Plant growth promoting *Bacillus* suppress *Brevicoryne brassicae* field infestation, but fail to trigger natural enemy responses

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Author Contribution Statement

KG and AG conceived and designed research. KG conducted experiments. PF and AG contributed 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 chemical defences against agricultural pests and as a component of integrated pest managements 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, (ii) changes in the profiles of aliphatic and indolic glucosinolates, (iii) 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). For the first time, we found that all *Bacillus* treatments efficiently suppressed *B. brassicae* field populations in varying magnitudes while *B. cereus*, *B. subtilis* and mixed treatments significantly changed foliar indole glucosinolate levels. The changes in glucosinolates were not, however, significantly associated with aphid and natural enemy population dynamics. None of the treated plants lured natural enemies, which responded in a density-dependent manner. Furthermore, all *Bacillus* treatments triggered early reproductive calabrese maturity, and *B. subtilis*, *B. amyloliquefaciens* and mixed treatments significantly improved calabrese biomass. Altogether, PGP *Bacillus* offer a multitude of plant benefits through accelerated maturity, increased biomass and suppressed pest infestation in the field. Therefore, they have great potential in future integrated pest management programmes.

Key-words *B. amyloliquefaciens, B. cereus, B. subtilis, Brassica oleracea* (calabrese), glucosinolates, 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 alter plant defensive chemistry and natural enemy responses in the field.
- We found that *Bacillus* spp. alter calabrese glucosinolate profiles, improve plant health, suppress the field infestation of the specialist, cabbage aphid (*B. brassicae*) and indirectly affect natural enemies in a density dependent manner.
- Thus, *Bacillus* species present 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. Those species that colonize plant roots and promote growth are termed Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper and Schroth 1978). *Bacillus* is one of the predominant genera of PGPR which colonizes plants exogenously as well as endogenously in a range of environments (Shishido et al. 1995; Reva et al. 2004; Fan et al. 2011). *Bacillus* spp. have 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) and are being widely exploited for their symbiotic associations with plants and antagonistic activities against plant pathogens (Compant et al. 2005; Ongena et al. 2007). Plant growth-promoting activity of *Bacillus* spp. can be ascribed to the production of diverse bioactive molecules with broad spectrum activities (Ongena and Jacques 2008), stable endospore formulations (Errington 2003), *in vitro* mass multiplication, rapid and extensive colonization and rhizosphere competence under stress conditions (Chowdhury et al. 2013). All these attributes add to the success of PGP *Bacillus* spp. as promoters of plant growth and potent microbial control agents, suppressing bacterial, nematode and fungal diseases. However, plant-mediated effects of these bacteria as a biocontrol agent of important agricultural pests remain virtually unexplored (Gange et al. 2012).

Several species of *Bacillus* including *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* are successful plant colonizers (Kloepper et al. 2004). For instance, *B. cereus* is a persistent and relatively abundant root colonizer (Halverson et al. 1993) and shows antifungal and plant growth promoting activities in a range of plants (Pleban et al. 1997; Chang et al. 2007; Dutta et al. 2013). *Bacillus subtilis* is one of the predominant rhizobacteria and offers a broad spectrum of plant benefits including antibacterial and antifungal activities (Asaka and Shoda 1996; Kinsella et al. 2009), reproductive yield increases (Flores et al. 2007; Sharaf-Eldin et al. 2008) and protection against the foliar-feeding whitefly, *Bemicia tabaci* (Valenzuela-Soto et al. 2010). *Bacillus amyloliquefaciens* is also abundant in the rhizosphere, colonizes roots successfully and promotes plant growth via plant pathogen suppression (Yu et al. 2002; Kim and Chung 2004) and yield benefits (Idriss et al. 2002; Chowdhury et al. 2013).

Bacillus spp. induce chemical changes in plants to promote plant growth and thus are likely to alter profiles of foliar primary and secondary metabolites in plants in response to herbivory (Gange et al. 2012). Foliar secondary metabolites of plants have been widely studied for their role in defence functions against biotic stresses (Rosenthal and Berenbaum 1992; Bennett and Wallsgrove 1994). One such class is the glucosinolates, secondary metabolic compounds produced in Brassicaceae plants as a major constitutive and induced defence against generalist and specialist feeders. They are also known to be used as recognition cues and feeding stimulants by specialist herbivores (Hopkins et al. 2009). To date, at least 120 glucosinolates have been identified from this plant family and grouped on the basis of structural similarities into three major classes *viz.* aliphatic, aromatic and heterocyclic (indolic) (Fahey et al. 2001; Hopkins et al. 2009). Aliphatic compounds constitute 50% of the identified chemical structures of glucosinolates and are known to affect insect growth and performance negatively (Hopkins et al. 2009). Brassicaceae plants show an enhanced accumulation of indolic glucosinolates (up to 20-fold) upon herbivory (Textor and Gershenzon 2009). Since both aliphatic as well as indolic glucosinolates play important roles against herbivory (Mewis et al. 2005; 2006) and act as feeding stimulants for specialists (Hopkins et al. 2009), their involvement in governing specialist herbivore population dynamics and in plant defences is of considerable interest.

The cabbage aphid (*B. brassicae*.), is a specialist feeder, and an economically important pest attacking different crops in the family Brassicaceae. The rapid colonization and multiplication on plants, adaptation to changing environmental conditions (Ruiz-Montoya et al. 2003) and development of rapid resistance to insecticides (Oduor et al. 1997; Ahmad and Akhtar 2013) cumulatively play significant roles in the ecological and economic importance of this species. This pest is cosmopolitan in distribution with extensive occurrence in temperate regions (Ellis and Singh 1993) and is present at higher altitudes in tropical countries (Hill 1983). 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 viral diseases. This aphid is a vector of 23 viral diseases in the Brassicaceae and thus affects cabbage production on a massive scale (Blackman and Eastop 2000). The use of pesticides is the most prevalent strategy to manage this pest (Lim et al. 1997) but the extensive use of insecticides 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, *Diaeretiella rapae* (Pike et al. 1999) and predatory syrphid flies (Jankowska 2005).

The upward or downward cascade of plant secondary metabolites in food chains lays the foundation for bottom-up and top-down regulation of herbivore population dynamics (Kessler and Baldwin 2002). Several studies in Brassicaceae show that glucosinolates affect normal growth and development of *B. brassicae* by both these approaches. The bottom-up approach highlights direct negative effects which include lowering of reproductive rates of individuals and thereby colony abundance and size (Cole 1997; Newton et al. 2009a; Newton et al. 2009b), whereas the top-down approach involves indirect processes such as emission of Herbivore Induced Plant Volatiles (HIPVs) that attract natural enemies of this aphid (Bradburne and Mithen 2000; Blande et al. 2007). The roles of different species of *Bacillus* in intervention of bottom-up and top-down regulation of *B. brassicae* population dynamics, by altering the levels of aliphatic and indolic glucosinolates is currently unknown.

The aims of this study were to determine whether the individual and mixed treatments 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, via changes in foliar glucosinolate profiles of calabrese, and (2) increase the reproductive and vegetative growth parameters of calabrese. We hypothesised that the different treatments of *Bacillus* will augment calabrese growth and alter glucosinolate profiles, which will eventually 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, biochemical 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. Five blocks were prepared each containing five ridges, with 4 m length of each ridge. 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 5 ml of 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. Randomly chosen seeds (n=630) were decanted on to five sterile petriplates, with 126 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. With sterile forceps, three randomly picked seeds from each treatment were sown 30 cm apart in the field site, in a randomized block design. After the emergence of seedlings, two of the three seedlings were removed, the more vigorous growing seedling per station being retained. Thereby, 40 replicates per treatment were produced, with eight replicates from each different treatment in each of five blocks. No pesticides and fertilizers were applied throughout the study but subsequent site management practices including irrigation, weeding and harvesting 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. Plants were harvested at physiological maturity, approximately 16 weeks after sowing.

Bacterial inoculants

All bacterial cultures were obtained from Dr B. Raymond (Imperial College London, UK) 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 allowed to be naturally colonized by *B. brassicae*. 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 six weeks after sowing. The observations were recorded for six subsequent (observation) weeks, thereby six repeated measures were obtained over a period of 45 days (5th August to 13th September 2013). Aphids and natural enemies in each block containing all five 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, and (2) number of mummified aphids (due to *D. rapae*), ladybird beetles (larvae, pupae and adults) and syrphid flies (larvae, pupae and adults) were counted. Production of reproductive structures was monitored over different observation sets, while fresh and dry shoot and root biomass were recorded immediately after harvest and after complete drying at 70ºC for 1 week, respectively. From oven dry root, reproductive and vegetative biomass, root weight ratios were computed.

LC/MS for calabrese glucosinolates analysis

Sample preparation

Ten randomly chosen plants per treatment were sampled for biochemical analyses. Calabrese glucosinolates were extracted by standardizing the previously reported procedures (Mellon et al. 2002; Tian et al. 2005). One un-infested middle leaf from each plant was picked 6 weeks after sowing, just prior to aphid and natural enemy bioassays and transferred to a 50 ml sterile Falcon tube. The tubes were immediately frozen in liquid nitrogen, stored at -80ºC and lyophilized for 48 hours to avoid the activity of myrosinase enzyme. After drying, each leaf was milled to a fine powder using a mortar and pestle and stored in 2 ml sterile Eppendorf Protein LoBIND S/L tubes (Eppendorf UK Ltd.) at -20ºC until further use. To a new Eppendorf tube containing 40 mg of milled sample, 750 µl of 70% aqueous methanol was added. The tubes were sealed and heated at 70ºC for 30 min with vortex mixing at every 5 min. The tubes were transferred to an ice bath and centrifuged at 15,000 rpm for 15 min. The supernatant was removed using a 1 ml glass syringe (Sigma-Aldrich Co. Ltd., Dorset, England), filtered through a 0.2 µm, 4 mm nylon syringe filter (VWR International Ltd., UK) and 450 µl supernatant was stored in an ice bath. The pellet was re-extracted twice with 750 µl of 70% methanol, heated and centrifuged as described above. Finally, all three 450 µl supernatants were combined in a 2 ml standard opening (8 mm) amber screw top glass vial (Agilent Technologies UK Ltd.) and concentrated under nitrogen. Concentrated samples were re-suspended in 1 ml deionized water and filtered through a 0.2 µm nylon filter.

LC/MS method

Glucosinolates were separated on an ACE 5 C_{18} 150 x 2.1 mm reversed-phase column (Hichrom Ltd., Theale, Berkshire) and eluted using a linear gradient of water containing 0.1% formic acid (phase A) and acetonitrile containing 0.1% formic acid (phase B) at a flow rate of 0.2 ml min^{-1}. Gradient elution was started at 95% A changing to 95% B in 9 min, and then from 95% B to 95% A in 6 min. The column was kept at 25˚C and the flow from the chromatograph was injected directly into the electrospray ionization source, with an acquisition time of 25 min. Mass spectrometry was performed using a BrukerMicrOTOF QII high resolution time of flight (TOF) mass spectrometer, in the negative ion mode. The electrospray capillary probe was

operated at +3.2kV, with 1 Bar nitrogen nebuliser gas. The dry gas temperature and flow rate were optimized at 180ºC and 8 l min^{-1} nitrogen respectively. The calibration range was set at 100-1000 Daltons and the reference standard used was Agilent Tunemix (Product Code G1969-85000, Agilent Technologies, UK). Extracted Ion Chromatograms (EICs) were used to selectively monitor glucosinolates in mass spectra: glucoiberin (422.02), glucoraphanin (436.04), progoitrin (388.03), glucobrassicanapin (388.07), glucobrassicin (447.05), 4-methoxyglucobrassicin (477.06) and neoglucobrassicin (477.06). The quantification of these seven glucosinolates was performed using sinigrin as an external standard. A calibration curve was obtained by using 2 µl duplicate injections of sinigrin in mobile phase at the levels of 100, 1000 and 10,000 pg μl^{-1}. Peak areas were integrated using BrukerQuantAnalysis 1.8 software.

Statistical analyses

Data analyses were performed in R version 3.0.2 (R Development Core Team) using Linear Model (LM) and Generalized Linear Mixed-Effects Models (GLMER) procedures. 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 random effect and interaction terms (treatments: time), was 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.

Differences in glucosinolate levels and plant biomass were analysed as a function of different treatments using a linear model procedure (linear regression), with treatments as a fixed factor. The degrees of association between glucosinolates and aphid counts, and between glucosinolates and natural enemy counts were determined by Pearson's product moment correlation, and r and p values for each combination of

glucosinolates with aphids and natural enemy numbers were determined. Discriminant analysis (DA) plots on each of aphid and natural enemy matrix were obtained using XLSTAT (version 2013.1) to highlight the differences between each of these (quantitative explanatory) variables across different treatments (categorical variables). The aphid and natural enemy counts across the six observation weeks were summed and represented on DA orthogonal plots to eliminate the variation over time and thereby simplify the analysis. Glucosinolate variations across different treatments were represented using Principal component analysis (PCA) biplot.

Results

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 below (Table 1).

Table 1 Accession numbers of representative query samples and their percent homology with 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

Plant parameters

The application of *Bacillus* treatments resulted in accelerated plant reproduction compared with control plants (Fig. 1). At 75 days after sowing (DAS) (week 6; 09/09-15/09), untreated plants attained 57.5% flowering, considerably lower than the treated plants; *B. cereus* (77.5%), *B. subtilis* (80%), *B. amyloliquefaciens* (67.5%) and mixed (75%). Thus, *Bacillus* treatments increased the onset of reproductive maturity in calabrese. *Bacillus cereus* tended to increase vegetative and total biomass (Fig. 2a,

Table 2), however, these effects were not statistically different at the 0.05 level. *Bacillus subtilis* increased total dry reproductive biomass (Fig. 2b, Table 2) offering plants dual benefits of earlier maturation as well as reproductive yield. *Bacillus amyloliquefaciens* treated plants had significantly higher total dry biomass whereas mixed treatment plants had higher vegetative, reproductive and total biomass but lowest root weight ratio (Fig. 2a,b,c,d) than control plants. Thus, calabrese displayed varied responses to different treatments, but all *Bacillus* treatments, except *B. cereus,* showed some significant positive impacts on vegetative as well as reproductive parameters over untreated plants (Table 2).

Table 2 Summary of statistical results for fresh and oven dry biomass of differentially treated calabrese (linear regression, LM procedure)

	B. c.		*B. s.*		*B. a.*		Mixed	
	t	P	t	P	t	P	t	P
Fresh biomass								
Reproductive	0.661	0.510	1.451	0.150	1.256	0.212	2.654	0.009**
Vegetative	1.227	0.223	0.257	0.797	0.986	0.326	2.131	0.035*
Root	1.834	0.069	0.997	0.321	1.957	0.053	1.641	0.103
Total	1.229	0.222	0.871	0.385	1.335	0.185	2.708	0.008**
Oven dry biomass								
Reproductive	1.116	0.267	2.049	0.043*	1.726	0.087	2.324	0.022*
Vegetative	1.947	0.054	0.679	0.499	1.793	0.076	2.518	0.013*
Root	1.639	0.105	0.338	0.736	1.231	0.221	0.712	0.478
Total	1.949	0.054	1.276	0.205	2.028	0.045*	2.709	0.008**
R:W ratio	-1.181	0.240	-0.636	0.526	-0.752	0.453	-3.313	0.001**

* P<0.05, ** P<0.01, *** P<0.001. *B. c.: B. cereus, B. s.: B. subtilis, B. a.: B. amyloliquefaciens*

Fig. 1 Calabrese reproduction. The percentage of calabrese plants flowering across all treatments over the last 3 observation weeks.

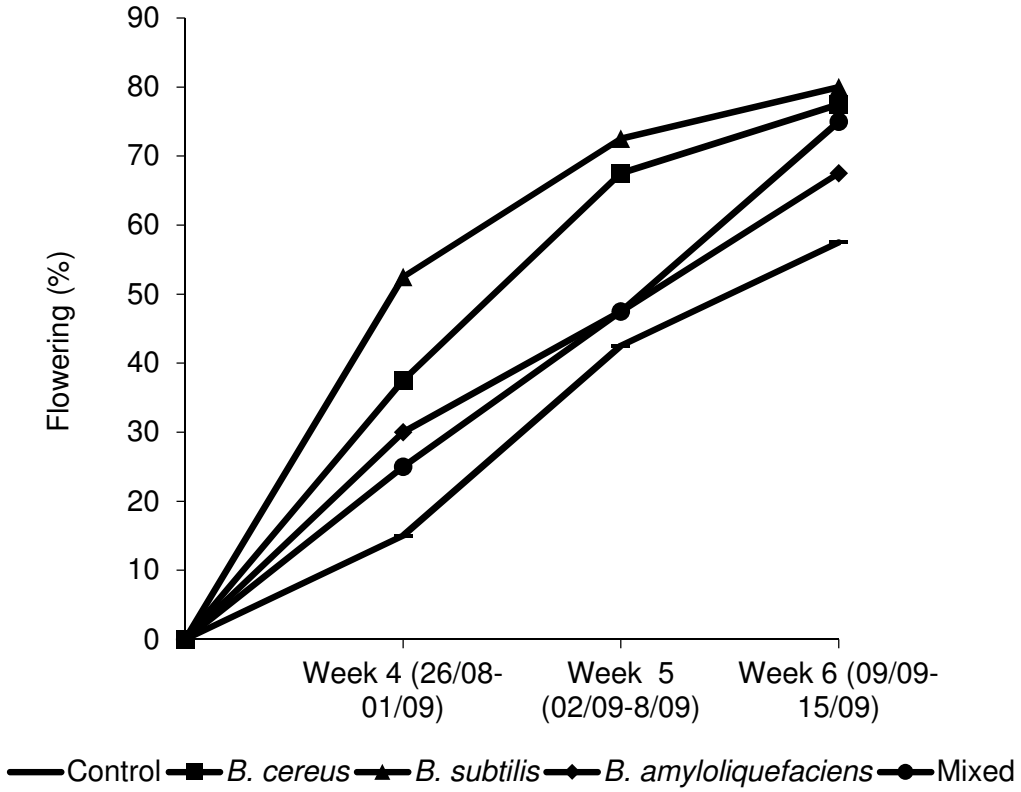
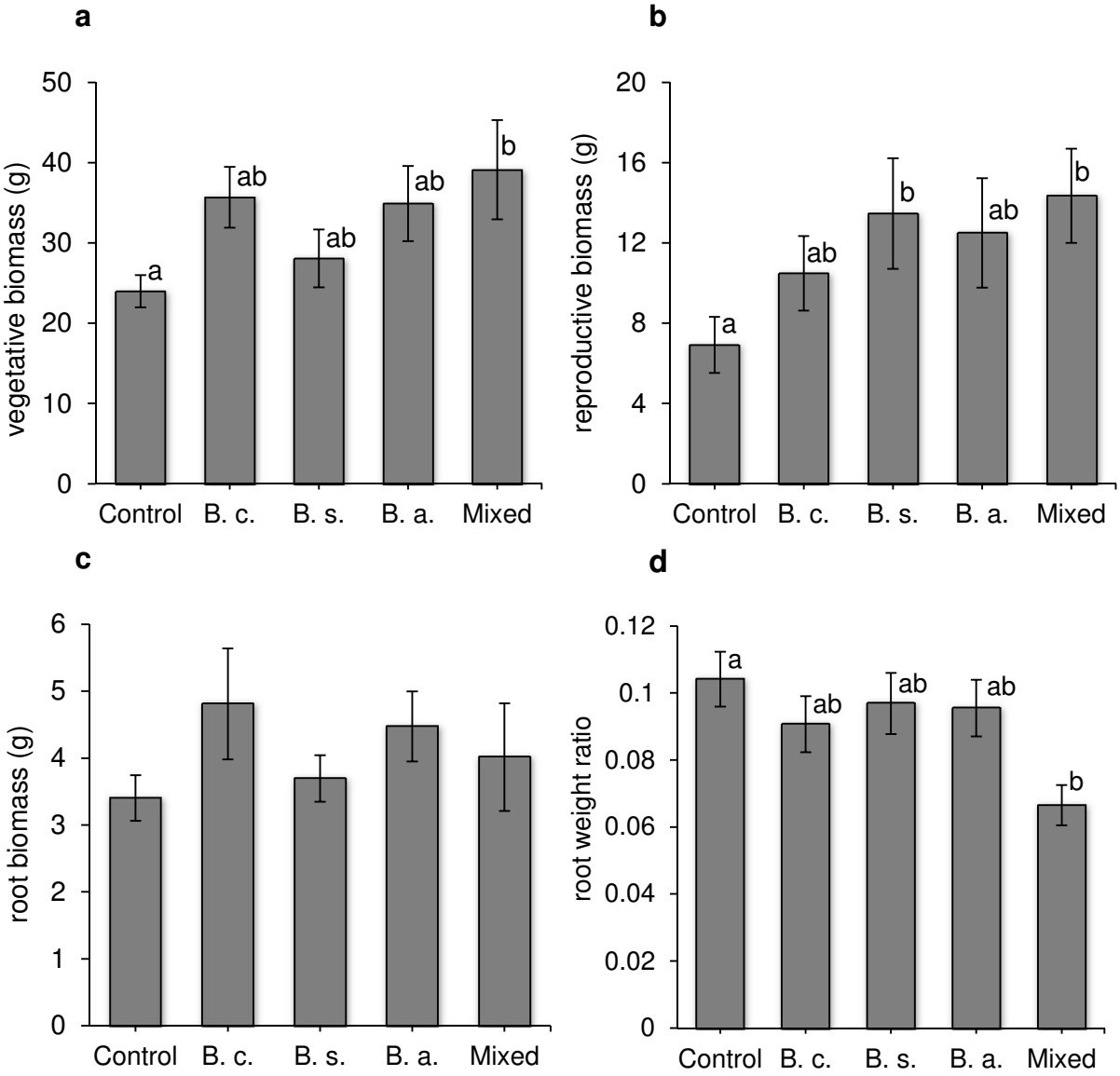


Table 3 The individual (treatments, time) and interaction terms (treatment: time) for aphid and natural enemy counts (GLMER procedure)

	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

Fig. 2 Effects of *Bacillus* spp. treatments on biomass. Oven dry biomass (Mean ± SE) of control and *Bacillus* treated calabrese; (a) total vegetative (leaf + stem), (b) reproductive, (c) root, and (d) root weight ratio. Different letters represent mean values that are significantly different and abbreviations; B. c., B. s. and B. a. represent *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* treatments, respectively.



Aphid bioassay

Nearly uniform natural colonization by *B. brassicae* across different treatments was observed in the first observation week (Fig. 3a). In observation Weeks 2 and 3, untreated plants showed rapid colonization and highest average aphid counts, whereas treated plants had substantially lower aphids (Table 3). 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 treated plants exhibiting a slower build-up of *B. brassicae* colonies, compared with untreated plants. 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 (111 aphids plant^{-1}) followed by the mixed treatment (82 aphids plant^{-1}), lower on *B. cereus* and *B. amyloliquefaciens* (42 aphids plant^{-1}) and lowest on *B. subtilis* treated plants (27 aphids plant^{-1}). On all plants, the nymphal form contributed most towards total aphid counts followed by winged and wingless adults (Fig. S1a,b,c). A discriminant analysis orthogonal plot indicated that aphid numbers differed across control, mixed and individual treatments (Fig. 3b). The centroid (mean of aphid counts on 40 plants in each treatment) values were much lower on individual treatments compared to control and mixed ones, with the lowest on *B. subtilis.* The cluster (scattering of aphids on all replicates in each treatment) of control plants was more spatially isolated from the mixed treatment and most from individual treatments, showing the increased degree of discordance for aphid numbers between these treatments.

Fig. 3 Population dynamics of *B. brassicae*. Differences in aphid populations across control and treated plants (a) changes in aphid population density (Mean ± SE) over time (b) DA plot of total aphid counts: the centroids and clusters of control and mixed treatments were distinct from those of the individual treatments.

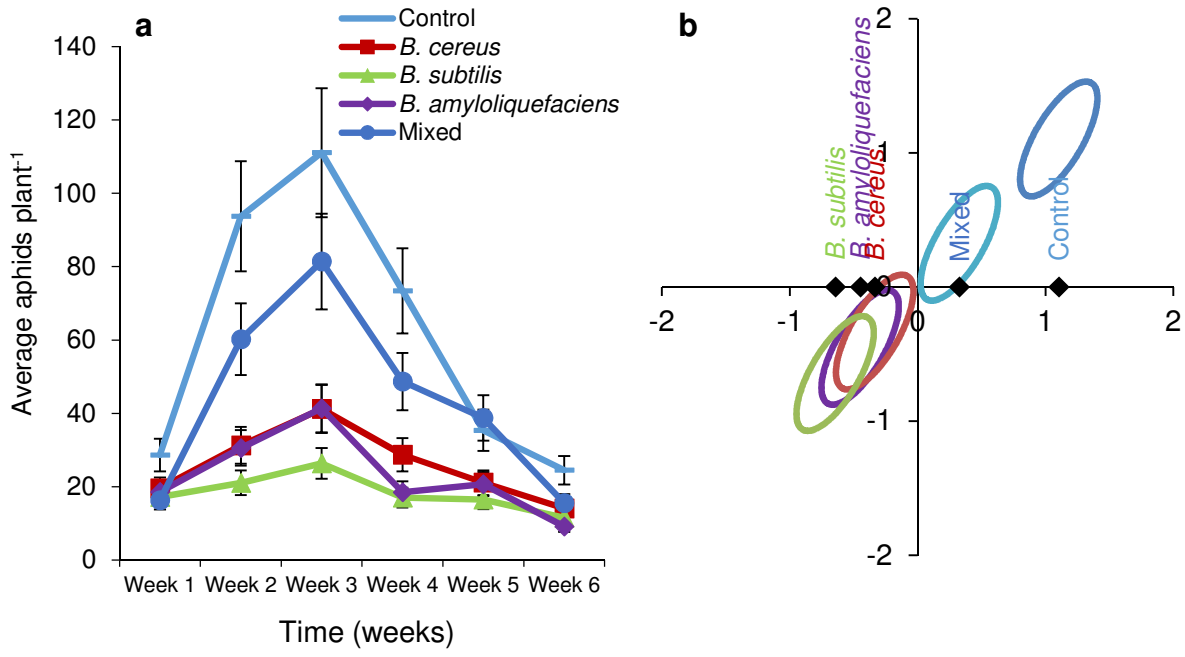
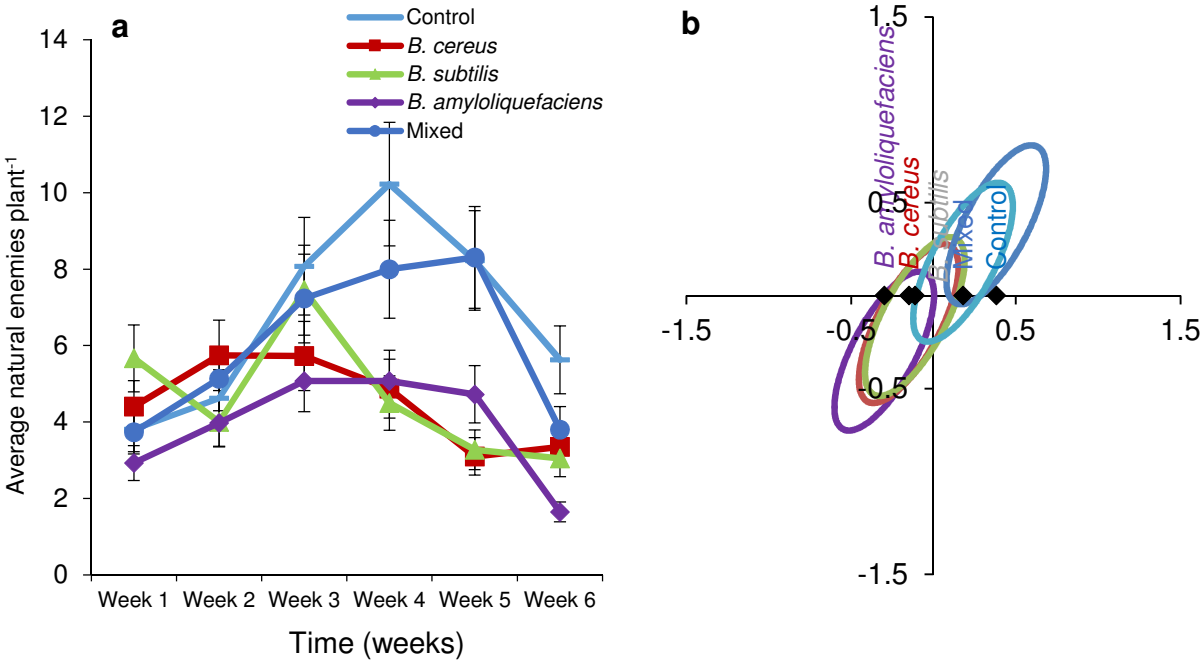


Fig. 4 Natural enemy population dynamics (a) Natural enemy population variations (Mean ± SE) on control and treated plants (b) DA plot of natural enemy counts: control and *B. amyloliquefaciens* treated plants had highest and lowest group means respectively which were also spatially separated on orthogonal plot while all other treatments had overlapped clusters.



Natural enemy bioassay

Treating plants with rhizobacteria showed similar natural enemy responses to untreated plants in the earliest stages of *B. brassicae* infestation, but highly dissimilar in later stages (Fig. 4a). Despite the varied responses in total natural enemies across all treatments, control plants had a significantly higher number of natural enemies on them when compared with all other treatments (Table 3). The time factor when analysed individually was significant along with the treatment: time interaction effects showing different time patterns followed by all treatments. Of the natural enemies, *D. rapae* was the most abundant followed by syrphid flies and ladybird beetles (Fig. S1d,e,f). 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 applied with individual bacteria had consistently lower average natural enemies than control plants and showed mixed trends in the first three observation weeks. The DA plot showed spatially distant separations of clusters between control and *B.*

amyloliquefaciens, with the highest grouped mean on control plants (Fig. 4b). Conversely, other treatments had overlapped clusters which suggests that the group means were not as distinct as in the aphid DA plot and application of *Bacillus* to calabrese did not have any significant direct influence on natural enemies.

Plant Biochemistry

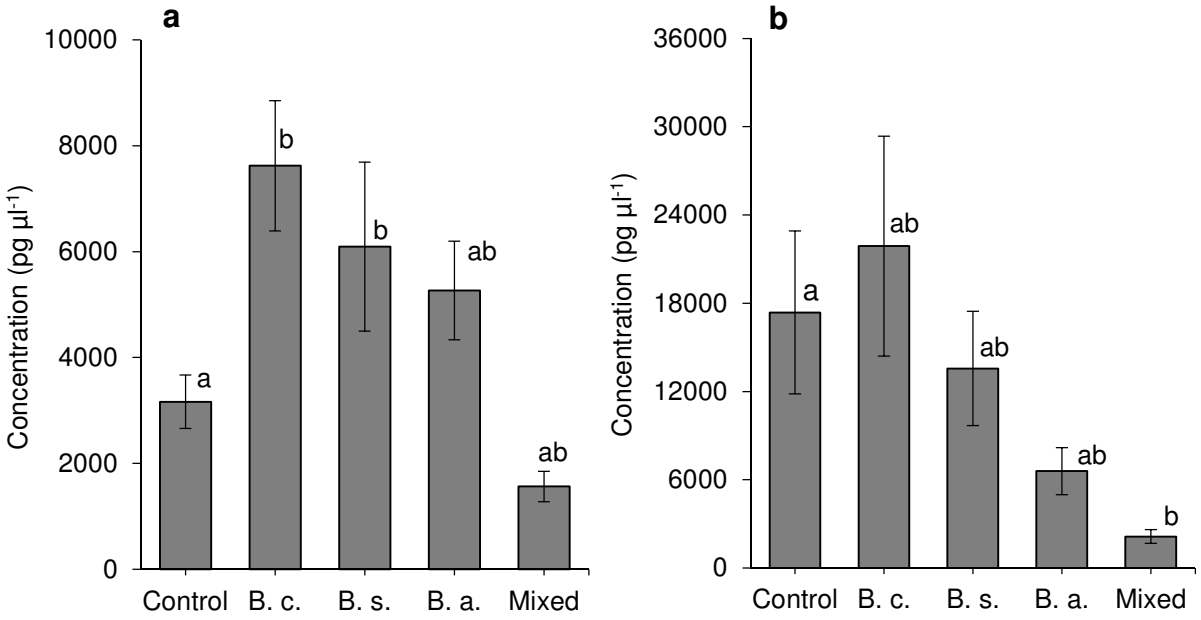
Two of seven quantified glucosinolates showed significant differences across the different treatments (Table 4). Furthermore, the levels of total glucosinolates were not significantly changed in any of the treatments. In *B. cereus* and *B. subtilis* treated plants, 4-methoxyglucobrassicin concentrations were increased (Fig. 5a), whereas in the mixed bacterial treatment, neoglucobrassicin concentrations were decreased significantly (Fig. 5b). No significant correlation between any of the glucosinolate concentrations including total glucosinolates, with aphid counts and with natural enemy counts was found in untreated and treated plants. The PCA of glucosinolate quantities across different treatments showed that *B. cereus* and *B. subtilis* treatments were significantly positively associated (Fig. 6). All treatments contributed towards variation across PC1. Control and mixed treatments accounted for large variation in neoglucobrassicin levels across PC2. No clear pattern of change in all other glucosinolates across different treatments was found.

Table 4 Effects of different *Bacillus* treatments on the levels of glucosinolates (linear regression, LM procedure)

	B. c.		*B. s.*		*B. a.*		Mixed	
	t	P	t	P	t	P	t	P
Glucoiberin	0.566	0.574	-0.443	0.659	-0.578	0.566	-0.898	0.374
Glucoraphanin	1.354	0.183	0.310	0.758	0.237	0.814	-0.051	0.960
Progoitrin	0.214	0.832	-0.327	0.746	1.282	0.207	-0.431	0.669
Glucobrassicanapin	0.693	0.693	-0.996	0.325	-0.399	0.692	-1.119	0.269
glucobrassicin	0.078	0.938	-1.051	0.299	-1.048	0.300	-1.362	0.180
4-methoxyglucobrassicin	3.498	0.001**	2.446	0.018*	1.721	0.092	-0.929	0.358
Neoglucobrassicin	0.826	0.413	-0.640	0.525	-1.599	0.117	-2.339	0.024*
Total	1.247	0.219	-0.426	0.672	-0.699	0.488	-1.515	0.137

* P<0.05, ** P<0.01, *** P<0.001. *B. c.: B. cereus, B. s.: B. subtilis, B. a.: B. amyloliquefaciens*

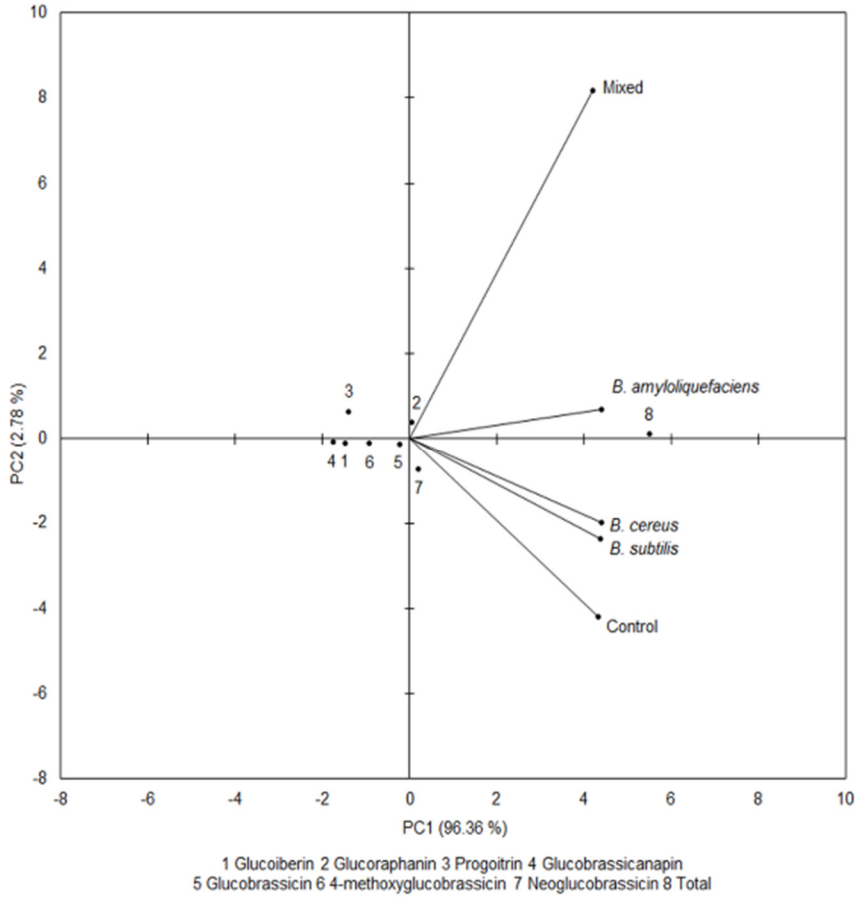
Fig. 5 Changes in calabrese glucosinolate profiles. The levels of glucosinolates in control and *Bacillus* treated plants (Mean ± SE); (a) *B. cereus* and *B. subtilis* treatments significantly increased 4-methoxyglucobrassicin whereas (b) mixed treatment significantly decreased neoglucobrassicin levels. Significant differences between means are represented by different letters.



Discussion

For the first time, we have shown that field application of PGP *Bacillus* can significantly suppress foliar populations of a specialist insect pest and can change foliar indole glucosinolate levels. These results are in contrast with studies in which PGP *Pseudomonas fluorescens* failed to enhance resistance to *Arabidopsis* against the specialists *Pieris rapae* L. (Van Oosten et al. 2008) and *B. brassicae* (Pineda et al. 2012) and PGP *Enterobacter radicincitans* DSM16656 colonization did not affect indole glucosinolate levels in *A. thaliana* (Brock et al. 2013). The individual and mixed bacterial treatments brought about varied degrees of responses in plants, aphids, glucosinolates and natural enemies. The mixed treatment was efficient in accelerating reproduction as well as boosting total plant biomass, but relatively less effective in suppressing cabbage aphid numbers as compared to the individual *Bacillus* treatments. On the contrary, all individual bacterial treatments, in varied magnitudes, decreased *B. brassicae* infestation, but were not as effective as the mixed treatment in promoting plant growth.

Fig. 6 PCA biplot showing variations in glucosinolate levels (scores) across different treatments (vectors). Glucobrassicanapin, glucoiberin, 4-methoxyglucobrassicin, glucobrassicin and total glucosinolates contributed towards variation across PC1 while progoitrin, glucoraphanin and neoglucobrassicin contributed most towards variation across PC2.



Root and plant surface colonization by PGPR is crucial to exert beneficial effects on plants. The exact mechanisms underlying successful colonization of bacteria on roots is not well understood, however, earlier studies on *Bacillus* showed that biofilm formation (Beauregard et al. 2013), plant expansin homolog protein synthesis (Kerff et al. 2008), biochemical alterations in cell walls (Dutta et al. 2013) and extracellular phytase activity (Idriss et al. 2002) play important roles in plant root colonization. *Bacillus* spp. are rhizosphere competent bacteria as they circumvent unfavourable environmental stresses by the production of stable endospores (Nicholson et al. 2000; Piggot and Hilbert 2004). Most of these attributes 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 a combination of PGP *B. subtilis* and *B. amyloliquefaciens* offered added yield benefits in several crop species. Herman et al. (2008) reported that these bacteria increased pepper yield, but failed to reduce the green peach aphid (*Myzus persicae* Sulzer) field populations. In another study, Saffron (*Crocus sativus*) corm sprouting times were significantly shortened and floral biomass was improved by *B. subtilis* (Sharaf-Eldin et al. 2008). In cabbage, growth promoting bacteria; *Bacillus megaterium* TV-91C, *Pantoea agglomerans* RK-92 and *B. subtilis* TV-17C increased growth, nutrient and hormone content (Turan et al. 2014). Furthermore, *B. amyloliquefaciens* FZB42 was reported to colonize lettuce rhizospheres effectively and improve growth and health under *Rhizoctonia solani* Kühn pathogen pressure (Chowdhury et al. 2013). Thus, we found similar results, in which *B. subtilis* and *B. amyloliquefaciens* encouraged reproduction and increased yield significantly together with suppression of the field populations of *B. brassicae*. The vegetative and total biomass in *B. cereus* applied plants were higher and this bacterium promoted early reproductive maturity over control plants and showed consistently higher suppression of *B. brassicae*. This accelerated maturity and increment in biomass caused by all *Bacillus* under study could be related to direct and indirect plant growth benefits that these bacteria may have facilitated. The direct benefits include the production of plant growth hormones, gibberellic acid (GA) and indole-3-acetic acid (IAA), increased uptake of nutrients (Vessey 2003; Yao et al. 2006) while indirect ones include protection of plants through induced systemic resistance (ISR) (Valenzuela-Soto et al. 2010) and plant stress control (Yang et al. 2009). Multiple species of *Bacillus* have apparently provided most of the above advantages and allowed maximum allocation of nutrients to shoot growth as evident by the significantly decreased calabrese root weight ratio.

Initially, the experimental plants were uniformly colonized by winged *B. brassicae* females, which were abundant early in the season. These reproduced viviparously and colonized new plants in the field. In the subsequent weeks, steady increases in aphid counts on control and mixed treated plants were due to rapid embryonic and nymphal development of *B. brassicae* on actively growing field calabrese. *Brevicoryne brassicae* reproduce best on 2-2.5 month old cabbage plants

(Markkula 1953), thus the peak reproductive phase synchronised with access to the best quality food source resulted in accelerated numerical increase of nymphs in observation Weeks 2-3. In peak infestation stages, wingless, viviparous aphid females exploit a rich food supply and reproduce as rapidly as possible (Hughes 1963), however, winged females were prominent in the field as compared to wingless, viviparous females in their respective peak growing periods. Thus, winged females producing wingless progenies played a significant part in rapid multiplication and dispersal at this stage. Similarly, these winged morphs may also have dispersed throughout the field on *Bacillus* treated plants, but could not establish themselves as successfully as on control plants.

Colonization of roots with PGPR leads to synthesis of proteins and secondary metabolites involved in elicitation of Induced Systemic Resistance (ISR) against pathogens and insects (Van Oosten et al. 2008; Van der Ent et al. 2009; Gange et al. 2012). An extensive study on the effects of *Bacillus* on ISR and plant growth promotion suggested that several strains of these bacteria elicit ISR by chemical alterations in plants (Kloepper et al. 2004). Thus, ISR triggered by PGP *Bacillus* likely primed calabrese and thereby eventually reduced the *B. brassicae* field infestation. The fertility of mother aphids and progenies is directly influenced by the quality of food allocated (Nevo and Coll 2001; Jahn et al. 2005) and types of fertilizer applied to host plants (Stafford et al. 2012), thus changes in amino acid profiles may have affected the feeding of *B. brassicae* and thereby reduced the infestation. A relevant study (Cole 1997) showed a strong positive correlation between *Brassica* phloem amino acids and the intrinsic rate of increase of *B. brassicae.* Four amino acids, *viz.* tyrosine, alanine, leucine and glutamic acid, accounted for 43% of the variation in intrinsic rate of increase of *B. brassicae.* This study suggests that the changes in calabrese amino acids may have affected *B. brassicae* fecundity in treated and untreated plants. Whether PGP *Bacillus* alter the plant amino acid profiles remains unknown.

Environmental factors such as temperature and precipitation significantly impact aphid population dynamics (Carver 1988). Heavy showers of rain for two consecutive days (19.2 mm on 24/08/2013 and 10.3 mm 25/08/2013) at the end of observation Week 4, followed by three infrequent rainfalls (3.3 mm on 06/09/2013, 6.8 mm on 09/09/2013 and 32.0 mm 13/09/2013) during observation Weeks 5 and 6 severely

reduced aphid populations. Thus, some intense showers of rain later in the season likely reduced aphid and natural enemy counts, and impeded dispersal.

In response to the fluctuations in the numbers of aphids, a parallel consistent pattern of change in natural enemy counts was observed in control plants. In previous studies, the populations of natural enemies were found to be higher on PGPR treated plants (Commare et al. 2002; Saravanakumar et al. 2008). Showing discrepancy with these studies but consistent with different work (Van Oosten et al. 2008; Kabouw et al. 2011), we did, however, find that PGPR-treated plants failed to attract natural enemies even though the aphid counts were similar to control plants in earlier measurements. Recent studies (Pineda et al. 2010; D'Alessandro et al. 2014), suggest that rhizobacteria increase HIPV emission, which trigger natural enemy responses. Similarly, *Bacillus* spp. may have influenced HIPVs and recruited the natural enemies to the entire plot rather than just to the treated plants, and so targeted natural enemy responses on treated plants were not observed.

Diaeretiella rapae was the most abundant natural enemy in the field and thereby contributed most to overall natural enemy counts and reducing *B. brassicae* field populations. The mummified aphid density is governed by the density of adult parasitoids, host aphids and climatic factors (Dhiman 2007). Thus, the highest population density of mummified aphids between observation Weeks 3 and 5 could be attributed to the highest *B. brassicae* counts between observation Weeks 2 and 3, on control plants. Heavy rain after observation Week 4 seems to have caused the sharp decline in the *D. rapae* population. Plants treated with individual *Bacillus* had insufficient aphids on them, thus the mummified aphid counts were much lower in later observation weeks. Aphid predators play an important role in suppression of *B. brassicae* populations (Hafez 1961), however, the average ladybird beetle and syrphid fly counts were very low on all plants and made no significant contribution towards final natural enemy counts.

Glucosinolates are the principal defence molecules in Brassicaceae (Hopkins et al. 2009), but whether PGPR colonization causes qualitative changes in plant glucosinolates profiles, and if such qualitative changes affect colonization and development of aphids, is less clear. In contrast, with earlier findings that PGPR colonization does not affect indole glucosinolate levels in *A. thaliana* (Brock et al. 2013), we found that *B. cereus* and *B. subtilis* treatments significantly increased 4-

methoxyglucobrassicin, whereas the mixed bacterial treatment significantly decreased neoglucobrassicin. Neither of these glucosinolates was, however, found to be associated with the levels of *B. brassicae* infestations and natural enemy counts in treated as well as untreated plants.

The large variation in the levels of individual glucosinolates and changes in those levels at different plant growth stages and in different plant parts makes the interpretation of biological activities associated with them difficult (Hopkins et al. 2009). In a previous study (Brock et al. 2013), *E. radicincitans* DSM16656 decreased foliar aliphatic glucosinolates in *A. thaliana* at a fast-growing stage, increased them at plant maturity stage, but did not change indole glucosinolate levels averaged over the life of the plant. Sampling at different stages in the life of the plants was beyond the scope of this study and could have had confounding effects on aphid infestation. It would, however, be instructive to do so, as aphid populations are likely to respond to temporal variation in plant defences. In our sample, we found no changes in aliphatic glucosinolates, but significant ones in the levels of two indole glucosinolates. These changes and the other minor changes in glucosinolate profiles were not in harmony with aphid or natural enemy dynamics. In another study, Schreiner et al. (2009) showed that *E. radicincitans* DSM16656 neither changed individual and total foliar glucosinolate concentrations nor triggered any plant defence responses in five cruciferous species. Thus, the effects of colonization of different PGPR on glucosinolate profiles appear to be specific to the plant species and plant developmental stage. Furthermore, they are likely to depend on the bacterial species inoculated and invading insect species as well.

The current management for *B. brassicae* involves the extensive use of pesticides, which is potentially detrimental to human and ecosystem health (Ellis 1996). Such excessive use has led to the development of resistance in *B. brassicae* (Ahmad and Akhtar 2013). Since various PGP *Bacillus* species suppress *B. brassicae* field infestations, making economic injury levels less likely, their inclusion in *B. brassicae* integrated pest management programmes is highly recommended. Furthermore, they have the added advantage of improving crop yield and maturity. This will ultimately reduce the necessity of pesticide and fertilizer applications, lower the probability of pesticide resistance development, and help preserve the natural enemies in crop ecosystems.

Conclusions

This study showed that the addition of PGPR to field-grown plants offers benefits as evidenced by reduced *B. brassicae* infestation, increment in yield and early flowering maturity. Except for the mixed treatment, these effects of *B. brassicae* suppression were consistent throughout the experimental duration. Conversely, on control and mixed treated plants this pest grew rapidly until density dependent responses of natural enemies occurred and climatic factors intervened. The *B. cereus* treatment improved the onset of reproductive maturity and in part, vegetative and total biomass; *B. subtilis* treated plants matured earliest and showed significantly improved reproductive yield. Both bacteria significantly elevated the indole glucosinolate, 4-methoxyglucobrassicin, levels in calabrese. *Bacillus amyloliquefaciens* also increased reproductive maturity, total biomass, but did not alter glucosinolate levels. Mixed treated plants significantly reduced another indole glucosinolate, neoglucobrassicin, partly improved reproductive maturity and offered the highest plant health benefits through significantly improved reproductive, vegetative and total biomass. None of these treatments, however, favoured natural enemy populations in the field. Thus, incorporation of PGP *Bacillus* in an integrated management programme for *B. brassicae* could reduce the use of chemical pesticides and conserve natural enemies in the field. 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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