

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

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20 **Author Contribution Statement**

21 KG and AG conceived and designed research. KG conducted experiments. PF and

22 AG provided new reagents. KG and TMG analyzed data. KG wrote the manuscript.

23 All authors approved the manuscript.

24 **Abstract**

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

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

48

49 **Key message**

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

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74 **Introduction**

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

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

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

99 23 viral diseases (Blackman and Eastop 2000). The use of pesticides, the most
100 prevalent strategy of suppressing field populations of this pest (Lim et al. 1997), on
101 directly consumed vegetable crops has increasing health and ecological concerns
102 (Ellis 1996). Under these circumstances, biological control involving predators,
103 parasitoids and microorganisms can be the best alternative pest management strategy.
104 The most widespread and important natural enemies of *B. brassicae* include the
105 braconid endoparasitoid, *D. rapae* (Pike et al. 1999) and predatory syrphid flies
106 (Jankowska 2005).

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

116 **Materials and Methods**

117 Land preparation, sowing and aftercare

118 The field experiment was undertaken at Royal Holloway's field experimentation site
119 (51.4247° N, 0.5669° W), with freely draining slightly acid (pH 5.4) loamy soil, from
120 June to October 2013. A site, measuring 20m × 10m was ploughed and five ridges
121 were prepared 90 cm apart, 40 cm wide and 30 cm high. The field was irrigated
122 before sowing to facilitate seed germination and early establishment. Seeds of

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

146 Bacterial inoculants

147 All bacteria used in the present study were originally isolated from the rhizospheres of
148 *Arabidopsis*, and were stored in 80% (v/v) glycerol stock at -80°C. At the beginning
149 of the experiment, the bacteria were recovered on 20 ml LB broth, allowed to incubate
150 at 37°C overnight on a rotary shaker, and serially diluted to 10⁻⁶ in 0.85% saline
151 water. After incubation, 50 µl of a 10⁻⁵ dilution of each bacterium was spread on LB
152 agar medium individually to determine the viable bacterial population count (colony
153 forming units ml⁻¹) after incubation. The concentrations of each bacterium applied
154 through seed treatment immediately after seed sterilization were 10⁸ cfu ml⁻¹ per plate.
155 To ensure bacterial colonization, one additional application of 200 ml (10⁸ cfu ml⁻¹) of
156 each *Bacillus* formulation was drenched to each treated plant after 1 month.

157 Field inoculations of all *Bacillus* species under study were confirmed 2 weeks
158 after sowing. Surface sterilized 1 cm root pieces from calabrese originally treated with
159 each *Bacillus* spp. were carefully excavated and plated on LB media plates following
160 the procedure of Sun et al. (2008). The resultant bacterial colony mixtures were sub-
161 cultured until single and distinct colonies of bacteria were obtained. The colony PCR
162 method was performed to amplify DNA from single bacterial colonies using universal
163 forward; 63f (5'-CAG GCC TAA CAC ATG CAA GTC-3') and reverse; 1387r (5'-
164 GGG CGG WGT GTA CAA GGC-3') primers. The PCR mixture (50 µl) contained:
165 2.5 µl bacterial colony, 1 µl deoxynucleoside triphosphate, 1 µl each of primers, 0.5
166 µl taq polymerase (Qiagen Ltd. UK), 5 µl 10x PCR buffer, 2.5 µl 25 mM magnesium
167 chloride and 36.5 µl water. The PCR program involved 30 cycles of 95°C for 1 min,
168 55°C for 1 min, and 72°C for 1.5 min followed by a final extension step of 5 min at
169 72°C. PCR products were separated using 1% agarose gel electrophoresis and purified
170 using a gel extraction kit (Qiagen Ltd. UK). Purified DNA samples were sequenced
171 by Eurofins MWG Operon (Eurofins MWG Operon, Germany). The partial

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

177 Aphid and natural enemy bioassays

178 Plants were not artificially infested by *B. brassicae*, to allow for natural colonization
179 of this pest to occur. These naturally occurring colonies were allowed to feed,
180 reproduce and disperse to new plants. As the natural enemies of *B. brassicae* are vital
181 in governing its population dynamics in field, their number on each replicate plant
182 was recorded along with aphids. These included the braconid endo-parasitoid, *D.*
183 *rapae*, seven-spotted ladybird beetle, *C. septempunctata*, and syrphid flies (all
184 observed species). Based on earlier experiments, an observation interval of 7 days was
185 considered as optimum for development of measurable variation in aphid parameters.
186 All experimental plants were monitored for aphid infestation, natural enemy and plant
187 growth parameters from 6 weeks after sowing for subsequent 6 (observation) weeks,
188 thereby 6 repeated measures were obtained over a period of 45 days (5th August to
189 13th September 2013). Aphids and natural enemies in each block containing all 5
190 treatments were counted on each day, thereby five blocks each week, to avoid bias
191 between treatments. For each plant (1) total number of aphid nymphs, winged and
192 wingless adults, (2) number of mummified aphids (due to *D. rapae*), ladybird beetles
193 (larvae, pupae and adults) and syrphid flies (larvae, pupae and adults) were counted.
194 Plants were harvested at physiological maturity, approximately 16 weeks after
195 sowing. Fresh and dry shoot and root biomass were recorded immediately after
196 harvest and after complete drying at 70°C for 1 week, respectively. From fresh and

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

199 Data analyses

200 Data analyses were performed in R version 3.0.2 (R Development Core Team).

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

215 **Results**

216 Plant parameters

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

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

225 Aphid bioassay

226 Nearly uniform natural colonization by *B. brassicae* across the different treatments
227 was observed in the first observation week (Fig. 1a). In weeks 2 and 3, untreated
228 plants showed rapid colonization and highest average aphid counts, whereas treated
229 plants had substantially fewer aphids. Thus, a large difference in *B. brassicae* counts
230 on untreated vs. treated calabrese plants was observed. The significant treatment: time
231 interactions suggested that certain treatments followed different temporal patterns
232 over the experimental duration, shown by *Bacillus*-treated plants exhibiting a slower
233 build-up of *B. brassicae* colonies, compared with untreated plants (Table 2). On all
234 treatments, the mean aphid population density reached a maximum at week 3 (Aug.
235 19-25), but varied in magnitude, being highest on control plants followed by the
236 mixed treatment, lower on *B. cereus* and *B. amyloliquefaciens* and lowest on *B.*
237 *subtilis* treated plants. On all plants, the nymphal form contributed most towards total
238 aphid counts followed by winged and wingless adults (Fig. 2a,b,c).

239 Natural enemy bioassay

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

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

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

259 Bacterial colonization

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

266 **Discussion**

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

269 been previously reported in a field situation. These results are in contrast with earlier
270 studies in which PGP *Pseudomonas fluorescens* failed to enhance resistance of
271 *Arabidopsis* against the specialists *Pieris rapae* L. (Van Oosten et al. 2008) and *B.*
272 *brassiccae* (Pineda et al. 2012). This is possibly due to differential expression of insect-
273 responsive genes and insect-derived volatiles that trigger priming in plants. For
274 instance, Pare et al (2005) highlighted the roles of volicitin, C₆ green-leaf and C₄
275 bacterial volatiles in mobilizing plant cellular defences and thus priming plants
276 against herbivores.

277 Stable endospore production (Nicholson et al. 2000; Piggot and Hilbert 2004) and
278 biofilm formation (Beauregard et al. 2013) may have played an important role in
279 attaining the successful calabrese root colonization by all *Bacillus* species in the
280 present study. This is important, given that successful field trials in this area are
281 remarkably few, due in most part to the failure of the inoculated bacteria to establish
282 in the rhizosphere (Gange et al. 2012). Previous studies have shown that *B. subtilis*, *B.*
283 *amyloliquefaciens* and *B. cereus* species offered added yield benefits in a variety of
284 crop species such as pepper (Herman et al. 2008), cabbage (Turan et al. 2014) and
285 lettuce (Chowdhury et al. 2013). However, we found that none of the *Bacillus* species
286 increased biomass and offered any direct plant growth benefits. This is possibly due to
287 spatiotemporal differences in the expression of *Bacillus* mediated plant beneficial
288 properties, which are governed by an interplay of biotic and abiotic factors e.g. root
289 exudates, rhizo-microbial guilds, pH, oxygen, soil nutrient status and structure.

290 Although *Bacillus* spp. failed to promote plant growth, their negative effects on insect
291 performance are likely to be due, in most part, to induction of systemic resistance that
292 primed plants against herbivores. Bacterial effects on plant biomass are not unusual as
293 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

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

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510 **Figure Legends**

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

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

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

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531 **Tables**532 **Table 1** Analysis of variance results comparing effects on calabrese plant growth533 (Mean \pm SE) following treatment of seeds with individual species of *Bacillus*, a534 mixture of three *Bacillus* species, or control solution. Each P value represents a

535 comparison over the control.

		Mean (\pm SE) ¹				
	F (P value)	Control	¹ <i>B. c.</i>	¹ <i>B. s.</i>	¹ <i>B. a.</i>	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)*

536 ¹*B. c.*, *B. s.*, *B. a.* and R:W ratio represent *B. cereus*, *B. subtilis*, *B. amyloliquefaciens*
537 and root: weight ratio respectively. Root weight ratios were calculated by dividing the
538 root mass by total plant biomass for each plant.

539 ²Means followed by asterisk (*) are significantly different at P<0.05 (Tukey's HSD
540 test) * P<0.05, ** P<0.01, *** P<0.001

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546 **Table 2** The GLMER results comparing the effects of treatment of seeds with
 547 individual species of *Bacillus*, a mixture of three *Bacillus* species, or control solution
 548 on aphid and natural enemy populations. Each χ^2 -value represents a comparison over
 549 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

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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	<i>Bacillus cereus</i> partial 16S rRNA gene, isolate BD17-R16 (HF584799.1)	99
2	KJ459079	<i>Bacillus subtilis</i> partial 16S rRNA gene, clone KH007 (GU413150.1)	99
3	KJ459080	<i>Bacillus amyloliquefaciens</i> partial 16S rRNA gene, strain CEN6 (KF822673.1)	99

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