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Use of Individual Strains of *Bacillus* spp. and Their Mixtures for Controlling Damping-off of Cotton Seedlings

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Abstract

Eight rhizobacterial strains of *Bacillus* spp. B_1 (*B. coagulans*), B_2 (*B. globisporus*), B_3 (*B. pumilus*), B_4 (*B. subtilis*), B_5 (*B. circulans*), B_6 (*B. cereus*), B_7 (*B. coagulans*), and B_8 (*B. cereus*) were used in the present study. The individual eight strains and their mixtures were tested for controlling damping-off of cotton seedlings in commercial fields of Sakha (North Delta), El-Gemmeiza (Middle Delta), Sirs El-Lian (Middle Delta), and Mallawy (Middle Egypt). Certain treatments (individual strains and mixtures) effectively controlled the disease in certain locations; however, there was a lack of uniformity among locations, i.e. in some locations, they were unsuccessful in controlling the disease or even expanding the disease. Thus, the treatment showed their best performance in controlling the disease at El-Gemmeiza and Sirs El-Lian while they failed in Sakha and Mallawy. Contrary to the common belief, the present study showed that, in most cases, the performance of mixtures was inferior compared to that of the individual strains regarding the attributes related to the efficiency of biological control such as the magnitude of effective treatments, stable performance, seedling stand counts, and yield. This inferiority in performance was due to incompatibility among individual strains involved in the mixtures. In most cases, there was no significant correlation between stand and yield after applying *Bacillus* treatments. Strains B_1, B_3, and B_6 were promising strains for commercialization for three reasons. Firstly, they were the only treatments, which effectively increased stand at three locations. Secondly, they significantly increased yield at Sirs El-Lian, and Thirdly, B_3 and B_6 significantly increased yield at El-Gemmeiza. Treatments T16, T33, and T37 were the only deleterious treatments as they significantly reduced stand at Mallawy.

Keywords – Biological control – cotton diseases – *Fusarium* spp. – plant-growth-promoting (PGP) – rhizobacterium – *Rhizoctonia solani*

Introduction

Cotton seedling damping-off is a disease of multiple causes. *Rhizoctonia solani* and *Fusarium* spp. are the pathogens most commonly involved in the disease in Egypt (El-Samawaty et al. 1999).

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Seed-dressing fungicides is the primary method by which the disease is controlled (Aly et al. 2017). The exclusive reliance of cotton growers in Egypt on chemically synthetic fungicides for controlling this disease could lead to some problems such as the development of pathogen isolates resistant to the applied fungicides, environmental pollution, contamination of surface and groundwater, the deleterious non-target effects on humans, beneficial soil organisms, insects, birds and fish (Liu et al. 2018). All these problems could be overcome by the application of some strains of *Bacillus* spp. (Aly et al. 2017). The genus *Bacillus* is described as a wide group of Gram-positive bacteria. They are rod-shaped, endospore-forming with aerobic or facultatively anaerobic metabolism and catalase-positive bacteria. *Bacillus* spp. are exceptionally ubiquitous since they can inhabit a large variety of ecological niches; they are found in soil, water, air, surfaces, and rhizosphere of plants and in many other extreme environments (Fira et al. 2018). From the biological control point of view, the spore-forming capacity of *Bacillus* spp. gives them prime importance in the field of biological control. This is because the spores produced by *Bacillus* spp. have the ability to endure extreme environmental conditions (Shafi et al. 2017).

Also, one of the most significant characteristics of *Bacillus* spp is their complex secondary metabolism and the ability to generate a wide range of antagonistic compounds that are structurally distinct, including bacteriocins, antimicrobial peptides, lipopeptides, polyketides, and siderophores. (Fira et al. 2018). *Bacillus* spp. also show plant growth-promoting properties (Fira et al. 2018). The diverse group of plant growth promoting and the antagonistic mechanisms developed by the genus *Bacillus* to suppress plant pathogens may differ from one species to another. For example, *B. subtilis* showed antagonistic capacities toward pathogenic fungi and bacteria by producing inhibitory protein (Liu et al. 2010) and several families of cyclic lipopeptides (Luo et al. 2015). *B. coagulans* has been found to produce several surfactants that are powerful lipopeptide surfactans (Huszcza & Burczykc 2006). *B. circulans* enhances plant growth through the production of hormones, especially IAA (Sheng & Huang 2002). It also increased the availability of potassium and hence plant growth (Khalil et al. 2018). Cultures of *B. globispora* showed fungistatic activity against plant-pathogenic fungi (Piotrowska-Cyplik et al. 2011). *B. cereus* is a plant-growth-promoting (PGP) rhizobacterium and known biocontrol agent. Its growth promotion is related to its capacity to dissolve inorganic and organic phosphorus and to produce IAA and gibberellin (Ku et al. 2018), while its biocontrol activity was due to the production of hydrolytic enzymes, comprising chitinase and protease, which inhibit spore germination and germ tube elongation of the pathogenic fungi (Chang et al. 2006).

The PGP trait of *B. pumilus* is associated with its capacity to solubilize phosphorus, Potassium, and zinc (Verma et al. 2016). *B. pumilus* inhibits growth of pathogenic fungi by the production chitinase and the antibiotic surfactin (Agarwal et al. 2017). In a previous field study (Aly et al. 2017), we used individual strains of *Bacillus* spp. for controlling damping-off of cotton seedlings. Despite the positive results obtained by many tested strains, they often exhibited inconsistent performance from one site to another. Many factors (Pierson & weller 1994) could be contributed to this inconsistent performance, such as the variability in root colonization by the introduced strains of *Bacillus* spp. Thus, population sizes varied from root to root by several orders of magnitude, and some roots may be completely unprotected. The variable development or inactivation in situ of bacterial metabolites responsible for controlling the disease is another factor. Also, the incidence of diseases caused by non-target pathogens can often result in inconsistent results. The introduction of *Bacillus* may fail to provide an increase in yield simply because the disease was not extreme enough to reduce yield. Further, for many root disease, areas in a field where the severe disease occurs may be unpredictable (Pierson & Weller 1994). Therefore, the use of mixtures of *Bacillus* strains for suppressing damping-off of cotton seedlings is a more ecologically sound approach than the classic approach, which depends on individual strains. The superiority of the mixtures is due to their long persistence in the rhizosphere and the use of a larger variety of biocontrol mechanism under a broader variety of environmental conditions (Pierson & Weller 1994). In the present study, we tested the hypothesis that mixtures of *Bacillus* strains will improve their efficiency in controlling cotton seedling damping-off.

Materials & Methods

Bacterial strains

Eight of rhizobacterial strains of *Bacillus* spp. B_1 (*B. coagulans*), B_2 (*B. globisporus*), B_3 (*B. pumilus*), B_4 (*B. subtilis*), B_5 (*B. circulans*), B_6 (*B. cereus*), B_7 (*B. coagulans*), and B_8 (*B. cereus*) (Bergey's Manual of Systematic Bacteriology 1985) were randomly selected from the bacterial culture collection of Cotton Diseases Research Section, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt. All strains were initially isolated from the rhizosphere of infected cotton seedlings collected from diverse locations in Egypt (Table 1).

Table 1 Classification and geographic origins of *Bacillus* strains used in the present study

Strain no.	Classification	Geographic origin	
		Region	Governorate
B_1	*B. coagulans*	Unknown	Unknown
B_2	*B. globisporus*	East Delta	Daqahliya
B_3	*B. pumilus*	Middle Egypt	Giza
B_4	*B. subtilis*	Upper Egypt	Assiute
B_5	*B. circulans*	West Delta	Beheira
B_6	*B. cereus*	Unknown	Unknown
B_7	*B. coagulans*	Middle Egypt	Minya
B_8	*B. cereus*	East Delta	Sharqiya

Preparation of bacteria inoculum

Different bacterial strains were grown on a shaker at 30˚C for 72 h in nutrient glucose broth. The growth was adjusted to 10^8 cfu/ml turbidimetrically utilizing spectro 2000 RSP 220 v, 50 HZ. Bacterial cultures were developed in powder form by combining 400 ml of cell suspension with 1 Kg of talc as a carrier that previously had been autoclaved for 30 minutes, for two consecutive days, 10 g of carboxy methyl cellulose (CMC) was applied to 1 kg of the carrier and mixed properly. By adding calcium carbonate, the pH was adjusted to 7.0 for all products. The population of bacteria was measured as 4×10^7 cfu/g talc.

Field trials

The individual eight strains and their mixtures were tested for controlling the disease in field plots established in 2020 in commercial fields of Sakha (North Delta), El-Gemmeiza (Middle Delta), Sirs El-Lian (Middel Delta), and Mallawy (Middle Egypt). It is assumed that these widely separated sites represent four distinct agroecosystems. Soil type was clay in all the experimental sites except Mallawy where the soil type was sandy clay. Each experiment was carried out as a randomized complete block design of three replicates in Sakha and Sirs El-Lian and four replicates in El-Gemmeiza and Mallawy, and each replicates consisted of two, five-meter long rows. In mixtures, each strain was added as the fraction of the tested amount of the mixture that is composed. Thus, strains in a mixture were applied in equal amounts. The tested mixtures included two-way, three-way, and four-way mixtures of strains.

Planting dates were 15 April in Sakha, 28 March in El-Gemmeiza, 26 March in Sirs El-Lian, and 20 March in Mallawy. Stand (healthy surviving seedlings) were recorded 45 days from sowing date. Seed cotton yield (cottonseed and lint before ginning) was picked from 10-25 October at each site.

Isolation and identification of fungi in soils of the experimental sites

Infected seedlings exhibited typical damping-off symptoms were removed from the field and washed under tap water to eliminate any adhering soil. Small pieces of necrotic root and hypocotyl tissue were sterilized with 10% Clorox solution for 2 minutes and washed with sterilized water several times. On sterilized filter paper, the surface-sterilized pieces were then dried and plated on

potato dextrose agar (PDA) medium amended with streptomycin sulphate and rose Bengal to remove bacterial contamination. The plates were then incubated for 3-7 days at 26±3°C. According to Gilman (1966) and Barnet & Hunter (1979), the growing colonies were characterized. Colonies of each fungus were determined as percentage of the total growing colonies.

Statistical analysis of the data

A randomised complete block of three or four replicates was the experimental design used in the current research. The least significant difference (LSD) or Duncan's multiple range test was used to compare the means of treatments. Analysis of variance (ANOVA) was carried out by means of the MSTAT-C statistical package. ANOVA was carried out to data of each field site separately due to variations in environmental conditions and site management. The correlation was done with the SPSS 10.0.0. software package

Results

Each of *B. coagulans* and *B. cereus* was represented by two strains (25%). Each of the strains of *B. globisporus*, *B. pumillus*, *B. subtilis*, and *B. circulans* was represented by only one strain (12.5%) (Table 1). Two strains (25%) were isolated from unknown origins. Three strains (37.5%) were isolated from the Nile Delta governorates (Daqahliya, Beheira, and Sharqiya). Each of Giza (Middel Egypt) and Assiut (Upper Egypt) was represented by only one strain (12.5%) (Table 1).

The infected seedlings used in isolation yielded *Fusarium* spp. and *R. solani* at all the experimental sites. The differences in isolation frequency between the two fungi were nonsignificant at all sites. *Chaetomium* and *Pythium* were isolated only from El-Gemmeiza. *Aspergillus* spp. were isolated from all sites except Sirs El-Lian. *Rhizopus* spp. were isolated from all sites except El-Gemmeiza (Table 2).

Table 2 Frequency of fungi isolated from cotton seedlings infected with post-emergence damping-off in the experimental plots

Fungus	Isolation frequency (%)[a]			
	Sakha (cv. Giza 94)	**El-Gemmeiza (cv. Giza 86)**	**Sirs El-Lian (cv. Giza 86)**	**Mallawy (cv. Giza95)**
Aspergillus spp.	32.99	22.03	0.00	18.75
Chaetomium spp.	0.00	18.45	0.00	0.00
Fusarium spp.	28.77	33.93	48.22	25.82
Pythium spp.	0.00	7.14	0.00	0.00
Rhizoctonia solani	20.55	18.46	44.64	36.61
Rhizopus spp.	18.95	0.00	7.14	18.75
LSD ($P \leq 0.05$)	18.17	15.57	16.76	20.61

[a] Colonies of each fungus were expressed as percentage of the total developing colonies. Each value is the mean of four replications (plates)

Certain treatments (individual strains and mixtures) did effectively controlled the disease at some sites; however, there was a lack of consistency among sites, that is, they were ineffective in controlling the disease in other sites or even increased it (Tables 3, 4). The sharp contrast between the performance of treatments at Sirs El-Lian and their performance at Mallawy is the best example for the inconsistency. Thus, at Sirs El-Lian, all treatments significantly increased stand while at Mallawy, all treatments failed in increasing stand (Table 3). As to deleterious treatments, T16, T33, and T37 at Mallawy significantly reduced stand compared to control 2 (Table 3). Seed cotton yield in Sakha loction was lost due to sever infection with boll worms (Table 4).

At Sakha, 37.5% of the individual strains significantly increased stand while all mixtures failed in increasing stand. At El-Gemmeiza, 87.5% of the individual strains significantly improved stand while only 62.5% of the three-way mixtures significantly improved stand. All treatments at Sirs El-Lian significantiy increased stand. At Mallawy, all treatments failed in improving stand (Table 5). At El-Gemmeiza and Sirs El-Lian, the single strains were superior to mixtures in the

percentages of the treatments, which significantly increased yield. At Mallawy, all individual strains and mixtures failed in increasing stand (Table 5).

Table 3 Effect of treating cotton seed with *Bacillus* strains or their mixtures on stand of cotton seedlings at four locations in 2020

Treatment no.	*Bacillus* strain(s)	Stand (%)			
		Sakha (cv. Giza 94)	El-Gemmeiza (cv. Giza 86)	Sirs El-Lian (cv. Giza 86)	Mallawy (cv. Giza 95)
T1	B1	62.27 e*	66.00 b-e*	50.00 bc*	55.44 g
T2	B2	55.27 a-e	66.13 b-e*	46.67 b*	46.38 a-g
T3	B3	59.00 de*	72.25 c-e*	46.67 b*	48.31 a-g
T4	B4	58.00 c-e	68.63 b-e*	46.67 b*	48.75 b-g
T5	B5	54.53 a-e	59.63 a-e	56.67 b-e*	49.94 c-g
T6	B6	62.80 e*	69.38 c-e*	60.00 c-f*	50.31 c-g
T7	B7	50.53 a-e	69.13 c-e*	63.33 d-g*	47.38 a-g
T8	B8	53.07 a-e	72.00 c-e*	63.33 d-g*	47.19 a-g
T9	B2+B1	57.47 c-e	68.00 b-e*	66.67 e-h*	54.81 fg
T10	B3+B1	53.27 a-e	64.38 b-e*	66.67 e-h*	46.94 a-g
T11	B4+B1	57.73 c-e	69.75 c-e*	63.33 d-g*	50.69 c-g
T12	B5+B1	53.27 a-e	55.13 a-c	63.33 d-g*	47.50 a-g
T13	B6+B1	55.20 a-e	61.75 a-e	56.67 b-e*	46.31 a-g
T14	B7+B1	52.93 a-e	61.88 a-e	56.67 b-e*	49.63 c-g
T15	B8+B1	54.07 a-e	56.38 a-d	56.67 b-e*	45.06 a-e
T16	B3+B2	47.87 a-e	63.38 b-e*	63.33 d-g*	39.69 ab*
T17	B4+B2	45.13 a-d	65.25 b-e*	63.33 d-g*	45.19 a-e
T18	B5+B2	53.67 a-e	64.13 b-e*	63.33 d-g*	44.06 a-e
T19	B6+B2	40.07 ab	59.00 a-e	60.00 c-f*	50.31 c-g
T20	B7+B2	54.73 a-e	73.63 de*	50.00 bc*	48.81 b-g
T21	B8+B2	55.73 a-e	66.38 b-e*	63.33 d-g*	54.94 fg
T22	B4+B3	43.47 a-d	61.63 a-e	53.33 b-d*	45.06 a-e
T23	B5+B3	53.73 a-e	63.88 b-e*	53.33 b-d*	48.25 a-g
T24	B6+B3	53.73 a-e	67.63 b-e*	63.33 d-g*	47.56 a-g
T25	B7+B3	54.20 a-e	60.13 a-e	53.33 b-d*	51.69 d-g
T26	B8+B3	54.73 a-e	65.13 b-e*	56.67 b-e*	44.88 a-e
T27	B5+B4	49.53 a-e	62.13 a-e	50.00 bc*	45.88 a-f
T28	B6+B4	49.47 a-e	64.38 b-e*	53.33 b-d*	46.25 a-f
T29	B7+B4	52.13 a-e	54.88 a-c	60.00 c-f *	44.25 a-e
T30	B8+B4	50.13 a-e	65.88 b-e*	66.67 e-h*	51.50 c-g
T31	B6+B5	57.27 c-e	70.25 c-e*	66.67 e-h*	49.13 c-g
T32	B7+B5	52.20 a-e	61.50 a-e	70.00 f-h*	51.31 c-g
T33	B8+B5	47.53 a-e	70.00 c-e*	73.33 gh*	39.44 a*
T34	B7+B6	50.67 a-e	50.88 ab	70.00 f-h*	46.56 a-g
T35	B8+B6	49.33 a-e	64.63 b-e*	66.67 e-h*	51.44 c-g
T36	B8+B7	46.33 a-e	69.00 b-e*	66.67 e-h*	46.50 a-g
T37	B3+B2+B1	48.40 a-e	55.75 a-d	63.33 d-g*	42.31 a-c*
T38	B4+B2+B1	52.13 a-e	65.63 b-e*	63.33 d-g*	51.94 d-g
T39	B4+B3+B1	55.13 a-e	66.25 b-e*	63.33 d-g*	44.81 a-e
T40	B4+B3+B2	53.93 a-e	70.13 c-e*	70.00 f-h*	45.19 a-e
T41	B7+B6+B5	50.60 a-e	67.50 b-e*	66.67 e-h*	52.31 e-g
T42	B8+B6+B5	56.53 b-e	54.50 a-c	63.33 d-g*	48.31 a-g
T43	B8+B7+B5	49.40 a-e	64.13 b-e*	63.33 d-g*	43.06 a-d
T44	B8+B7+B6	47.33 a-e	61.25 a-e	60.00 c-f*	44.81 a-e
T45	B4+B3+B2+B1	42.07 a-c	62.38 a-e	63.33 d-g*	48.63 b-g
T46	B8+B7+B6+B5	39.27 a	68.75 b-e*	56.67 b-e*	50.31 c-g
T47	C_1[a]	41.73 a-c	76.38 e*	76.67 h*	48.81 b-g
T48	C_2[b]	41.87 a-c	45.38 a	26.67 a	52.00 d-g

[a] C_1 was cotton seeds treated with the fungicide Monceren at a rate of 3 g/Kg seeds

[b] C_2 was untreated cotton seeds

*Significant difference from control 2

Table 4 Effect of treating cotton seed with *Bacillus* strains or their mixtures on seed cotton yield at four locations in 2020

Treatment no.	*Bacillus* strain(s)	Seed cotton yield (Kentar[a]/Feddan[b])			
		Sakha[c] (cv. Giza 94)	El-Gemmeiza (cv. Giza 86)	Sirs El-Lian (cv. Giza 86)	Mallawy (cv. Giza 95)
T1	B1	---	7.31 a-f	5.93 e-g*	6.68 a-c
T2	B2	---	6.93 a-f	4.93 ef*	6.46 a-c
T3	B3	---	8.85 d-f*	5.45 ef*	5.98 a-c
T4	B4	---	7.95 a-f	5.17 ef*	6.26 a-c
T5	B5	---	7.06 a-f	6.41 fg*	6.33 a-c
T6	B6	---	8.34 b-f*	6.30 fg*	6.74 a-c
T7	B7	---	7.18 a-f	4.59 de*	7.71 a-c
T8	B8	---	8.72 c-f*	5.54 ef*	6.88 a-c
T9	B2+B1	---	7.83 a-f	4.86 ef*	6.67 a-c
T10	B3+B1	---	6.54 a-f	5.92 e-g*	6.88 a-c
T11	B4+B1	---	7.70 a-f	3.28 b-d*	5.84 a-c
T12	B5+B1	---	7.57 a-f	3.38 cd*	6.40 a-c
T13	B6+B1	---	7.44 a-f	2.60 bc*	5.91 a-c
T14	B7+B1	---	6.80 a-f	1.63 ab	5.84 a-c
T15	B8+B1	---	7.95 a-f	3.18 b-d*	6.54 a-c
T16	B3+B2	---	5.77 ab	3.01 bc*	6.05 a-c
T17	B4+B2	---	6.28 a-e	3.11 b-d*	6.18 a-c
T18	B5+B2	---	6.93 a-f	2.33 bc*	5.98 a-c
T19	B6+B2	---	7.18 a-f	2.80 bc*	7.44 a-c
T20	B7+B2	---	8.98 ef*	2.55 bc*	6.67 a-c
T21	B8+B2	---	7.44 a-f	2.33 bc*	6.33 a-c
T22	B4+B3	---	7.82 a-f	2.24 a-c	5.98 a-c
T23	B5+B3	---	7.83 a-f	3.01 bc*	6.26 a-c
T24	B6+B3	---	7.31 a-f	1.90 a-c	7.09 a-c
T25	B7+B3	---	6.67 a-f	2.48 bc*	7.79 bc
T26	B8+B3	---	6.29 a-e	3.05 bc*	6.67 a-c
T27	B5+B4	---	6.03 a-c	2.32 bc*	6.12 a-c
T28	B6+B4	---	7.44 a-f	2.89 bc*	7.51 a-c
T29	B7+B4	---	7.18 a-f	2.48 bc*	6.25 a-c
T30	B8+B4	---	5.77 ab	2.50 bc*	6.12 a-c
T31	B6+B5	---	8.20 a-f	2.06 a-c	7.16 a-c
T32	B7+B5	---	7.59 a-f	2.18 a-c	5.91 a-c
T33	B8+B5	---	8.47 b-f*	2.13 a-c	6.74 a-c
T34	B7+B6	---	7.18 a-f	2.52 bc*	6.05 a-c
T35	B8+B6	---	6.16 a-d	2.58 bc*	7.23 a-c
T36	B8+B7	---	8.21 a-f	2.93 bc*	8.00 c
T37	B3+B2+B1	---	8.08 a-f	2.05 a-c	7.23 a-c
T38	B4+B2+B1	---	7.83 a-f	2.03 a-c	7.30 a-c
T39	B4+B3+B1	---	7.31 a-f	1.81 a-c	6.25 a-c
T40	B4+B3+B2	---	8.47 b-f	2.08 a-c	6.19 a-c
T41	B7+B6+B5	---	7.31 a-f	2.78 bc*	5.42 a
T42	B8+B6+B5	---	7.18 a-f	2.36 bc*	7.23 a-c
T43	B8+B7+B5	---	8.08 a-f	1.88 a-c	5.56 ab
T44	B8+B7+B6	---	8.08 a-f	2.67 bc*	5.77 a-c
T45	B4+B3+B2+B1	---	6.93 a-f	2.94 bc*	6.40 a-c
T46	B8+B7+B6+B5	---	7.31 a-f	1.92 a-c	7.16 a-c
T47	C_1[d]	---	9.24 f*	7.17 g*	6.67 a-c
T48	C_2[e]	---	5.52 a	0.67 a	7.02 a-c

[a] One Kentar = 157.50 Kg

[b] One feddan = 4200 m^2

[c] Seed cotton yield was lost due to sever infection with boll worms

[d] C_1 was cotton seeds treated with the fungicide Monceren at a rate of 3 g/Kg seeds
[e] C_2 was untreated cotton seeds
*Significant difference from control 2

Table 5 Effect of application method of *Bacillus* strains on the magnitude of the effective treatments as biocontrol agents

Location	Application method	Effective treatments in increasing stand		Effective treatments in increasing yield	
		No.	%	No.	%[d]
Sakha	Single strains	3	37.5[a]	…	…
	Two-way mixtures	0.0	0.0[b]	…	…
	Three-way mixtures	0.0	0.0[c]	…	…
El-Gemmeiza	Single strains	7	87.5	3	37.5
	Two-way mixtures	24	85.7	2	7.1
	Three-way mixtures	5	62.5	0	0.0
Sirs El-Lian	Single strains	8	100	8	100
	Two-way mixtures	28	100	22	78.6
	Three-way mixtures	8	100	3	37.5
Mallawy	Single strains	0.0	0.0	0.0	0.0
	Two-way mixtures	0.0	0.0	0.0	0.0
	Three-way mixtures	0.0	0.0	0.0	0.0

[a] $\frac{\text{Number of effective treatments}}{8} \times 100$
[b] $\frac{\text{Number of effective treatments}}{28} \times 100$
[c] $\frac{\text{Number of effective treatments}}{8} \times 100$
[d] Not determined due to severe infection with boll worms

At Sakha, seedling stand counts of individual strains was significantly greater than those of the mixtures. At both El-Gemmeiza and Mallawy, mean of single strains did not significant differ from those of the mixtures. At Sirs El-Lian, the single strains showed the least stand (Table 6). Single strains and mixtures gave the same yield at both El-Gemmeiza and Mallawy. At Sirs El-Lian, the single strains gave the highest yield (Table 6). It is worth noting that the four-way mixtures of strains were excluded because they were represented by only two treatments, which were too low to draw reliable conclusions.

In most cases, the correlation between stand and yield was nonsignificant after applying *Bacillus* treatments; however, the single strains showed a significant positive correlation between stand and yield ($r = 0.78$, $p = 0.02$). All methods of application showed significant positive correlation ($r = 0.35$, $p = 0.2$) at El-Gemmeiza and significant negative correlation ($r = -0.33$, $p = 0.03$) at Sirs El-Lian (Table 7).

Table 6 Effect of application method of *Bacillus* strains on the performance of treatments in enhancing cotton stand and yield

Location	Application method	Stand (%)	Yield (kentar/feddan)
Sakha	Single strains (n = 8)[a]	54.59 b	….[b]
	Two-way mixtures (n = 28)	51.63 a	….
	Three-way mixtures (n = 8)	49.33 a	….
El-Gemmeiza	Single strains (n = 8)	68.92 a	7.83 a
	Two-way mixtures (n = 28)	63.61 a	7.23 a
	Three-way mixtures (n = 8)	64.17 a	7.83 a
Sirs El-Lian	Single strains (n = 8)	53.81 a	4.85 b
	Two-way mixtures (n = 28)	61.31 b	2.80 a
	Three-way mixtures (n = 8)	63.81 b	1.52 a
Mallawy	Single strains (n = 8)	49.26 a	6.92 a
	Two-way mixtures (n = 28)	47.63 a	6.56 a
	Three-way mixtures (n = 8)	46.64 a	6.66 a

[a] Within a location, means followed by the same letter are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test
[b] Not determined due to severe infection with boll worms

Table 7 Effect of application method of *Bacillus* strains on the correlation between cotton stand (%) and yield (kentar/feddan)

Location	Application method	Linear correlation coefficient (r) between cotton stand and yield
Sakha	Single strains	….[a]
	Two-way mixtures	….
	Three-way mixtures	….
	All methods	….
El-Gemmeiza	Single strains	0.777 (P = 0.023, n = 8)
	Two-way mixtures	0.274 (P = 0.158, n = 28)
	Three-way mixtures	0.225 (P = 0.592, n = 8)
	All methods	0.354 (P = 0.018, n = 44)
Sirs El-Lian	Single strains	0.127 (P = 0.764, n = 8)
	Two-way mixtures	0.149 (P = 0.450, n = 28)
	Three-way mixtures	-0.064 (P = 0.880, n = 8)
	All methods	-0.327 (P = 0.030, n = 44)
Mallawy	Single strains	-0.099 (P = 0.816, n = 8)
	Two-way mixtures	0.143 (P = 0.468, n = 28)
	Three-way mixtures	0.082 (P = 0.847, n = 8)
	All methods	0.125 (P = 0.419, n = 44)

[a] Not determined due to the loss of yield

Discussion

Most of the studies reporting high efficiency of the biocontrol agents were conducted under controlled environments (greenhouse or growth chambar). However, under field conditions, where the environment is poorly controlled or totally uncontrolled, the biocontrol agents are only moderately effective or sometimes are totally ineffective. This discrepancy in the performance of biocontrol agents between controlled and uncontrolled commercial fields is due to several reasons. Under field conditions, rhizosphere is subjected to fluctuating physical and chemical edaphic factors, which affect rhizosphere microflora (Guetsky et al. 2001). Thus, biological control is more problematic under field conditions. However, despite all these limitations, field evaluation is a more realistic approach and leads to more accurate evaluation of the performance of the biocontrol agents. Thus, in the present study, treatments of *Bacillus* spp. were evaluated under field conditions at four widely separated agroecosystems represent almost all cotton-growing areas in Egypt.

The findings of many previous studies demonstrated that mixtures of rhizobacterial strains exhibited better biocontrol and plant-growth promotion than the individual strains (Jetiyanon & Kloepper 2002, de Boer et al. 2003, Liu et al. 2018). Contrary to the common belief, the results of the present study showed that, in most cases, the performance of the mixture was inferior compared to that of the individual strains regarding the attributes related to efficient biocontrol such as the magnitude of effective treatments, stable biocontrol, seedling stand counts, and yield. Therefore, at this point, the question that may arise is why the results of the present study are not in agreement with the widely accepted hypothesis. In some cases, the mixture of biocontrol agents does not result in improved suppression than the individual antagonists. This case is called incompatability and can arise because biocontrol agents may inhibit each other as well as the target pathogen or pathogens. Thus, an important prerequisite for successful development of strain mixture appears to be compatability of the coinoculated strains (Raupach & Kloepper 1998). Therefore, it seems reasonable to assume that the inferior performance of the mixtures was due to incompatability among the individual strain involved in these mixture. Some published reports lend support to this assumption (Raupach & Kleopper 1998, de Boer et al. 2003). The infected seedlings used in isolation yielded *Fusarium* spp. and *R. solani* at all sites. These fungi are considered major causes

of cotton seedling damping-off while the other isolated fungi are considered minor disease agents except when cotton seedlings are weakened (Watkins 1984).

The results of the present study showed that the performance of the tested treatments (individual strains and mixtures) was site specific. Thus, the treatments showed their best performance in controlling the disease at El-Gemmeiza and Sirs El-Lian and this may be a varietal response. On the other hand, the same treatments failed in controlling the disease at Sakha and Mallawy because the disease may not be severe enough to limit stand (Pierson & Weller 1994). Another possibility may be a rapid decrease in the size of populations of active cells to levels that are inadequate to obtain antagonism after incorporation into the soil. This rapid decline (microbiostasis) occurs when the bacterial strains introduced are unable to cope with the adverse and fluctuating biotic and abiotic soil conditions to survive and remain active. For example, in newly introduced populations of typical soil bacteria like fluorescent pseudomonads and *B. subtilis* declines were observed (Veen et el. 1997). The lack of correlation between seedling stand counts and seed cotton yield as we have shown here, after applying treatments, was also observed by Hagedorn et al. (1993), Aly et al. 2000, 2017). The abundance of iron in soil is inversely associated with soil pH (Misaghi et al. 1988). The pH values of the soils of Egypt ranged from 7.92 to 9.15 (Aly & Kandil 1999). Iron is anticipated to be sparingly soluble in these alkaline soils and too low in concentration to sustain microbial growth. Siderophores are one of the most significant bioactive molecules formed by the genus *Bacillus* (Fira et al. 2018). Therefore, it seems reasonable to speculate that the operative mechanism of damping-off suppression, at least by some of the effective individual strains, could be siderophore-mediated iron deprivation. However, it should be keep in mind that certain factors, such as the specific pathogen, the specific strain, the type of soil, and the affinity of the siderophore for iron, affect the degree of disease suppression as a result of siderophore production by *Bacillus* strains (Bashan & de-Bashan 2005). On the other hand, siderophore-mediated competition for iron may limit the biocontrol activity of the individual strains of *Bacillus* spp. involved in mixtures resulting in incompatability. Hence the inferior performance of the mixtures (de Boer et al. 2003).

Chitin is an essential structural feature of most fungal cell walls (Sahai & Manocha 1993). Several *Bacillus* species have been shown to secrete chitinase (Shafi et al. 2017). There is also a huge number of data supporting the essential role of chitinolytic enzymes in antagonistic interactions between bacteria and fungi (Haran et al. 1996, Shafi et al. 2017). Chitinase exhibited activity over the 4.5-7.5.5 pH range (Pleban et al. 1997). Chitinase is therefore unlikely to play an important role in the active antagonism displayed by the effective individual strains of *Bacillus* spp. since the pH value range is unfavorable for its activity in the Egyptian soils. It is noteworthy that B_1, B_3, and B_6 strains effectively controlled the disease under the broadest range of environmental conditions as they were the only treatments, which significantly increased stand at three sites. From practical stand point, B_1, B_3, and B_6 seem to be promising strains for commercialization for three reasons. Firstly, they were the only treatments, which effectively increased stand at three sites. Secondly, they significantly increased yield at Sirs El-Lian. Thirdly, B_3 and B_6 significantly increased yield of El-Gemmeiza. However, the confirmation of this possibility may require further comprehensive evaluation at as many sites as possible. Treatment T16, T33, and T37 significantly reduced stand at Mallawy. This deleterious effect of rhizobacteria on cotton stand was also reported under field conditions (Hagedorn. et al. 1993, Aly et al. 2017) and under greenhouse conditions in autoclaved soil (Khiyami et al. 2014).

In the present study, we did not investigate the specific mechanisms involved in the biological control of cotton seedling damping-off by the individual strains of *Bacillus* spp. Therefore, further work is needed to elucidate these mechanisms.

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