Plant growth promoting *Bacillus* suppress *Brevicoryne brassicae* field infestation and trigger density dependent and independent natural enemy responses

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KG and AG conceived and designed research. KG conducted experiments. PF and AG provided new reagents. KG and TMG analyzed data. KG wrote the manuscript. All authors approved the manuscript.

Abstract

Soil-dwelling Plant Growth Promoting (PGP) *Bacillus* live in intimate associations with plants; some species offer direct benefits via plant growth promotion while others confer protection against various pathogens. However, the roles of PGP *Bacillus* as elicitors of plant defences against agricultural pests and as a component of integrated pest management systems remain virtually unexplored. The effects of three major ubiquitous gram positive rhizobacteria; *Bacillus cereus*, *Bacillus subtilis* and *Bacillus amyloliquefaciens* were studied individually and in admixture on (i) calabrese (sprouting broccoli, *Brassica oleracea*) vegetative and reproductive growth parameters and (ii) the population dynamics of the specialist cosmopolitan pest, cabbage aphid (*Brevicoryne brassicae*) infestation, and its important natural enemies; the braconid endoparasitoid (*Diaeretiella rapae*), ladybird beetle (*Coccinella septempunctata*) and syrphid fly (all species). We found that all *Bacillus* treatments efficiently suppressed *B. brassicae* field populations in varying magnitudes. *B. cereus* and *B. subtilis* significantly increased the rates of parasitism by *D. rapae*, however, none of the other treated plants lured natural enemies, which responded in a density-dependent manner. Although the mixed *Bacillus* treatment significantly reduced root weight ratio, none of the *Bacillus* spp. treatments produced significant effects on calabrese growth. Taken together, PGP *Bacillus* may offer multiple plant benefits through suppressed pest infestation and increased percent parasitism in the field, with potential applications in integrated pest management.

Key-words *B. amyloliquefaciens, B. cereus, B. subtilis, Brassica oleracea* (calabrese), multitrophic interactions, natural enemy

Key message

- We explored whether soil-dwelling plant growth promoting *Bacillus* can suppress the population of a foliar-feeding pest and trigger natural enemy responses in the field.
- We found that *Bacillus* spp. suppress the field infestation of the specialist, cabbage aphid (*B. brassicae*) and directly as well as indirectly affect natural enemies.
- Thus, *Bacillus* species may offer a novel, cost-effective and sustainable approach to suppress field pests and could be a valuable resource in integrated pest management programme against foliar-feeding insects.

Introduction

Rhizobacteria are a major component of the soil microbial community and live in intimate associations with plants. *Bacillus* is one of the predominant genera of Plant Growth Promoting Rhizobacteria (PGPR) and has the potential to play physiologically and functionally diverse roles in multispecies interactions in plant ecosystems (Kloepper et al. 2004; Ryu et al. 2004; Pieterse and Dicke 2007; Van der Ent et al. 2009). Attributes include production of diverse bioactive molecules with broad spectrum activities (Ongena and Jacques 2008), stable endospore formulations (Errington 2003), extensive colonization, and rhizosphere competence under stress conditions (Chowdhury et al. 2013). These add to the success of PGP *Bacillus* spp. as potent microbial control agents, suppressing bacterial, nematode and fungal diseases. However, plant-mediated effects of these bacteria as a form of biocontrol of agricultural pests remains virtually unexplored (Gange et al. 2012).

Several species of *Bacillus* including *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* are successful plant root colonizers (Kloepper et al. 2004). They show a broad spectrum antifungal and plant growth promoting activities in a range of plants (e.g. *B. cereus*; Pleban et al. 1997; Chang et al. 2007; Dutta et al. 2013, *B. subtilis*; Asaka and Shoda 1996; Flores et al. 2007; Sharaf-Eldin et al. 2008, *B. amyloliquefaciens*; Idriss et al. 2002; Kim and Chung 2004; Chowdhury et al. 2013). Furthermore, *Bacillus* spp. have potential to induce chemical changes in plants and trigger natural enemy responses in response to herbivory (Gange et al. 2012).

The cabbage aphid (*B. brassicae*.), is a specialist feeder, and an economically important pest attacking different crops in the family Brassicaceae. The direct damage caused by *B. brassicae* to infested plants results in losses in yield and marketability (Strickland 1957; Liu et al. 1994), whereas indirect damage includes the dispersal of

23 viral diseases (Blackman and Eastop 2000). The use of pesticides, the most prevalent strategy of suppressing field populations of this pest (Lim et al. 1997), on directly consumed vegetable crops has increasing health and ecological concerns (Ellis 1996). Under these circumstances, biological control involving predators, parasitoids and microorganisms can be the best alternative pest management strategy. The most widespread and important natural enemies of *B. brassicae* include the braconid endoparasitoid, *D. rapae* (Pike et al. 1999) and predatory syrphid flies (Jankowska 2005).

The aims of this study were to determine whether individual and mixed application of *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* to calabrese (1) suppress the field colonization, reproduction and development of the specialist feeder, *B. brassicae* and its natural enemies, and (2) increase the reproductive and vegetative growth parameters of calabrese. We hypothesised that the different treatments of *Bacillus* would augment calabrese growth, and result in the reduction of *B. brassicae* field infestation, together with altered natural enemy responses. To explore this, we took a holistic approach in which we studied microbial, morphological and ecological aspects of multitrophic, *Bacillus-Brassica-Brevicoryne*-natural enemy interactions.

Materials and Methods

Land preparation, sowing and aftercare

The field experiment was undertaken at Royal Holloway's field experimentation site (51.4247° N, 0.5669° W), with freely draining slightly acid (pH 5.4) loamy soil, from June to October 2013. A site, measuring 20m × 10m was ploughed and five ridges were prepared 90 cm apart, 40 cm wide and 30 cm high. The field was irrigated before sowing to facilitate seed germination and early establishment. Seeds of

calabrese cv. Green Sprouting (Country Value Seeds, UK) were surface sterilized using sodium hypochlorite, following the procedure of Bhalla and Singh (2008). In brief, approximately 1000 seeds were placed in a 50 ml sterile screw cap tube containing 40 ml of 2% sodium hypochlorite and this tube was then shaken for 20 minutes. In a laminar flow cabinet, sodium hypochlorite was discarded and seeds were subsequently washed with 40 ml sterile distilled water five times. Six hundred randomly chosen seeds were decanted on to 5 sterile petriplates, with 120 seeds per plate and were subjected to five different treatments; (1) 'control', seeds without bacterial treatment; seeds that were inoculated individually with (2) *B. cereus* No. 8 FW Athal; (3) *B. subtilis* NRRLB23051 and (4) *B. amyloliquefaciens* subsp. plantarum FZB42BGSC10A6 and (5) seeds inoculated with all three species of bacteria ('mixed' treatment). Untreated and treated seeds were swirled and imbibed for 4 hours in sterile distilled water and bacterial suspensions respectively. The plot was laid out in a randomized block design, with five 4.5 × 2.7 m blocks, each having five rows. In each row, 3 randomly picked seeds per hill were sown 30 cm apart using sterile forceps, with eight replicates from each different treatment in each of five blocks (3 seeds per hill × 8 hills per row × 5 rows [1 block^{-1}]= 120 seeds). After the emergence of seedlings, two of the three seedlings were removed and one vigorous seedling per hill was retained, thereby producing 40 replicates per treatment. No pesticides and fertilizers were applied throughout the study but subsequent site management practices including irrigation and hand weeding were carried out. Plants were irrigated regularly, with an interval of 1 day during dry spells and weeding was practised thrice with an interval of 20 days.

Bacterial inoculants

All bacteria used in the present study were originally isolated from the rhizospheres of *Arabidopsis*, and were stored in 80% (v/v) glycerol stock at -80ºC. At the beginning of the experiment, the bacteria were recovered on 20 ml LB broth, allowed to incubate at 37ºC overnight on a rotary shaker, and serially diluted to 10^{-6} in 0.85% saline water. After incubation, 50 µl of a 10^{-5} dilution of each bacterium was spread on LB agar medium individually to determine the viable bacterial population count (colony forming units ml^{-1}) after incubation. The concentrations of each bacterium applied through seed treatment immediately after seed sterilization were 10^8 cfu ml^{-1} per plate. To ensure bacterial colonization, one additional application of 200 ml (10^8 cfu ml^{-1}) of each *Bacillus* formulation was drenched to each treated plant after 1 month.

Field inoculations of all *Bacillus* species under study were confirmed 2 weeks after sowing. Surface sterilized 1 cm root pieces from calabrese originally treated with each *Bacillus* spp. were carefully excavated and plated on LB media plates following the procedure of Sun et al. (2008). The resultant bacterial colony mixtures were sub-cultured until single and distinct colonies of bacteria were obtained. The colony PCR method was performed to amplify DNA from single bacterial colonies using universal forward; 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse; 1387r (5'-GGG CGG WGT GTA CAA GGC-3') primers. The PCR mixture (50 µl) contained: 2.5 µl bacterial colony, 1 µl deoxynucleoside triphosphate, 1 µl each of primers, 0.5 µl taq polymerase (Qiagen Ltd. UK), 5 µl 10x PCR buffer, 2.5 µl 25 mM magnesium chloride and 36.5 µl water. The PCR program involved 30 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min followed by a final extension step of 5 min at 72°C. PCR products were separated using 1% agarose gel electrophoresis and purified using a gel extraction kit (Qiagen Ltd. UK). Purified DNA samples were sequenced by Eurofins MWG Operon (Eurofins MWG Operon, Germany). The partial

nucleotide (query) sequences were identified on the basis of homology percentage with the existing accessions in the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). The identified sequences were submitted to NCBI Genbank and accession numbers for bacteria were obtained.

Aphid and natural enemy bioassays

Plants were not artificially infested by *B. brassicae*, to allow for natural colonization of this pest to occur. These naturally occurring colonies were allowed to feed, reproduce and disperse to new plants. As the natural enemies of *B. brassicae* are vital in governing its population dynamics in field, their number on each replicate plant was recorded along with aphids. These included the braconid endo-parasitoid, *D. rapae*, seven-spotted ladybird beetle, *C. septempunctata*, and syrphid flies (all observed species). Based on earlier experiments, an observation interval of 7 days was considered as optimum for development of measurable variation in aphid parameters. All experimental plants were monitored for aphid infestation, natural enemy and plant growth parameters from 6 weeks after sowing for subsequent 6 (observation) weeks, thereby 6 repeated measures were obtained over a period of 45 days (5^{th} August to 13^{th} September 2013). Aphids and natural enemies in each block containing all 5 treatments were counted on each day, thereby five blocks each week, to avoid bias between treatments. For each plant (1) total number of aphid nymphs, winged and wingless adults, (2) number of mummified aphids (due to *D. rapae*), ladybird beetles (larvae, pupae and adults) and syrphid flies (larvae, pupae and adults) were counted. Plants were harvested at physiological maturity, approximately 16 weeks after sowing. Fresh and dry shoot and root biomass were recorded immediately after harvest and after complete drying at 70ºC for 1 week, respectively. From fresh and

oven dry root, reproductive and vegetative biomass, fresh and dry root weight ratios were calculated by dividing the root mass by total plant biomass for each plant.

Data analyses

Data analyses were performed in R version 3.0.2 (R Development Core Team). Differences in plant biomass and percent parasitism by *D. rapae* between control and treated plants were analysed using single factor ANOVA and means separated with Tukey's HSD posthoc test ('aov' and 'TukeyHSD' functions in R). As the relationships between response (aphid and natural enemy number) and explanatory (treatment and time) variables were non-linear, the polynomial regression procedure (GLMER procedure, nlme and lme4 libraries in R) using treatments as a fixed effect parameter, time as a random effect and interaction terms (treatments: time), were used to determine if there was a significant effect of treatments over time. A model selection, to determine the better of two GLMER models, was performed using Akaike Information Criterion (AIC) values (Bolker et al. 2009). The data were analysed with a Poisson distribution with a log link mode because the response variables were count data, with skewed observation values. Along with GLMER procedure, the repeated measures 'Anova' function from the 'car' package in R was used to report Chi-squared and p-values for treatment, time and interaction effects.

Results

Plant parameters

The mixed treatment tended to increase reproductive (fresh), vegetative (oven dry) and total (fresh and dry) biomass, however, these effects were not statistically different at the 0.05 level. For fresh reproductive and dry vegetative biomass, the mixed treatment means were much higher (almost twice) than the control. However,

these were not significant, due to large variability in the data set. Significantly lower root weight ratio was observed in mixed treated plants. Calabrese displayed varied responses to different treatments, however, none of the individual treatments showed any significant positive impacts on any of the biomass types studied (Table 1).

Aphid bioassay

Nearly uniform natural colonization by *B. brassicae* across the different treatments was observed in the first observation week (Fig. 1a). In weeks 2 and 3, untreated plants showed rapid colonization and highest average aphid counts, whereas treated plants had substantially fewer aphids. Thus, a large difference in *B. brassicae* counts on untreated vs. treated calabrese plants was observed. The significant treatment: time interactions suggested that certain treatments followed different temporal patterns over the experimental duration, shown by *Bacillus*-treated plants exhibiting a slower build-up of *B. brassicae* colonies, compared with untreated plants (Table 2). On all treatments, the mean aphid population density reached a maximum at week 3 (Aug. 19-25), but varied in magnitude, being highest on control plants followed by the mixed treatment, lower on *B. cereus* and *B. amyloliquefaciens* and lowest on *B. subtilis* treated plants. On all plants, the nymphal form contributed most towards total aphid counts followed by winged and wingless adults (Fig. 2a,b,c).

Natural enemy bioassay

Of the natural enemies, *D. rapae* was the most abundant followed by syrphid flies and ladybird beetles (Fig. 2d,e,f). Application of rhizobacteria to plants resulted in similar natural enemy responses to untreated plants in the earliest stages of *B. brassicae* infestation, but highly dissimilar in later stages. Despite the varied responses in total natural enemies across all treatments, control plants had a significantly higher number

of mummified aphids and syrphid flies on them when compared with all other treatments. Furthermore, there were significant treatment: time interaction effects, showing that the treatments followed different temporal patterns (Table 2). The average number of natural enemies on control plants increased gradually until observation week 4 and decreased afterwards. In the mixed treatment, this number increased until observation week 5 and decreased in observation week 6.

Plants with individual bacteria applied had consistently lower average natural enemies than control plants and showed varying trends in the first 3 observation weeks. Despite having lower aphid counts as compared with control plants in week 2 and 3, *B. cereus* and *B. subtilis* treated plants had the greatest number of natural enemies in weeks 2 and 3 respectively. Furthermore, the percentage of aphids parasitized by *D. rapae* was significantly higher in *B. cereus* and *B. subtilis* treated plants, when compared with control [$F(4, 205)= 8.17$, $P<0.001$], suggesting density independent effects of these treatments on natural enemies (Fig. 3).

Bacterial colonization

All three originally applied bacteria from each respective treatment were successfully recovered from roots of plants after two weeks. Most isolates showed 95-99% homology with existent *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* accessions in NCBI Genbank and thus, for each identified bacterial isolate, accession number was obtained. Three representative accessions, one from each different treatment, along with closest NCBI accession matches are specified in Table S1.

Discussion

Our results demonstrate that field application of PGP *Bacillus* can significantly suppress foliar populations of an insect pest. So far as we are aware this effect has not

been previously reported in a field situation. These results are in contrast with earlier studies in which PGP *Pseudomonas fluorescens* failed to enhance resistance of *Arabidopsis* against the specialists *Pieris rapae* L. (Van Oosten et al. 2008) and *B. brassicae* (Pineda et al. 2012). This is possibly due to differential expression of insect-responsive genes and insect-derived volatiles that trigger priming in plants. For instance, Pare et al (2005) highlighted the roles of volicitin, C_6 green-leaf and C_4 bacterial volatiles in mobilizing plant cellular defences and thus priming plants against herbivores.

Stable endospore production (Nicholson et al. 2000; Piggot and Hilbert 2004) and biofilm formation (Beauregard et al. 2013) may have played an important role in attaining the successful calabrese root colonization by all *Bacillus* species in the present study. This is important, given that successful field trials in this area are remarkably few, due in most part to the failure of the inoculated bacteria to establish in the rhizosphere (Gange et al. 2012). Previous studies have shown that *B. subtilis, B. amyloliquefaciens* and *B. cereus* species offered added yield benefits in a variety of crop species such as pepper (Herman et al. 2008), cabbage (Turan et al. 2014) and lettuce (Chowdhury et al. 2013). However, we found that none of the *Bacillus* species increased biomass and offered any direct plant growth benefits. This is possibly due to spatiotemporal differences in the expression of *Bacillus* mediated plant beneficial properties, which are governed by an interplay of biotic and abiotic factors e.g. root exudates, rhizo-microbial guilds, pH, oxygen, soil nutrient status and structure. Although *Bacillus* spp. failed to promote plant growth, their negative effects on insect performance are likely to be due, in most part, to induction of systemic resistance that primed plants against herbivores. Bacterial effects on plant biomass are not unusual as earlier studies showed similar results wherein PGPR reduced root biomass (Walley

294 and Germida 1997) and increased shoot biomass (Chowdhury et al. 2013; Turan et al.
295 2014).
296 Initially, the experimental plants were uniformly colonized by winged *B. brassicae*
297 females, however, in the subsequent weeks, steady increases in aphid counts on
298 control and mixed bacterial-treated plants were due to rapid embryonic and nymphal
299 development of *B. brassicae* on actively growing field calabrese. The overall alate
300 population was reduced in subsequent weeks, mostly as a result of alates leaving to
301 colonize new plants, and thereby showing density-dependent aphid population
302 development. The induced systemic resistance triggered by PGP *Bacillus* through the
303 intervention of plant defensive signalling pathways likely primed calabrese and
304 thereby eventually reduced the *B. brassicae* field infestation. Two similar studies
305 reported the negative effects of PGP *Bacillus* inoculation on the growth and
306 development of generalist insect herbivores (Vijayasamundeeswari *et al.*, 2009;
307 Valenzuela-Soto *et al.*, 2010) through the induction of systemic resistance. The
308 bioformulation containing *B. subtilis* showed detrimental effects against *H. armigera*
309 in cotton (Vijayasamundeeswari *et al.*, 2009), and against virus free *Bemisia tabaci* in
310 tomato (Valenzuela-Soto *et al.*, 2010).
311 A parallel consistent pattern of change in natural enemy counts was observed on
312 control, *B. amyloliquefaciens* and mixed bacterial-treated plants. *D. rapae* contributed
313 most to overall natural enemy counts and reducing *B. brassicae* field populations. The
314 mummified aphid density is governed by the density of adult parasitoids, host aphids
315 and climatic factors (Dhiman 2007). Thus, the highest population density of
316 mummified aphids between observation weeks 3 and 5 may be attributed to the
317 highest *B. brassicae* counts between observation weeks 2 and 3, on control plants. As
318 in previous studies showing that populations of natural enemies were higher on PGPR

319 treated plants (Commare et al. 2002; Saravanakumar et al. 2008), we found *B. cereus*
320 and *B. subtilis* treated plants had the highest percent parasitism. This may be
321 attributed to the higher natural enemy counts in week 2 on *B. cereus* treated plants,
322 and in week 3 on *B. subtilis* treated plants, despite having less than half of the aphids
323 present on both of these plants during those weeks. Recent studies (Pineda et al. 2010;
324 D'Alessandro et al. 2014) suggest that rhizobacteria increase herbivore induced plant
325 volatiles (HIPV) emission, which trigger natural enemy responses. Thus, *B. cereus*
326 and *B. subtilis* may have influenced HIPVs and recruited the natural enemies, and so
327 targeted natural enemy responses were observed in the first three weeks. Such effects
328 were not observed in control, *B. amyloliquefaciens* and mixed treated plants, possibly
329 due to the lack of adequate HIPV emissions or masking of such effects by aphid-
330 density dependent responses. Our results show some consistencies with others who
331 showed failure of PGPR-treated plants to attract natural enemies (Van Oosten et al.
332 2008; Kabouw et al. 2011).
333 Aphid predators play an important role in suppression of *B. brassicae* populations
334 (Hafez 1961), however, the average ladybird beetle and syrphid fly counts were very
335 low on all plants and made no significant contribution towards final natural enemy
336 counts. Environmental factors such as temperature and precipitation significantly
337 impact aphid and natural enemy population dynamics (Carver 1988). Heavy showers
338 of rain for 2 consecutive days (19.2 mm on 24/08/2013 and 10.3 mm 25/08/2013) at
339 the end of observation week 4, followed by 3 infrequent rainfalls (3.3 mm on
340 06/09/2013, 6.8 mm on 09/09/2013 and 32.0 mm 13/09/2013) during observation
341 weeks 5 and 6 severely reduced aphid and natural enemy populations.

342 Conclusions

While application of PGP rhizobacteria had little effect on plant growth, all individual bacterial treatments, in varying magnitudes, decreased *B. brassicae* infestation, while application of *B. cereus* and *B. subtilis* increased the rates of *D. rapae* parasitism in the first three weeks. The parasitization was subsequently decreased on these treatments as a result of fewer aphids on them. On control and mixed treated plants, aphid populations grew rapidly until density dependent responses of aphids and natural enemies occurred and climatic factors intervened. The incorporation of PGP *Bacillus* in an integrated management programme for *B. brassicae* could reduce the use of chemical pesticides, lower the probability of pesticide resistance development, and help conserve natural enemies in the field. However, further investigations are needed to establish the mechanisms of these treatments in diverse environmental conditions and to unravel the complexity of different metabolic pathways and bio-molecules involved in induction of plant defences. Nevertheless, it is clear that exploration of effects of PGPR against major pests infesting commercially important crops could contribute towards the development of novel, cheap and sustainable pest management strategies.

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Figure Legends

Fig. 1 Changes in **(a)** *B. brassicae* and **(b)** natural enemy populations (Mean ± SE) in the field after treatment of seeds with individual species of *Bacillus*, a mixture of three *Bacillus* species, or control solution.

Fig. 2 Changes in the populations of (a) nymph (b) wingless adult (c) winged adult (d) ladybird beetle (e) mummified aphid and (f) syrphid fly larvae (Mean ± SE) on field grown calabrese plants (n=40) treated with control solution, and individual or mixed *Bacillus* species inocula.

Fig. 3 Changes in percent parasitism by *D. rapae* (Mean ± SE) on *Bacillus* treated (3 species individually or in admixture) and untreated field grown calabrese plants (n=40). Significant differences between means are represented by different letters.

Tables

Table 1 Analysis of variance results comparing effects on calabrese plant growth (Mean ± SE) following treatment of seeds with individual species of *Bacillus*, a mixture of three *Bacillus* species, or control solution. Each P value represents a comparison over the control.

		Mean (± SE)[1]				
	F (P value)	Control	[1]*B. c.*	[1]*B. s.*	[1]*B. a.*	Mixed
Fresh biomass						
Vegetative	1.42 (0.23)	123 (13.7)	165 (19.6)	132 (19.7)	157 (25.5)	196 (36.1)
Reproductive	1.95 (0.10)	45 (9)	59.6 (9.5)	77.1 (15.4)	73.1 (16.8)	103.7 (23.2)
Root	1.31 (0.27)	10.2 (0.9)	15.6 (2.4)	13.1 (1.35)	16 (1.8)	15 (3)
[1]R:W ratio	3.33 (0.013)	0.064 (0.006)	0.069 (0.005)	0.073 (0.006)	0.076 (0.006)	0.048 (0.004)
Oven dry biomass						
Vegetative	2.09 (0.08)	23.9 (2)	35.6 (3.7)	28 (3.5)	34.9 (4.6)	39.1 (6.1)
Reproductive	1.71 (0.15)	6.92 (1.3)	10.4 (1.8)	13.4 (2.7)	12.5 (2.7)	14.3 (2.3)
Root	0.87 (0.48)	3.4 (0.3)	4.8 (0.8)	3.6 (0.3)	4.4 (0.5)	4.0 (0.8)
[1]R:W ratio	3.20 (0.01)	0.10 (0.008)	0.09 (0.008)	0.09 (0.009)	0.09 (0.008)	[2]0.06 (0.005)*

[1]*B. c*., *B. s*., *B. a*. and R:W ratio represent *B. cereus*, *B. subtilis*, *B. amyloliquefaciens* and root: weight ratio respectively. Root weight ratios were calculated by dividing the root mass by total plant biomass for each plant.
[2]Means followed by asterisk (*) are significantly different at P<0.05 (Tukey's HSD test) * P<0.05, ** P<0.01, *** P<0.001

Table 2 The GLMER results comparing the effects of treatment of seeds with individual species of *Bacillus*, a mixture of three *Bacillus* species, or control solution on aphid and natural enemy populations. Each χ^2-value represents a comparison over the control.

	χ^2	df	P
Total aphids			
Treatment	7827.32	4	<0.001
Time (quadratic)	53.059	2	<0.001
Treatment: time (quadratic)	693.58	8	<0.001
Natural enemy			
Treatment	252.78	4	<0.001
Time (quadratic)	59.85	2	<0.001
Treatment: time (quadratic)	179.97	8	<0.001

562 **Table S1** The partial 16S rRNA sequencing results showing percent homology with
563 existing accessions in NCBI Genbank.

Query sample	Accession Nos.	Closest NCBI match	Homology (%)
1	KJ459078	*Bacillus cereus* partial 16S rRNA gene, isolate BD17-R16 (HF584799.1)	99
2	KJ459079	*Bacillus subtilis* partial 16S rRNA gene, clone KH007 (GU413150.1)	99
3	KJ459080	*Bacillus amyloliquefaciens* partial 16S rRNA gene, strain CEN6 (KF822673.1)	99

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