificant difference between them. PHYB9 suspension also provided the highest seed production (27.97 g/plant), whereas PHYB9 as a formulation gave the lowest (24.08 g/plant). Studies on the interaction between *Bacillus* spp. on *F. camptoceras* by scanning electron microscope (SEM) revealed that both caused a complete mycoparasitism on the fungal growth. The bacterial growth was seen to adhere and colonize the hyphae, resulting in hyphal tissue maceration. Therefore, the use of both isolates of *Bacillus* spp. to control root rot disease of black cumin under greenhouse and field conditions can be recommended.

**Keywords:** Biological control, Black cumin, *Bacillus* spp., *Fusarium* wilt, Rhizobacteria

## Background

Black cumin (*Nigella sativa* L., Family: Ranunculaceae) seeds have great economic importance as they occupy an export priority in the first rank. It has especial importance among the other traditional crops in the Middle and Upper Egypt, especially in Giza, Fayoum, Beni-Suef, El-Minya, and Assiut Governorates (FAO 2016). It is an annual herb possessing a wide range of medicinal uses; the seeds are used in folk herbal medicine all over the world for the treatment and prevention of a number of diseases (Chevallier 1996). Root rot is one of the most serious diseases affecting black cumin. It is caused by *Fusarium* spp. (Sharma and Meena 2012). Fungal disease control is based mainly on the use of fungicides (Al-Sman et al. 2017). Fungicides may be toxic to animals, crops, and humans and

could lead to the development of fungicide-tolerant pathogen strains (Abo-Elyousr et al. 2014). Biological control of soil-borne diseases, using bioagents, is an alternative method to the chemical control (Abo-Elyousr et al. 2019). It offers a way to effectively control pathogens without harmful effects on animals, humans, plants, and/or the environment (Sallam et al. 2013). In recent years, various *Bacillus* spp. were used as potential biocontrol agents against different *Fusarium* spp. The use of plant growth-promoting rhizobacteria (PGPR) has become a common practice in many countries of the world (Abo-Elyousr and Mohammed 2009 and Alamri et al. 2019).

The objective of this study was to determine the effect of two *Bacillus simplex* isolates, PHY1 and PHYB9 isolates, to control the root rot disease of black cumin caused by *Fusarium camptoceras* (PHYF1) under greenhouse and field conditions in Assiut Governorate, Egypt.

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## Materials and methods

### Source of microorganisms

Pathogenic fungi isolate *Fusarium camptoceras* (PHYF1), the causal agent of root rot disease, was isolated from black cumin (Al-Sman et al. 2017). The isolate was grown in Petri dishes (9 cm in diameter) which contained potato dextrose agar medium (PDA) (20 g dextrose, 15–20 g agar, 200 g potato, and 1 L water). Petri dishes were incubated for 7 days at 27 °C. Two isolates of *Bacillus simplex* (PHYB1 and PHYB9), isolated from the rhizosphere of black cumin, at Assiut Governorate, Egypt, were used. The isolates were identified according to their morphological and physiological characteristics (Dye 1968; Schaad 1988, and Holt et al. 1994).

### Inoculum preparation and soil infestation with the pathogenic fungi

Inoculum of pathogenic isolate were prepared by inoculating of discs 0.5 cm in diameter of 5-day-old cultures of PHYF1 in bottles which contained the autoclaved barley medium (100 dried coarse sand, 75 g dried barley grains, 75 ml tap water). Bottles were incubated for 15 days at  $25 \pm 1$  °C (Abo-Elyousr et al. 2009). After this period, the contents were mixed together and used as a source of inoculum. Pots (30 cm diameter) filled with 5 kg soil were infested by mixing about 150 g of the inoculums with the soil and then irrigated directly. For control treatments, sterilized uninoculated soil were used (Gabr et al. 1998). Ten disinfested seeds of black cumin were seeded in each pot, 7 days after soil infestation with the pathogen (Hilal et al. 2000). Pots were irrigated directly after sowing and subsequently as needed for 8 weeks.

### Inoculum preparation of suspension antagonistic of *Bacillus simplex*

The antagonistic bacterial isolates PHYB1 and PHYB9 were used in this study. Inoculum of each bacterial isolate was prepared by growing it in a nutrient yeast extract broth, incubated at 25 °C on an orbital shaker at 200 rpm for 24 h. Bacteria were afterward polluted by centrifugation at 15,000 rpm for 5 min and washed in distilled water and the concentration of the bacteria was adjusted to  $10^8$  cfu.

### Preparation of formulation antagonistic *Bacillus simplex*

To prepare the *Bacillus* spp. formulation, the method of Abo-Elyousr and El-Hendawy (2008) was followed. In brief, each isolate was grown separately at 20 °C in 250-ml flask each containing 100 ml of tryptic soya broth liquid medium and shaking at 1000 rpm for 48 h. A 400-ml aliquot of the bacterial suspension containing ( $10^8$  cfu ml<sup>-1</sup>) was mixed by 1 kg of the talc powder (dry-sterilized at 105 °C for 12 h), 15 g CaCO<sub>3</sub> (to adjust the pH to 7), and 10 g carboxymethylcellulose.

### Effect of *Bacillus simplex* as suspension/or formulation on disease severity under greenhouse conditions

Inoculums and pot infestation of the tested pathogenic isolate PHYF1 were prepared as previously mentioned. Each pot was planted by ten sterilized seeds of black cumin after they were soaked in the antagonistic bacterial suspension for 20 min and was also treated with the formulation bacteria; they were left to dry then grown directly. Two controls were used in this experiment: one was healthy control (untreated with bioagents or pathogens) and the second was infected control (untreated with bioagents but infected with the pathogen). The treatments were arranged in a randomized complete block design with five replicates. Results were recorded as disease severity percentage after 80 days of planting. The root rot was scored on 0–3 scale, where:

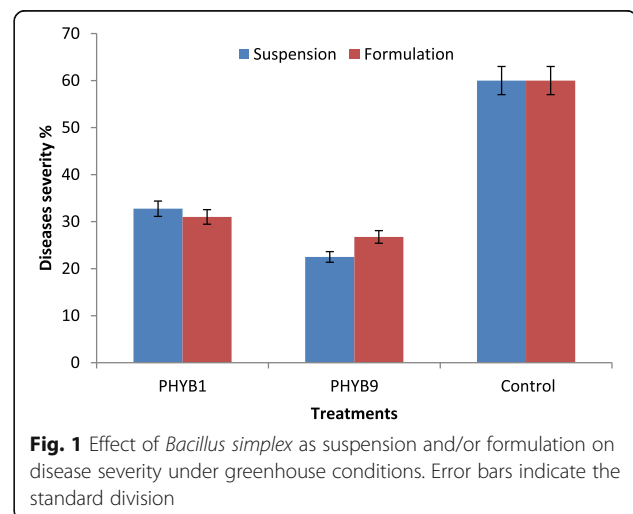
0 = healthy, creamy white on discoloration of sub crown internode and crown roots, 1 = light brown discoloration of sub crown internode and crown roots, 2 = brown discoloration of sub crown internode and crown roots, and 3 = dark brown to black discoloration of sub crown internode and crown roots and/or roots mostly decayed (Tinline et al. 1975). The disease severity (disease index) was estimated according to the following equation (Seleim et al. 2011):

$$\text{Root rot index} = \frac{(n \times 1) + (n \times 2) + (n \times 3)}{T \times 3} \times 100.$$

$n$  = number of plants in each scale of disease plants (1, 2, 3)

$T$  = total number of plants

At the end of the experiment, plants from various treatments were removed, washed thoroughly with running water, then oven dried at 65 °C for 72 h for dry weight. The experiment was repeated twice.



**Table 1** Effect of *Bacillus simplex* as suspension and/or formulation on root and foliar dry weight under greenhouse conditions

Treatments	Root dry weight (gm/plants)			Foliar dry weight (gm/plants)		
	Suspension	Formulation	Mean of treatments	Suspension	Formulation	Mean of treatments
PHYB1	0.29 ab	0.28 abc	0.28 a	2.11 b	2.46 a	2.02 a
PHYB9	0.21 c	0.22 bc	0.21 b	1.76 c	2.45 a	1.72 b
Infected control	0.08 d	0.10 d	0.09 c	0.72 d	0.75 d	0.73 c
Healthy control	0.30 a	0.30 a	0.30 a	2.46 a	2.42 a	2.44 a
Mean	0.22 a	0.22 a		1.73 a	1.80 a	

Values in the column followed by a similar letter are not significantly different as determined by the LSD test ( $P = 0.05$ )

### Control of the disease using *Bacillus simplex* under field conditions

The field trials were conducted at the Experimental Farm of Plant Pathology Department, Faculty of Agriculture, Assiut University, Egypt, during 2014/2015 and 2015/2016 growing seasons. The plots were divided into two rows: length/row 2 m and five holes/row; three plots were used for each treatment. The distance between holes is 30 cm and the infestation was carried out by adding 10 g substrate containing inocula of the pathogen PHYF1. Seeds were treated by bioagents as in greenhouse experiment. Disease severity percentage was recorded after 80 days from planting, and seed production (g/plants) was recorded at the end of experiment.

### Scanning electron microscopic (SEM) examination and photography

The method of Zhou et al. (2011) for preparing the samples was followed. After growing the bacterial suspensions of PHYB1 and PHYB9 with the pathogen PHYF1 in Petri dishes, 2–3 samples, size 3–5 mm, from the direct interactions were cut and fixed in 5% cold buffered glutaraldehyde for 2 min. The samples were scanned at 15 kV, using a JEOL JSM 5300 Lv scanning electron microscope, and photographed.

### Evaluation of fixed and volatile oils

#### Distillery fixed oils

Soxhlet extractor was used to get fixed oils of black cumin seeds, according to Salea et al. (2013).

#### Distillery volatile oils

Clevenger type device was used to get an oil pilot from black cumin seeds. In this method, steam was passed through the seeds containing the volatile oils; 100 g of the seeds were placed in flasks with 500 ml water and left for 3 h for the seeds boiling. The water evaporated carrying the volatile oil and dried over anhydrous sodium sulphate (Khalid and Shedeed 2016).

#### Statistical analysis

Analyses of variance were carried out, using MSTAT-C program version 2.10 (1991) and least significant difference (LSD) at  $P \leq 0.05$  was employed to test significant difference between treatments (Gomez and Gomez 1984). All experiments were performed twice.

### Results and discussion

#### Effect of *B. simplex* on disease severity under greenhouse conditions

The two isolates significantly reduced the disease severity than the control when treated as suspension or formulation. Isolate PHYB9 was more effective to the disease severity than PHYB1 (22.5 and 32.75%, respectively, Fig. 1). There was an insignificant difference between the two methods of application, suspension or formulation. Biocontrol of plant diseases usually involve interactions between the host, pathogen, and antagonist. *Bacillus* sp. is one of the important biocontrol agents that are potentially useful for biological control of plant diseases due to ability to form endospores and produce different biologically active compounds with a broad host range and a broad spectrum of activity (Chen et al.

**Table 2** Effect of *Bacillus simplex* as suspension and/or formulation on disease severity under field conditions in 2015 and 2016 growing seasons

Treatments	Disease severity percentage in field 2015			Disease severity percentage in field 2016		
	Suspension	Formulation	Mean of treatments	Suspension	Formulation	Mean of treatments
PHYB1	26.13 c	11.13f	18.63 b	13.75 b	14.75 b	14.25 b
PHYB9	25.63 c	14.38 ef	20.00 b	8.75 bc	10.00 bc	9.37 b
Infected control	58.13 a	39.38 b	48.75 a	49.38 a	49.38 a	49.38 a
Mean	26.73 a	16.83 b		17.97 a	18.53 a	

Values in the column followed by a similar letter are not significantly different as determined by the LSD test ( $P = 0.05$ )

**Table 3** Effect of *Bacillus simplex* as suspension and/or formulation on seed production under field conditions in 2015 and 2016 growing seasons

Treatments	Seed production 2015			Seed production 2016		
	Suspension	Formulation	Mean of treatments	Suspension	Formulation	Mean of treatments
PHYB1	26.30 b	26.42 b	18.63b	24.83 a	31.42 a	28.13 a
PHYB9	27.97 b	24.08 b	20.00 b	31.45 a	28.40 a	29.92 a
Infected control	8.80 c	8.80 c	48.75 a	10.18 b	10.18 b	10.18 b
Healthy control	39.70a	39.70 a	39.7 b	34.20 a	34.20 a	34.20 a
Mean	25.70 a	24.75 a		25.16 a	26.05 a	

Values in the column followed by a similar letter are not significantly different as determined by the LSD test ( $P = 0.05$ )

2013 and Fira et al. 2018). They screened 60 isolates isolated from China, and 6 strains from these isolates exhibited above 50% biocontrol efficacy on tomato wilt caused by *Ralstonia solanacearum* under greenhouse conditions. It is plant growth-promoting rhizobacteria that can have beneficial effects on plant growth (Gamez et al. 2019). The use of *Bacillus* against *Fusarium* wilt in different plant species including horticultural crops has been widely documented (Hashem and Abo-Elyousr 2011 and Alamri et al. 2019).

#### Effect of *B. simplex* on dry weight of root and foliar dry weight under greenhouse conditions

The data presented in Table 1 showed that seeds treated with PHYB1 and PHYB9 as a suspension or a formulation increased the root dry weight. PHYB1 gave the highest effect in the dry weigh (0.29 g/plant) than that by PHYB9 (0.21 g/plant) and then the control (0.08 g/plant). The results showed that bacteria PHYB1, as a formulation gave the highest effect on the root dry weight (0.28 g/plant), followed by PHYB9 with insignificant difference between them. Also, applications of PHYB1 and PHYB9 as a suspension and formulation gave the best foliar dry weight (2.11 and 1.76 g/plant), with insignificant difference between PHYB1 and PHYB9 when used as a formulation

Also, obtained results reported herein showed that bacterial suspension (PGPR) had the greatest efficacy regarding the morphological parameters of the seeds. Principally, the two treatments (formulation and suspension) were not different in most of the main studied traits of

plant growth. The results agree with previous studies of Miao et al. (2018). Biocontrol agents enhanced the plant growth by production of phytohormones, which are usually believed to be involved in plant growth promotion (Glickmann and Dessaux 1995). Gopalakrishnan et al. (2012) stated that the PGPR enhanced the morphological parameters of the plant by their production of some hormone, such as indole acetic acid (IAA), siderophore production, and phosphate solubilization. IAA-producing microorganisms are known to promote root elongation and plant growth

#### Effect of *B. simplex* on disease severity and seed production under field conditions

##### Disease severity percentage

Both tested bacterium isolates were effective against disease severity of black cumin in field when treated as a suspension. PHYB1 and PHYB9 gave highest reduction of disease severity (26.13 and 25.63%, respectively) than the control (58.13%). Treatment with PHYB1 as a formulation gave the highest reduction of disease severity (11.13%) than PHYB9 (14.84%) (Table 2). Also, in the 2016 season, the results indicated that both bacterial isolates were effective on disease severity when treated as suspension or formulation, with insignificant difference between them. PHYB9 as a suspension gave the best reduction of disease severity (8.75%), while PHYB1 as a formulation gave lower effect (14.75%) (Table 2). Under greenhouse or field experiments, both bacteria significantly decreased the disease severity percentage than the untreated control. Obtained results are in harmony with those reported by Sallam et al. (2013). The results showed that both treatments increased seed yield and plant growth in terms of root and stem dry weight. Many researchers demonstrated that PGPR significantly increased the yield and yield components (Yadegari and Rahmani 2010; Ekinci et al. 2014).

##### Seed production

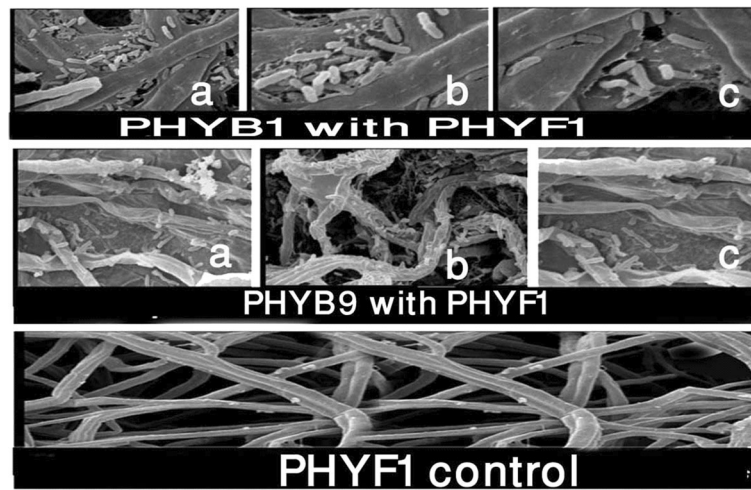
Seeds treated with both isolates PHYB1 and PHYB9 as a suspension or formulation improved its production.

**Table 4** Effect of *Bacillus simplex* as suspension and/or formulation on volatile oils and fixed oils

Treatments	Volatile oils (100 gm) seeds		Fixed oils (100) gm seeds	
	Suspension	Formulation	Suspension	Formulation
PHYB1	0.34 b	0.27 b	0.7 b	0.4 c
PHYB9	0.32 c	0.22 c	0.6 c	0.9 b
Infected control	0.16 d	0.13 d	0.4 d	0.4 c
Healthy control	0.38 a	0.37 a	1.0 a	1.0 a

Values in the column followed by a similar letter are not significantly different as determined by the LSD test ( $P = 0.05$ )





**Fig. 2** Interaction between *Bacillus simplex* strain (PHYB1 and PHYB9) with mycelia of *F. camptoceras* (PHYF1). Electron microscopic scanning photographs

PHYB1, as a suspension, gave the higher seed production (27.97 g/plants) than PHYB9 as a formulation (24.08 g/plants) with insignificant difference between them (Table 3).

#### Effect of *B. simplex* on volatile oils and fixed oils

Seeds of black cumin infected with *F. camptoceras* and treated with PHYB1 as a suspension gave a higher amount of volatile oil (0.34 mg/100 g seeds) than the PHYB9 and the less amount recorded by PHYB9 as formulation (0.22 mg/100 g seeds) and then the control (0.13 mg/100 g seeds) (Table 4). Also, the data show that PHYB9, as a formulation, gave the highest rate of fixed oil (0.9 mg/100 g seeds) and PHYB9, as a suspension, gave 0.7 mg/100 g seeds.

#### Interaction between *B. simplex* strain and mycelia of *F. camptoceras*

The studies revealed the nature of antagonism involved in the interaction between the isolated PHYB1 and PHYB9 with *F. camptoceras*. A clear distortion of fungal mycelium following lysis and bursting of the hyphae was observed at the interaction zone in dual cultures. The burst sites were surrounded by bacteria, some of which were found attached to the hyphae (Fig. 2b). Ultrastructural studies scanning electron microscope showed that this bioagents' mode of action against *F. camptoceras* includes two types of antagonism, antibiosis, and parasitism. Loss of pathogen hyphae structural integrity and reduction in spores developed due to antibiosis of strain were observed. Further, hyperparasitism of PGPR on fungal hyphae was evident through maceration, lysis of hyphal filaments, shriveling, shrinking, and abnormal coiling (Fig. 2a). These results are consistent with Lim et al. (1991). Antagonistic effect could be due to direct

influence of bioagents against pathogens. The interaction between *B. simplex* on *F. camptoceras* revealed that the *Bacillus* caused a complete mycoparasitism on the fungal growth. The bacterial growth was seen colonizing the hyphae and adhering, thus leading to maceration of hyphal tissues or antagonistic effect may be due to direct effect of antagonistic bacteria against *F. camptoceras* through cloning their hyphae to prevent their continued growth (Patel and Saraf 2017).

#### Conclusion

The plant growth-promoting rhizobacteria (PGPR) *B. simplex* PHYB 1 and 9 proved significant reductions of disease severity of *F. camptoceras* (PHYF1), when used as a suspension or formulation. They also improved the vegetative growth and seed productivity and quantity of fixed and volatile oils in black cumin.

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#### Authors' contributions

All authors contributed equally in the manuscript. AKM participated in the planning and implementation of the experiments, sampling, lab work, writing of the draft, and writing of the manuscript. AMK participated in the bacterial identification, implementation of the experiments, and writing of the manuscript. AE participated in the planning and implementation of the experiments, analyzing of data, and writing of the draft. AE participated in the implementation of the experiments and collection of data. All authors read and approved the final manuscript.

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**Consent for publication**

All authors approve to publication

**Competing interests**

The authors declare that they have no competing interests.

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