Soil-dwelling *Bacillus* spp. affects aphid infestation of calabrese and natural enemy responses in a context-specific manner

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**Abstract**

1. Plant growth-promoting rhizobacteria in the genus *Bacillus* have been shown to reduce growth and increase parasitism of some aphids, but the generality of these interactions is unknown. All previous studies have taken place in temperate conditions.

2. We studied the effects of seed application of three *Bacillus* species, singly and in mixture, on three aphid pests and their natural enemies, on field-grown calabrese (green sprouting broccoli) in the subtropical climate of South West India.

3. All three bacteria reduced populations of *Brevicoryne brassicae* and *Myzus persicae*, but had no effect on numbers of *Lipaphis erysimi*. Chewing insects (flea beetles and diamondback moth larvae) were also unaffected by the treatments. However, parasitism rates of aphids were higher on plants treated with the bacteria.

4. We conclude that *Bacillus* spp. shape above ground interactions in a context-specific manner, directly via altered field infestations of some pests and indirectly via natural enemy responses.

**KEYWORDS**

*Bacillus amyloliquefaciens*, *Bacillus cereus*, *Bacillus subtilis*, *Brevicoryne brassicae*, *Lipaphis erysimi*, multitrophic interactions, *Myzus persicae*

**INTRODUCTION**

Plants form symbiotic associations with numerous plant growth-promoting rhizobacteria (PGPR) species, deriving a multitude of growth and protection benefits. Many of these species improve plant biomass, augment flowering and enable toleration of biotic and abiotic stresses (Lugtenberg & Kamilova, 2009). Species of *Bacillus* in particular are considered to have an important role in the development of sustainable agricultural systems by providing benefits to plant growth (Dhayalan & Karuppasamy, 2021). For instance, *B. cereus* Frankland & Frankland is an effective root colonizer which can suppress plant diseases (Shameer & Prasad, 2018) and enhance biomass in different plants (Dutta et al., 2013), as can *B. subtilis* Ehrenberg (Blake et al., 2021) and *B. amyloliquefaciens* Priest (Ngalimat et al., 2021). Synthesis of various bioactive molecules that have a wide spectrum of anti-pathogenic activities and effective rhizosphere competence for nutrients and niches are some of the many attributes that contribute to the success of *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* as effective suppressors of various plant diseases (Ali et al., 2020). However, relatively little is known about how these three ubiquitous soil bacterial species extend their effects up to foliage-feeding insects and their natural enemies (Gange et al., 2012).

Aphids, including cabbage aphid (*Brevicoryne brassicae* L.), green peach aphid (*Myzus persicae* [Sulzer]) and mustard aphid (*Lipaphis erysimi* [Kaltenbach]) reduce the biomass and quality of many Brassicaceae plants on a large scale and spread epidemic viral diseases, throughout the world (Blackman & Eastop, 2000). *B. brassicae* is a specialist aphid that causes curling, distortion and yellowing of foliage. In severe infestations, damage can be seen in the inner core of cabbage heads and plants may completely wither and die.

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Furthermore, *B. brassicae* is a vector of many viral diseases such as cauliflower and turnip mosaic, cabbage black ringspot and cabbage ring necrosis (Broadbent, 2015). *M. persicae* is a generalist feeder that attacks a wide range of plants worldwide and transmits over 100 viral diseases (Gadhave et al., 2020). *L. erysimi* is also one of the most damaging pests of brassicas and is a vector of several viral diseases. Depending upon plant species, plant growth stage and the extent of infestation, *L. erysimi* can cause up to 90% loss in some crop plants (Pate et al., 2004).

There is some evidence that *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* can reduce the growth, reproduction and population build up of *B. brassicae* on calabrese (*Brassica oleracea* L.) in both laboratory and field conditions (Gadhave, Finch, et al., 2016a; Gadhave & Gange, 2016), though effects varied between bacterial species. Meanwhile, a mixture of other PGPR species (including *Pseudomonas*, *Azotobacter* and *Azospirillum*) reduced fecundity of *B. brassicae* feeding on canola *Brassica napus* L. in glasshouse conditions (Nasab et al., 2019). In contrast, the effects of PGPR on *M. persicae* appear to be more variable. Application of *B. cereus* reduced aphid fecundity on bell pepper (*Capsicum annuum* L.) in a glasshouse (Mardani-Talaei et al., 2017), but this species and *B. amyloliquefaciens* had no effect on aphids on the same plant in field conditions (Herman et al., 2008). Meanwhile, another PGPR, *Pseudomonas fluorescens* (Flügge) increased the growth of *M. persicae* but had no effect on *B. brassicae* (Pineda et al., 2012). To date, there have been no studies of the effects of PGPR applications on *L. erysimi*.

Taken together, these variable results show that PGPR-aphid interactions are likely to be context dependent, with outcomes determined by the identity of the PGPR, the target insect, the conditions in which the experiments take place and the composition of the indigenous bacterial community (Gadhave, Hourston, et al., 2016b; Gadhave et al., 2018). Given that the interactions between PGPR and other foliar-feeding insects are equally variable (Friman et al., 2021), there is a clear need for more studies that might enable us to search for patterns and thereby unravel these complex interactions. Doing so is critical if PGPR are to fully realize their potential as alternative forms of pest control in sustainable agriculture (Basu et al., 2021).

Studies of the effects of PGPR on predators and parasitoids of herbivorous insects are rarer but show much variation as those with the herbivores themselves. A laboratory study showed that the volatile blend of *Arabidopsis thaliana* (L.) Heynh. plants treated with *P. fluorescens* was less attractive to *Diaeretiella rapae* (McIntosh) (Hymenoptera: Braconidae), thereby reducing parasitism of *M. persicae* (Pineda et al., 2013). However, parasitism of *Mamestra brassicae* L. larvae by another Braconid, *Microplitis mediator* (Haldyay), was increased when feeding on the same plant when treated with this bacterium (Pangesti et al., 2015). Meanwhile, *B. cereus* and *B. subtilis* were found to increase rates of parasitism of *B. brassicae* by *D. rapae* (McIntosh), through density-dependent responses (Gadhave, Finch, et al., 2016a).

Studies of PGPR effects on predators of herbivorous insects are even rarer but equally inconclusive. For example, Boutard-Hunt et al. (2009) found limited evidence that natural enemy numbers (not specified, but including Coccinellidae, Neuroptera, Diptera and Anthocoridae) were higher on pepper plants treated with *B. amyloliquefaciens*. Saravanakumar et al. (2008) found that field applications of *Pseudomonas* to rice reduced leaffolder, *Cnaphalocrocis medinalis* (Guenée) incidence and lured its natural enemies, whereas Gadhave, Finch, et al. (2016a) found no effect of the aforementioned *Bacillus* species on predators (*Coccinellids* and *Syphid larvae*) of *B. brassicae* in field-grown calabrese.

The aim of this field experiment, conducted in the subtropical climate of India, was to investigate the effects of seed application of *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* on the growth of calabrese and its infestation by the aphids *B. brassicae*, *M. persicae*, *L. erysimi* and colonization by their most abundant natural enemies; *D. rapae* and syrphid fly larvae. We also recorded attack by flea beetles (*Phyllotreta cruciferae* [Goeze]), diamondback moth larvae (*Plutella xylostella* L.) and cabbage whitefly (*Aleyrodites proletella* L.) over the course of one growing season. The plant biomass data were given in Gange and Gadhave (2018), and the insect data are reported here. Application of *B. amyloliquefaciens* and a mixture of *B. amyloliquefaciens*, *B. cereus* and *B. subtilis* increased the yield of plants by 48% and 70%, respectively, whereas all bacterial treatments significantly increased the variability in the size of plants, relative to controls (Gange & Gadhave, 2018).

It is a fundamental principle of science that experiments should be repeatable (Baker, 2016), something which is particularly important in the soil environment (Bond-Lamberty et al., 2016). The work reported here was designed to be similar to our previous study (Gadhave, Finch, et al., 2016a) on a *Bacillus*-calabrese-cabbage aphid-natural enemy model system which was conducted in temperate conditions (UK). As the effects of PGPR on plants and herbivores are often strain specific (Dhayalan & Karuppasamy, 2021), it is important to know if the effects seen in that study (above) are context specific or whether other locally isolated strains of similar *Bacillus* spp. also have detrimental effects on insects in a different climate such as the tropics, where local soil microbial communities would differ. The current study differs from that of Gadhave, Finch, et al. (2016a) because treatments were applied only to the seeds, to determine if this simple application method could influence levels of pests and their natural enemies in field conditions. Due to the limited shelf life of liquid products in warm climates, seed coating is currently the most viable method of PGPR delivery to roots (Arriel-Elias et al., 2018). This has relevance in determining the efficacy and applicability of using bacterial seed coating technology, which is becoming increasingly popular for its field applications (Rocha et al., 2019). We have already shown that some seed applications, particularly a mix of *B. cereus*, *B. subtilis* and *B. amyloliquefaciens*, resulted in increased root and shoot biomass in the tropical environment (Gange & Gadhave, 2018). Here, we hypothesized that the colonization of roots with plant growth-promoting *Bacillus* spp. would facilitate an array of additional benefits to plants via suppressed aphid numbers and *Bacillus*-mediated natural enemy responses.
MATERIALS AND METHODS

Seed sterilization and bacterial inoculation

Rhizobacterial cultures, *B. cereus* 2028, *B. subtilis* N11 and *B. amyloliquefaciens* TFRI4 were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), India, and subcultured immediately following the MTCC culture revival guidelines. All these *Bacillus* spp. have been previously reported to colonize an array of crops as epiphytes as well as endophytes. Freeze-dried bacterial cultures were suspended with 400-μl nutrient agar broth and a loop from each bacterial suspension was transferred to 50-ml sterile falcon tubes, each containing 20-ml sterile NA broth. These tubes were incubated overnight at 37°C on a rotary shaker and serially diluted to 10^{-3} in sterile 0.85% saline water. Approximately 500 seeds of broccoli cv. Imperial (Sakata Seed Corporation, Japan) were placed in a sterile 50-ml falcon tube and surface sterilized using 2% 40-ml sodium hypochlorite. The tube was tightly closed and vigorously shaken for 20 min. Thereafter, we followed an identical procedure to that described in Gadhave, Finch, et al. (2016), wherein seeds were rinsed in sterile water and divided into equal batches on five Petri plates, in 5 treatments, (1) control (in sterile water), (2) *B. cereus*, (12 ml of a 10^{-3} dilution of bacteria) (3) *B. subtilis*, (4) *B. amyloliquefaciens* and (5) mixture of all three bacterial species (4 ml of each). Seeds were left to imbibe for 4 h before sowing.

Land preparation, sowing and aftercare

The field experiment was carried out at Khandala, India (18°3′N 74°2′E), from March to June 2014. The experimental plot had black cotton soil, with 162 kg/ha available nitrogen, 5.6 kg/ha available phosphorus and soil pH of 6.7. Five raised seedbeds, each measuring 0.91 × 0.91 m, were prepared and watered the night before sowing. Seeds from each treatment were sown separately on each of five beds (2 March 2014) and seedbeds were watered every alternate day. Transplanting was done 20 days after sowing when seedlings attained 8–10-cm height (22 March 2014). For transplanting, a plot measuring 32 × 10 m was ploughed and five 6 × 8.5 m blocks were prepared. Twenty seedlings per treatment were planted out in a randomized block design.

The plot was irrigated every alternate day, no pesticides and fertilizers were applied throughout the experiment and hand weeding was performed once every 3 weeks. Plants were harvested 14 weeks after sowing, and data for fresh vegetative, root and total biomass were given in Gange and Gadhave (2018). The average daily temperature increased from 25°C in April to 35°C in May, with light but infrequent showers of rain during this time.

Aphid and natural enemy monitoring

The insects recorded were cabbage aphid (*B. brassicaceae*), green peach aphid (*M. persicae*), mustard aphid (*L. erysimi*), flea beetle adults (*P. cruciferae*), larvae of diamondback moth (*P. xylostella*) and adult whitefly (*A. proletella*), and natural enemies: the braconid endoparasitoid *D. rapae* and syrphid fly larvae (mixed species). All plants were allowed to become naturally infested and all insects were counted on each plant every 2 weeks, with final insect population counts when plants completed their vegetative growth 10 weeks after transplanting. Almost all plants were uncolonized by insects 2 weeks after transplanting. Therefore, the observations taken in this week were not taken into account for the final data analyses.

Statistical analyses

Statistical analyses were performed in R version 4.0.2. As the data consisted of repeated measures from the same plants, we used linear mixed effects models to examine the effects of treatments over time on insect and parasitized aphid numbers. As these were count data, we used a Poisson error structure with a log link function and χ^2 values are reported for treatment, time and interaction effects, after investigation of model simplification to produce minimal adequate models and testing for overdispersion. The packages “lme4”, “car” and “multcomp” were used to perform these analyses.

It was impossible to verify the identity of individual aphids that were parasitized by *D. rapae*. Total mummified aphid counts were thus divided by total aphid numbers on each sampling occasion, to obtain a rate of parasitism. As these were percentage data, we used a logit transformation (Warton & Hui, 2011) before examination of treatment and time effects with a repeated measures analysis of variance.

RESULTS

Aphid numbers

Populations of *B. brassicaceae* on control plants were higher than those on plants treated with PGPR (χ^2 = 647.5, df = 4, p < .001), though the only pairwise difference was between control and *B. cereus*-treated plants (χ^2 = 4.74, p < .01) (Figure 1a). Populations built up more slowly over time on treated plants, leading to a significant interaction term (χ^2 = 589.2, df = 12, p < .001).

PGPR treatment also significantly reduced numbers of *M. persicae* (χ^2 = 1942.1, df = 4, p < .001) (Figure 1b). Indeed, bacterial treatments effectively prevented population build up, resulting in very different patterns of aphid abundance over time, compared with those on control plants (χ^2 = 611.8, df = 12, p < .001).

Numbers of *L. erysimi* were lower than those of the other two aphids and showed different patterns of abundance (Figure 1c). Although there was a significant effect of treatment (χ^2 = 287.6, df = 4, p < .001), this was not because populations on control plants were higher. Indeed, all populations were of similar size, apart from those on plants whose seeds had been treated with all three bacteria. In this case, numbers remained more or less static, compared with more dramatic seasonal changes seen in the other treatments (χ^2 = 199.5, df = 12, p < .001; Figure 1c).
Other insects

None of the treatments had any effect on flea beetle numbers ($\chi^2 = 4.7, \text{df} = 4, p > .05$), which were low and tended to decrease as the crop matured (Figure 2a). A similar situation occurred with diamondback moth larvae, where patterns of abundance were similar across all treatments ($\chi^2 = 5.8, \text{df} = 4, p > .05$; Figure 2b). However, whitefly responses were more similar to those of the aphids, in that seed treatment with PGPR did reduce numbers on plants compared with controls ($\chi^2 = 21.6, \text{df} = 4, p < .01$; Figure 2c). Numbers on treated plants also declined more steeply as the plants aged, leading to a significant interaction term between treatment and time ($\chi^2 = 49.7, \text{df} = 12, p < .01$).

Natural enemy responses

Although aphid numbers were highest on control plants, the number of mummies caused by $D.\ rapae$ was lowest on these plants ($\chi^2 = 29.2, \text{df} = 4, p < .001$; Figure 3a). A similar pattern was seen with the rate of parasitism ($F_{3,304} = 5.01, p < .001$; Figure 3b). Overall, parasitism rates were significantly higher on plants treated with $B.\ amyloliquefaciens$ and all three PGPR, compared with controls ($q = 4.67$ and $3.92$, respectively, $p < .001$). Meanwhile, syrphid larvae seemed to show no response to treatment or varying numbers of aphids. Although there was a suggestion that numbers were higher for a time on $B.\ subtilis$-treated plants, no significant effect of treatment was found ($\chi^2 = 1.7, \text{df} = 4, p > .05$; Figure 3c).

DISCUSSION

Perhaps, the most encouraging aspect of this study is that treatment of seeds with PGPR can result in reduced numbers of some pest insects on the mature plants. The aphids $B.\ brassicae$ and $M.\ persicae$ and whitefly $A.\ proletella$ all produced lower populations on treated plants, whereas the exceptions to this were the aphid $L.\ erysimi$ and the two species of chewing insect. Furthermore, such effects can propagate to higher trophic levels, as the rate of aphid parasitism was considerably higher on plants growing from treated seeds. Therefore, our original hypothesis was partially upheld, as PGPR effects were not consistent across pest species or natural enemies.

In this experiment, all PGPR treatments, with the exception of $B.\ cereus$, increased root and shoot biomass of the calabrese plants, with plants being over 50% larger when $B.\ amyloliquefaciens$ or the PGPR mixture was applied (Gange & Gadhave, 2018). It is reasonable...
to suggest that the increased yield was due to the observed reductions in pest numbers, given that in insect-free laboratory conditions, no direct effects of PGPR inoculation on biomass were found (Gange & Gadhave, 2018). Seed treatment with PGPR may, therefore, represent a cost-effective and sustainable method of pest management in brassica crops. However, results such as these may also go some way towards explaining the inconsistent results of PGPR seen in field conditions, if insect numbers were low in previous experiments (Herrmann & Lesueur, 2013).

Measurements of plant biochemistry were beyond the scope of this study, but it is possible that PGPR-induced changes in glucosinolates, defensive secondary metabolites commonly found in crucifers, may have accounted for the reductions in B. brassicae numbers (Cole, 1997). The number of studies investigating the effects of PGPR on glucosinolates is still too small to generalize, with negative, null and positive effects being found (Gadhave & Gange, 2018; Nasab et al., 2019; Nosheen et al., 2016). The behaviour of the insects themselves may also play an additional role that has hitherto not been considered. B. brassicae is a gregarious, mostly sedentary aphid, in contrast to L. erysimi, which is much more mobile (Åhman, 1990). Thus, if certain PGPR affected the palatability of plants to the latter aphid, it is perfectly capable of moving from one plant to another, thereby negating any apparent treatment effects (Ghosh, 1980).

Perhaps surprisingly for a generalist species, M. persicae seems to be far less responsive to such chemicals (Cole, 1997; Kordan et al., 2021), instead, this species seems to be most affected by the nutrient content of phloem sap (Cao et al., 2018; Van Emden & Bashford, 1971). Studies of PGPR effects on the nutrient content of cabbage foliage are exceedingly few and determined by the identity of the bacteria concerned (Ekinci et al., 2014; Turan et al., 2014). It is possible that the presence of the inoculated bacteria changed the amino acid content of plants, to the detriment of M. persicae (Ekinci et al., 2014).

It is interesting that amongst the non-aphid pests, only the sucking species A. proletella was negatively affected by PGPR treatments. This may be due to similarities in dietary requirements (e.g., free amino acids and soluble carbohydrates) of this species and B. brassicae and M. persicae, as they belong to the same phloem-feeding guild. Furthermore, PGPR-mediated induced systemic resistance may also have contributed to the reduced populations of A. proletella and B. brassicae, via phloem transport (Singh et al., 2017). Neither of the two chewing insects was affected by the treatments, though the abundance of both species was relatively low. Inoculation of cabbage plants with Pseudomonas simiae WCS417r did reduce the biomass of P. xylostella larvae, but population numbers were not measured (Friman et al., 2021). Although there are few studies, in general, PGPR reduce the growth of chewing insects, though the majority of these

![Figure 2](image-url)
effects come from controlled experiments in glasshouses, where other insects are absent (Gadhave & Gange, 2018).

The presence of other insects, particularly natural enemies, in field-grown brassica crops is a key component of pest management (Fidelis et al., 2018). We found that *B. amyloliquefaciens* and the mixture of all three PGPR increased rates of parasitism, even though aphid numbers were lower on these plants. In a similar experiment in the UK, treatment with *B. cereus* and *B. subtilis* increased parasitism, whereas *B. amyloliquefaciens* had no effect (Gadhave, Finch, et al., 2016a). Thus, as in the UK experiment, parasitoid responses to aphid numbers appeared to be inverse density independent; numbers of parasitized aphids were lowest on control plants which had highest aphid numbers. Furthermore, in the present study, the higher parasitism rates on plants treated with *B. amyloliquefaciens* or the mixture occurred on plants which were largest and showed most variability in biomass (Gange & Gadhave, 2018). It has long been known that parasitism rates of herbivores can be reduced on larger, more structurally complex plants (Gingras et al., 2002), so PGPR-induced changes in plant size also do not account for the differences in parasitism rates. Therefore, the most plausible explanation is one involving a PGPR-induced change in plant chemistry. Certain glucosinolates can serve as attractants for this parasitoid and sequestering of these by *B. brassicae* can influence parasitism rates (Le Guigo et al., 2011). Why different effects of bacterial inoculation were seen in the UK and India is open to speculation, but perhaps, the most plausible explanation lies in the nature of the indigenous soil microbial community. In the UK, the application of the different treatments had a variety of effects on soil bacterial composition, but those of *B. amyloliquefaciens* were the most pronounced (Gadhave et al., 2018). It is highly likely that different species-specific effects occurred in the present study. Such effects can translate into altered volatile emissions, which have consequences for parasitoid attraction (Pangesti et al., 2015; Pineda et al., 2013). Meanwhile, our results with syrphid predators were similar to those in the UK, in that PGPR treatments did not seem to lure these natural enemies.

**CONCLUSIONS**

We found that the seed applications of PGPR can reduce numbers of some aphid pests on mature calabrese plants, in a subtropical climate.
Some of these effects were similar to those in a previous experiment in the UK (Gadhave, Finch, et al., 2016a). This suggests that if locally sourced inoculants are used, there may be some consistency in experimental outcomes, meaning that these bacteria could have a vital role to play in the management of pests in sustainable agricultural systems (Dhayalan & Karuppasamy, 2021). Furthermore, these effects can propagate to higher trophic levels, increasing rates of parasitism. Overall, reductions in pest numbers can translate into increased yields, via this simple and sustainable technique. Careful tailoring of species in rhizobacterial inoculants with local conditions would maximize their benefits in agriculture and further experiments over several seasons are required to establish general patterns of interactions.

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CONFLICT OF INTEREST
The Authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS
Kiran R. Gadhave and Alan C. Gange designed the experiment, which was executed by Kiran R. Gadhave. Both authors analysed the data and wrote the manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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