Interactions between plant growth promoting rhizobacteria, foliar-feeding insects and higher trophic levels

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Declaration of Authorship

These doctoral studies were conducted under the supervision of Prof. Alan C. Gange. The work presented in this thesis is the result of original research carried out by myself, while enrolled in the School of Biological Sciences as a candidate for the degree of Doctor of Philosophy. This work was conducted independently and has not been submitted for any other degree of award in any other university or educational establishment. Where I have consulted the work of others, this is always clearly stated.

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Abstract

Several rhizobacteria promote plant growth and suppress numerous plant diseases; however, the roles of these bacteria in altering plant chemical defences against foliar insects, changing plant-associated microbial community and in shaping plant-insect-natural enemy multi-trophic interactions have been sparsely explored. The aim of this project was to study the effects of different *Bacillus* species on calabrese (*Brassica oleracea*) growth parameters, biochemistry and endophytic bacterial community, the life history traits and population dynamics of different foliar-feeding insects and their common natural enemies. The magnitude of effects of *Bacillus* spp. on a variety of these parameters varied between and within control and treated plants. Preliminary experiments showed that a commercial *Bacillus* mixture was not as effective as native *Bacillus* species in promoting plant growth. All *Bacillus* treatments significantly altered the life history traits of *B. brassicae* in the laboratory conditions. *B. subtilis*, *B. amyloliquefaciens* and mixed treatments significantly increased the onset of reproductive maturity and plant biomass, and changed foliar indole glucosinolate levels. However, the effects of all *Bacillus* species on insect herbivores were contrasting; the infestation of *B. brassicae* was significantly reduced in a temperate climate, and that along with two aphids; *Myzus persicae* and *Lipaphis erysimi* was increased in the tropics. The natural enemy responses on all experimental plants, in both field studies, were aphid density dependent. The diversity, evenness and abundance endophytic bacteria differed across control and treated plants. It is recommended that discrepancies in the results of bacterial inoculants use in different conditions can be reduced through a careful tailoring of bacterial species in inocula with soil and crop. It is concluded that Plant Growth Promoting *Bacillus* accelerate calabrese maturity and increase biomass, alter endophytic bacterial diversity, evenness and abundance, and shape above ground interactions via significant changes in aphid field infestation levels, which are site-specific and which indirectly extend up to the natural enemy level.

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Chapter 1

Introduction

The World has an ever growing demand for food which needs to be satisfied in a sustainable manner. Pest attack is a serious threat to agricultural industries and leads to significant quantitative and qualitative loss of food commodities (Oerke, 2006). Modern crop production systems in many countries are heavily reliant on the use of synthetic pesticides and fertilizers. Intensive use of these agrochemicals has increased human health and environmental concerns. Moreover, such use exploits land and water resources, and will likely increase input demand to sustain the current yield levels. In contrast, natural ecosystems are more sustainable in the context of productivity, insect pest resistance and nutritional aspects (Ewel, 1999). Plants in natural ecosystems often suffer far lower levels of pest attack. Why should this be so? There are many reasons, such as crop monocultures representing large targets for pests to attack, resistance mechanisms being lost during plant breeding programs and the lack of natural enemies in crop situations. Since none of these problems can be solved easily, one potentially important solution, which is sparsely explored in biological pest control, is the soil microbial community.

The soil microbial community plays vital role in determination of the soil health. Soil microbes comprise a vast array of bacteria and fungi. Some of these are easily culturable and thus have potential to be used in experiments and subsequently mass-produced. The rhizosphere is the root-soil zone with highest activity of microorganisms and is therefore the largest ecosystem present in the nature (Hinsinger *et al.*, 2009). Due to the direct influence of plant root exudates, a wide variety of microorganisms co-exist in the rhizosphere and perform numerous potential roles in multitrophic interactions. Amongst them, bacteria form the predominant group that influences soil health and plant biology to a significant extent. The diversity of rhizobacteria has been studied by many researchers to determine their functional role in management of soil borne diseases, soil health improvement and yield enhancement in major food crop systems (Johri *et al.*, 2003; Lugtenberg & Kamilova, 2009). Recent studies have begun to investigate how certain bacteria in the soil can alter the chemistry of above-ground tissues (Selvaraj *et al.*, 2008; Brock *et al.*, 2013).

1.1 Plant growth promoting rhizobacteria

Soil bacteria that colonize plant roots and promote plant growth are termed as Plant Growth Promoting Rhizobacteria (PGPR) (Kloepper & Schroth, 1978). Bacteria belonging to the genera *Rhizobium, Brachyrhizobium, Azatobacter, Azospirillum, Pseudomonas, Bacillus* and *Serratia* are the predominant groups promoting plant growth. The direct plant growth promotion includes stimulation of plant growth through biofertilization, root growth stimulation, rhizoremediation and plant stress control while indirect includes protection of plant through antibiosis, Induced Systemic Resistance (ISR) and Competition for Nutrients and Niche (CNN) (Lugtenberg & Kamilova, 2009). The bacterial traits; colonization competitiveness (e.g. plant carbon, sugars and space) and persistence (e.g. maintenance of stable contact with plants) determine the success of plant growth promotion (Lugtenberg & Kamilova, 2009).

1.1.1 Plant growth promoting Bacillus

Bacillus is a predominant group of rhizobacteria and has diverse species that are intensively exploited for their commercial applications in the agricultural industry. The use of Plant Growth Promoting (PGP) *Bacillus* spp. as biological suppressors of herbivorous insects can mitigate the current challenges of excessive pesticide use, evolution of insecticidal resistance and secondary pest outbreak in a relatively easy and inexpensive way. The key attributes of *Bacillus* as a potential bio-control agent include the capacities to form stable endospore formulations and to maintain stable contact with roots, which facilitate successful and persistent colonization (Nicholson *et al.*, 2000; Chowdhury *et al.*, 2013). A variety of these bacteria synthesise bioactive molecules including hormones that promote plant growth. These molecules have a broad spectrum of activities against diverse pathogens (Nagórska *et al.*, 2007). They also increase the uptake of essential nutrients and thus increase plant quality and yield (Choudhary & Johri, 2009). The addition of certain *Bacillus* species changes the composition of rhizobacterial community (Probanza *et al.*, 2002), with a prospect of favouring rhizobacteria that are highly beneficial to plants.

The *Bacillus* species; *B. cereus*, *B. subtilis* and *B. amyloliquefaciens* are abundant in rhizospheres and have been shown to produce plant growth promotion *viz*. plant biomass increase, early reproductive maturity, and biotic and abiotic stress tolerance

(Kloepper *et al.*, 2004). Their effects on foliar feeding insects are, however, sparsely explored. *Bacillus cereus* has abilities to colonize plant roots extensively and persist in rhizospheres until harvest (Halverson *et al.*, 1993). Furthermore, this bacterium enhances nodulation in legumes (Halverson *et al.*, 1993) and thus facilitates nutrient availability, suppresses plant diseases (Handelsman *et al.*, 1990) and enhances biomass in different plants (Osburn *et al.*, 1995; Dutta *et al.*, 2013). *Bacillus subtilis* is a prime soil bacterium and is one of the widely studied bacteria for plant growth promotion (Lugtenberg & Kamilova, 2009). It successfully colonizes plant through biofilm formation (Beauregard *et al.*, 2013) and offers a multitude of advantages to crops including increase in yield (Sharaf-Eldin *et al.*, 2008) and induction of systemic resistance against a variety of fungal and bacterial pathogens, and a few phloem feeders (Asaka & Shoda, 1996; Valenzuela-Soto *et al.*, 2010). *Bacillus amyloliquefaciens* is also one of the most persistent and abundant bacteria in rhizospheres (Kröber *et al.*, 2014) and shows anti-pathogen activities (Yu *et al.*, 2002). It helps plant derive essential nutrients and therefore reduces the rates of fertilizer applications (Adesemoye & Kloepper, 2009), and improves yield in lettuce and other vegetables (Idriss *et al.*, 2002; Chowdhury *et al.*, 2013).

1.1.2 Endophytic bacterial diversity and abundance

Endophytic bacteria live inside plants and are often the most successful subset of rhizosphere, rhizo- and phylloplane bacterial community (Berg *et al.*, 2005; Rosenblueth & Martínez-Romero, 2006). They form symbiotic, commensal and pathogenic relationships with plants. Endophytes produce an array of compounds that facilitate nutrient acquisition, qualitative and quantitative trait enhancement and offer fitness against biotic and abiotic stresses (Ryan *et al*., 2007). Earlier studies have explored the endophtic bacterial diversity using a classical culture-dependent approach which involves surface sterilization of plant pieces, plating, isolation and sequencing of 16S rRNA amplicons (Hallmann & Berg, 2006; Rosenblueth & Martínez-Romero, 2006). Since only a minor fraction of any bacterial community is culturable (Amann *et al.*, 1995), this approach, however, fails to represent the majority of the bacterial community. Recent studies have used metagenomic approaches for taxonomic and functional profiling of plant associated bacteria (de Campos *et al.*, 2013; Kröber *et al.*, 2014; Schmidt *et al.*, 2014; Shi *et al.*, 2014). These

studies suggest that *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteriodetes* are the most abundant bacterial phyla in rhizo- and endospheres. Whether the application of common rhizobacterial species to plants influence the endophytic bacterial community remains to be seen.

1.1.3 Bacterial molecules in PGPR mediated induced systemic resistance

An increase in host plant defence mechanisms resulting from an external stimulus like a pathogen or pest is termed Induced Systemic Resistance (ISR) (Hammerschmidt & Kuc, 1995). PGPR trigger ISR that helps plant effectively suppress invading pathogens and deleterious insect herbivores (Van Oosten *et al.*, 2008). Colonization of roots with PGPR leads to the synthesis of proteins and secondary metabolites involved in elicitation of ISR. Earlier studies proposed that several bacterial traits like flagella, synthesis of lipopolysaccharides (LPS) and siderophores trigger ISR (Leeman *et al.*, 1995; Van Wees *et al.*, 1997; Van Loon *et al.*, 1998; Ramamoorthy *et al.*, 2001). Further studies on understanding root-microbial communication also revealed the roles of airborne organic volatile compounds, 2, 3-butanediol and acetoin in ISR induction in *Arabidopsis* (Ping & Boland, 2004; Ryu *et al.*, 2004). In later studies, the individual bacterial compounds such as cyclic lipopeptides (Ongena *et al.*, 2007), antifungal factor, 2, 4-diacetyl phloroglucinol (Phl) (Iavicoli *et al.*, 2003) and signalling molecules *N*-acyl homoserine lactone (AHLs) (Schuhegger *et al.*, 2006) were also found to be associated with ISR.

1.1.4 PGPR and plant signalling

Upon pathogen attack and insect damage, plants undergo priming; a rapid physiological response generated by plants to deal with invaders. This response is facilitated by an array of plant hormones through individual and cross-talk of different plant signalling pathways (Van der Ent *et al.*, 2009). Despite extensive molecular studies on phytohormone pathways, very little is known about the effects of bacterial treatments on elicitation of plant defence mechanisms against insects in a multitrophic context. A recent study showed that PGPR modulated host plant resistance via manipulation of jasmonic acid (JA)-dependent and JA-independent responses (Valenzuela-Soto *et al.*, 2010). At the transcriptional level, *P. fluorescens* primed *Arabidopsis* for changes in the expressions of genes that are involved in the activation of jasmonic and abscisic acid defensive signalling pathways against *Myzus persicae.*

Conversely, no such changes were observed against *B. brassicae* suggesting that PGPR modify plant-aphid interactions at the transcript level. The effects of soil bacteria on the infesting pests may, however, depend upon the host, microbial culture, pest feeding nature, plant developmental stage and degree of insect specialism (Pineda *et al.*, 2010).

1.1.5 PGPR and plant secondary metabolites

Foliar secondary metabolites play important roles in host plant defence against invading herbivores (Bennett & Wallsgrove, 1994). In *Brassicaceae* plants, glucosinolates are the major constitutive defence against generalist and specialist feeders (Hopkins *et al.*, 2009). In total, 120 glucosinolates have been identified and classified on the basis of structural similarities into three major groups; aliphatic, aromatic and heterocyclic (e.g. indole) glucosinolates (Fahey *et al.*, 2001). Despite the nature of glucosinolates as plant constitutive defences, their expression in plants is induced by insect damage, plant pathogenic microorganisms and other abiotic stresses (Chew, 1988). The effects of rhizobacterial colonization on plant glucosinolate profiles are far less explored. Two earlier studies have reported that the inoculation of plant growth promoting bacterium, *Enterobacter radicincitans* DSM 16656 in *Arabidopsis* slightly decreased aliphatic glucosinolates (Brock *et al.*, 2013), whereas in cruciferous plants, glucosinolate levels remained unaffected (Schreiner *et al.*, 2009). The effects of colonization of major plant growth promoting rhizobacteria such as *Bacillus* and *Pseudomonas* on aliphatic and indolic glucosinolate content have not been elucidated so far. Since both of these metabolites offer resistance to plants against herbivory (Mewis *et al.*, 2005; 2006) and stimulate feeding for specialists (Hopkins *et al.*, 2009), it is essential to study the effects of these rhizobacteria in changing glucosinolate levels and thus in determining insect and natural enemy population dynamics.

1.1.6 PGPR and insect herbivores

The potential effects of PGPR on a variety of insect herbivores, compared with those on plant pathogens, remain sparsely explored. So far, a few species belonging to the selective genera of PGPR *viz*. *Pseudomonas*, *Bacillus* and *Serratia* have been studied against insect herbivores (Gange *et al.*, 2012). Of those species, most belong to the genus *Pseudomonas*, which shown to have negative consequences on the majority of

targeted pests. For instance, Qingwen *et al.* (1998) revealed that *P. gladioli* treatment to cotton plants reduced the growth, consumption rates and digestive ability of cotton bollworm, *Helicoverpa armigera*. In rice, the different strains of fluorescent *P. fluorescens* effectively suppressed leaf-folder *Cnaphalocrocis medinalis* infestation in field conditions due to increased accumulation of defence molecules; chitinase and proteinase inhibitors (Saravanakumar *et al.*, 2007). Pineda *et al.* (2010) revealed that *Arabidopsis* roots colonization with *P. fluorescens* elicits ISR against different herbivores (Van der Ent *et al.*, 2009). The mixture of *Pseudomonas* spp. with other rhizobacterial and fungal species was also found to be significantly effective in reducing pests. Zehnder *et al.* (1997) showed that a PGPR mixture (*Pseudomonas putida* 89B-61, *Serratia marcescens* 90-166, *Flavomonas oryzihabitans* INR-5 and *B. pumilus* INR-7) significantly reduced the populations of cucumber beetles. The effects of combinations of entomopathogenic fungi, *Beauveria bassiana* and *P. fluorescens* were studied in two different studies on groundnut and rice crops. Both of these studies showed significant reduction in the incidences of leaf miner and collar rot in groundnut (Senthilraja *et al.*, 2010) and leaf-folder pest and sheath blight disease in rice (Karthiba *et al.*, 2010).

Two recent studies reported the negative effects of individual species inoculation of PGP *Bacillus* on the growth and development of generalist insect herbivores (Vijayasamundeeswari *et al.*, 2009; Valenzuela-Soto *et al.*, 2010). The bioformulation containing *B. subtilis* showed detrimental effects against *H. armigera* in cotton (Vijayasamundeeswari *et al.*, 2009), and against virus free *Bemisia tabaci* in tomato (Valenzuela-Soto *et al.*, 2010). However, unlike Pseudomonads, the mixture of two PGP *Bacillus* species showed no effects against plant herbivores. A soil amendment based application of pepper plants with two *Bacillus* species, *B. subtilis* and *B. amyloliquefaciens*, significantly increased pepper yields, but failed to suppress green peach aphid (*Myzus persicae*) populations (Herman *et al.*, 2008). These studies with *Bacillus* spp. highlighted that the use of individual and mixture of microbial species can have varied effects on invading insects. Furthermore, these effects are likely to be specific to plants, pest species and degree of insect specialism. More studies are needed to explore the potential of various *Bacillus* species individually and in mixture in reducing pests in the field conditions. Irrespective of pest resistance, the majority of studies with PGP *Pseudomonas* and *Bacillus* spp. have shown to offer the multiple

advantages to plants through elevated crop yield, conservation of natural enemies in crop ecosystem and alteration of plant defence signalling.

1.2 Insect pests and natural enemies

Brassicaceae crops are widely cultivated globally, primarily because of their high nutritional status and profitability, and medicinal properties (Warwick, 2011). Calabrese (*Brassica oleracea*) is a short-duration crop (3-4 months) sown during summer months in UK; from May to July in a well-drained soil. The pests, diamondback moth (*Plutella xylostella*), cabbage looper (*Trichoplusia ni*), cabbage aphid (*Brevicoryne brassicae*), cabbage moth (*Mamestra brassicae*), turnip aphid (*Lipaphis erysimi*) and green peach aphid (*M. persicae*) cause up to 70% yield loss to *Brassicaceae* crops in the absence of control measures (Webb, 2004).

1.2.1 Cabbage moth

M. brassicae is one of the devastating pests of crucifers and causes severe defoliation and quantitative as well as qualitative yield loss. This pest is cosmopolitan in distribution and has major pest status throughout the Europe, temperate countries in Asia and few countries in Africa (Devetak *et al*., 2014). Upon infestation, the adult moths lay eggs beneath the leaf surface. The neonate caterpillars scrap the outer portions of cabbage head and make numerous irregular holes. This is followed by inward progression towards core in the later stages. Such damage facilitates secondary infection by bacteria and fungi causing decay of infested tissues.

1.2.2 Cabbage aphid

The cabbage aphid, *B. brassicae* attacks different crops from the cabbage family, *Brassicaceae* and causes serious losses in cabbage, cauliflower, broccoli and Brussels sprout (Blackman & Eastop, 2000). The aphid is predominantly a cosmopolitan pest, but occurs extensively in temperate regions and sporadically at higher altitudes in the tropical countries. Typically, this aphid colonizes and feeds on the lower surface of younger leaves and floral parts, and causes losses in yield and marketability. This aphid also spreads 23 viral diseases in the *Brassicaceae* family (Kessing & Mau, 1991). The primary mode of reproduction in aphids is parthenogenesis (vivipary) wherein neonate nymphs are produced from females without any sexual intervention. The life cycle of aphids is largely determined by changing environmental conditions.

The alterations in photoperiod and temperature are the key factors that determine swap over of the modes of reproduction and thus ultimately govern aphid population dynamics. Based on the utilization of host plants and egg laying behaviour, the cabbage aphid can typically be classified as monoecious, with host alternation confined within *Brassicaceae* family and holocyclic, and with overwintering eggs on plant debris (Blackman & Eastop, 2006). At the termination of the overwintering phase, hatched parthenogenic alate females colonize and multiply swiftly on summer plant hosts (Dixon, 1998). Rapid nymphal development is facilitated by the unique mechanism of parthenogenesis termed as 'telescoping of generations'. This is characterized by concurrent embryonic development within mother aphid and its embryos. The alate forms are produced in response to high population density, changing environmental conditions and depletion of existing host plants, to disperse the colony to new host plants (Lamb, 1961; Hughes, 1963). The distinct modes of reproduction, short multiplication span, rapid dispersal to new plants and large population density makes aphids some of the most noxious and economically important pests across the globe.

1.2.3 Green peach and turnip aphids

Green peach aphid, *M. persicae* is the generalist pest that attacks plants from over forty plant families. This aphid has holocyclic as well as anholocyclic strains, and heterocious feeding habits requiring several hosts to complete life cycle (Tamaki *et al*. 1982). *Myzus persicae* causes plant wilting and distortion, and reduces plant growth, yield and marketability via transmission of about 100 viral diseases (Castle and Berger, 1993). This aphid produces over 20 annual generations in mild climates, and develops rapidly, with often 10 to 12 days for a complete generation (van Emden *et al*., 1969). *Lipaphis erysimi* is the specialist feeder and is one of the prevalent pests of *Brassicaceae*. *L. erysimi* can cause up to 90% loss in some crop plants (Rana, 2005) and transmit several viral diseases (Prasad and Phadke, 1980). The life cycle of this aphid is similar as cabbage aphid (i.e. monoecious and holocyclic), with the longevity of 20-40 days, producing up to 35 generations per year (Capinera, 2008).

1.2.4 Natural enemies

The most widespread and important natural enemies of *B. brassicae* include ladybird beetles, syrphid flies, lacewing and parasitic wasps, e.g. *Diaeretiella rapae*

(McIntosh). Of these natural enemies, *D. rapae* is the most prevalent solitary endoparasitoid of *B. brassicae* and accounts for 82.5% of all aphid parasitoids collected in *Brassicaceae* fields (Pike *et al.*, 1999). The females of *D. rapae* oviposit inside second to fourth instar nymphs of *B. brassicae* and upon hatching, the larva feeds on the inner body content. As a result of feeding, a light brown hollow case of the host aphid called a 'mummy' is produced. The pupation takes place inside the host and adults pierce a hole to emerge from such mummified aphids. All developmental stages; from oviposition to adult emergence, take place inside the host and the life cycle is typically completed in 9-10 days. The intensity of parasitism and predation is, however, low in early season and thus mostly natural enemies fail to maintain aphid populations below economic injury level (Nunnenmacher & Goldbach, 1996). A previous study has shown that plant growth promoting rhizobacteria can attract natural enemies through a release of volatile organic compounds (Saravanakumar *et al.*, 2008) and thus can be potentially used to overcome the limitations of use of predators in aphid management.

Despite possessing several attributes necessary for plant growth promotion, the potential of the extensive rhizobacteria, *Bacillus* in pest management has been sparsely explored. In this thesis, I attempt to study the potential plant-mediated effects of application of *Bacillus* on the performance of a variety of generalist and specialist foliage-feeders, and their common natural enemies. I used the *B. oleracea* plant as a study system primarily because of its (i) non-mycorrhizal nature; thus avoiding avoid any biases arising from mycorrhizal colonization, (ii) well-studied defensive chemistry, which could be linked with insect population dynamics, (iii) natural infestation by a variety of pests and natural enemies; thus allowing more insects to be incorporated in the field assays and (iv) experimental suitability (e.g. short duration, wide cultivation). The overall hypothesis was that *Bacillus* species influence the soil microbial community, alter foliar chemistry, suppress pest populations and trigger natural enemy responses. The overall aim is ultimately to develop crop protection systems for the future that are sustainable and that reduce the need for pesticides. The project aim was to determine the appropriate individual or mixed *Bacillus* species that need to be added to model crops (*B. oleracea*), so as to provide maximum insect resistance, with increased natural enemy responses. In so doing, I aim to increase the

yield of crops, so as to meet the population needs and to improve the quality of human food, by reducing the likelihood of chemical residues being present.

1.3 Objectives

1. To determine if it is possible to use plant growth promoting rhizobacteria as biological control agents of foliar-feeding insect pests in calabrese (*B. oleracea*).
2. To determine if plant growth promoting rhizobacteria alter the plant secondary metabolite profiles to enhance host plant resistance against invading insect herbivores and to attract natural enemies of these pests.
3. To determine if the addition of certain species of plant growth promoting rhizobacteria can affect the diversity and abundance of other naturally occurring endophytic bacteria in the field conditions.

Chapter 2

Material and Methods

2.1 General methods

2.1.1 Cultivation of host plants

Seeds of calabrese (*Brassica oleracea*) variety, Green Sprouting (Country Value Seeds, UK) were used in the laboratory studies. Plants were grown in 1.5 l plastic plots in a constant environment room maintained at 20°C temperature, 60% humidity and 16 hrs photoperiod. The preliminary experiments were performed in partial nutrient limiting conditions. Three seeds were sown in pots filled with the medium containing John Innes potting compost No. 3 and horticultural grit sand on 50:50 (v/v) basis. Before sowing, seeds were soaked in water for 4 hours to improve germination and aid early plant establishment.

2.1.2 Bacterial inoculation

The five species mixture of PGP *Bacillus* (*B. subtilis, B. laterosporus, B. licheniformis, B. megaterium, and B. pumilus*) was obtained from Symbio Ltd, Wormley, Surrey, UK. Plants were arranged in two treatments: 'control', plants that received 200 ml distilled water; and 'bacteria', plants that received 200 ml of a 2% aqueous solution containing 10^8 cfu/ml bacterial seeds treatments. To ensure proper inoculation, two treatments a week were allocated in which 10^8 cfu/ml bacterial suspension was drenched in the root zone. Ten replicates per treatment were maintained and plants were randomised for their position under light. The application rates of formulations were kept higher than recommended field doses to ensure adequate inoculation. Plants were watered as required and thinned out twice, 7 and 10 days after emergence to retain the most vigorous seedling.

2.1.3 Statistical analysis

The data analyses on all preliminary experiments were performed using R version 3.0.1 (The R Foundation for Statistical Computing). The repeated measures (*viz.* plant height, number of leaves, number of aphid nymphs, and adults and number of leaves infested) were analysed using generalized linear model (glm function in R) procedure, with treatment and time as linear predictors. The data on non-repeated measures (*viz.* calabrese biomass and insect growth parameters) were analysed using a linear model

(LM) procedure and corresponding t values for each parameter were reported. While analysing *M. brassicae* nutritional indices, initial larval weight was used as a covariate to take into consideration any differences in larval weights before the bioassay was carried out. The mean values of pre-reproductive periods, aphid adult weights, number of embryos and intrinsic growth rates of first three developing adults were considered while analysing data.

2.2 Preliminary experiments

2.2.1 Experiment 1 (Calabrese growth parameters)

The aim of this experiment was to study the effects of application of the different treatments described above on calabrese growth parameters. Calabrese growth was monitored at four time points. At 15, 30 and 45 days after sowing (DAS), plant height and number of leaves were recorded; whereas at 60 DAS, total, shoot, root biomass and root: shoot ratio were noted.

2.2.1.1 Results & Discussion

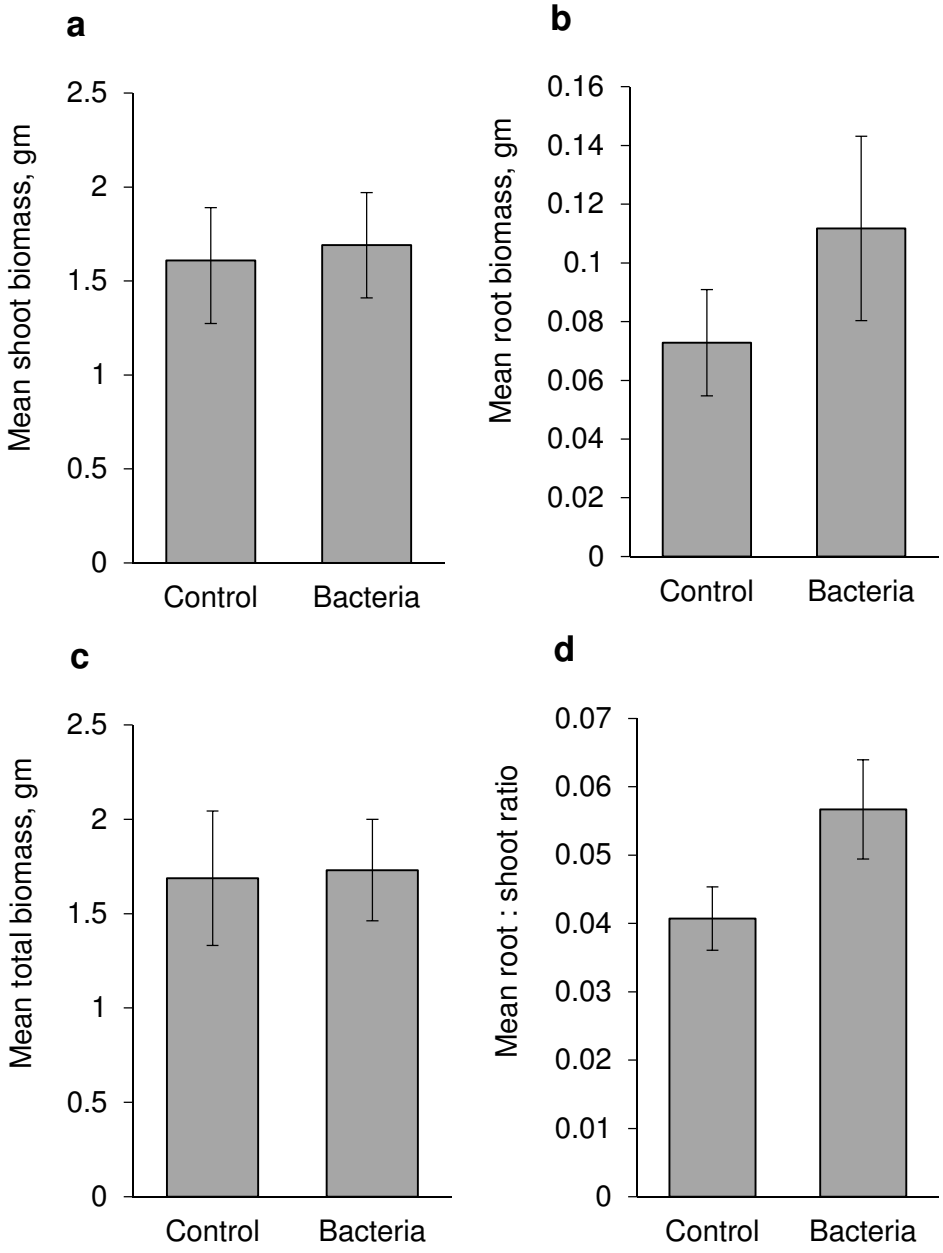
The bacterial treatment favoured root growth as evidenced by increased root biomass and root: shoot ratio in calabrese (Fig. 1). These parameters were not, however, significant at 0.05 level (Table 1). Furthermore, treatment and interaction effects were insignificant for plant height and number of leaves showing that bacterial treatment did not have significant linear effect on these plant parameters over the time. None of the other plant biomass was significantly improved.

Table 1 Effects of bacterial treatment on growth response of calabrese

No.	Parameter		Bacteria	
			t	**P**
1	Height	Treatment	0.47	0.64
		Time	**15.2**	**<0.001**
		Treatment: time	0.59	0.55
2	No. of leaves	Treatment	0.27	0.78
		Time	**8.02**	**<0.001**
		Treatment: time	-0.14	0.88
3	Biomass	Total	0.01	0.925
		Shoot	0.18	0.857
		Root	1.07	0.297
		Root/shoot ratio	1.85	0.08

Significant effects are in bold.

Figure 1 Effects of mixed bacterial treatment on calabrese biomass: (a) shoot (b) root (c) total (d) root: shoot ratio



These results showed discordance with earlier studies showing that *Bacillus* spp. improve vegetative and reproductive plant parameters (Kloepper *et al.*, 2004). Most of these studies used one to three microbial species and showed that bacteria facilitate nutrient availability (Adesemoye & Kloepper, 2009) and stimulate plant growth through production of growth hormones (Gutiérrez-Mañero *et al.*, 2001). Thus, the observed discrepancy could be attributed to the addition of a bacterial inoculant containing five species, which may have encouraged antagonistic interactions in the

rhizosphere, without producing any favourable effects on plant growth. Further investigations were carried out to link the effects of the bacterial treatment on generalist and specialist feeders through different insect bioassays.

2.2.2 Experiment 2 (Cabbage moth bioassay)

The effects of bacterial treatment on the growth of the generalist cabbage moth, *M. brassicae* were studied using different indices. Late third instar caterpillars of cabbage moth, *M. brassicae* were obtained from the Centre for Ecology and Hydrology, Wallingford, UK. Third instar caterpillars were carefully picked using a hair brush and transferred to the 9 cm petri dishes. The dishes were lined with new, damp filter papers daily to maintain appropriate sanitation and humidity. Before feeding, caterpillars were starved for 24 hours, and seven replicate caterpillars per treatment were maintained. After starvation, initial individual larval weights were determined and then caterpillars were allowed to feed on the excised leaves with known weights. The plates were incubated in the controlled laboratory conditions; 20°C temperature, 60% RH and observations on larval weight, initial leaf weight, leaf weight after feeding, and weight of larval excreta were recorded daily for seven consecutive days until pupation. Nutritional indices were calculated based on insect food consumption and utilization studies by Waldbauer (1968), with a slight modification. While calculating indices, we used the initial fresh weights of *M. brassicae* caterpillars instead of mean larval weight during the experiment to minimise inaccuracies and misleading effects due to a short observation period confined to one or two instars (Farrar *et al.*, 1989). The following different indices were recorded: overall gain in larval weight, quantity of food metabolized, relative growth rate, relative consumption rate, assimilation efficiency, net growth efficiency and gross growth efficiency.

2.2.2.1 Results & Discussion

The generalized linear model analysis showed that bacterial treatment did not significantly influence any of the nutritional indices studied (Table 2). However, the covariate, initial larval weight, had significant influence on overall gain in larval weight, relative growth rate and relative consumption rate. Furthermore, the additions of bacteria to calabrese slightly reduced larval weight gain and pupal weight (Fig. 2), however, these were not statistically significantly different.

Table 2 Nutritional and growth indices of *M. brassicae* fed on bacterial treated calabrese

No.	Indices		Bacteria	
			t	**P**
1	Overall gain in larval weight	Bacteria	0.38	0.71
		Initial larval weight	**7.86**	**<0.001**
2	Quantity of food metabolized	Bacteria	-1.01	0.33
		Initial larval weight	0.46	0.65
3	Relative growth rate	Bacteria	0.18	0.86
		Initial larval weight	**-4.40**	**0.001**
4	Relative consumption rate	Bacteria	-0.08	0.93
		Initial larval weight	**-2.74**	**0.01**
5	Assimilation efficiency	Bacteria	1.72	0.11
		Initial larval weight	0.72	0.48
6	Net growth efficiency	Bacteria	1.55	0.14
		Initial larval weight	2.04	0.06
7	Gross growth efficiency	Bacteria	0.45	0.65
		Initial larval weight	-0.78	0.45
8	Pupal weight	Bacteria	0.73	0.47
		Initial larval weight	1.32	0.21

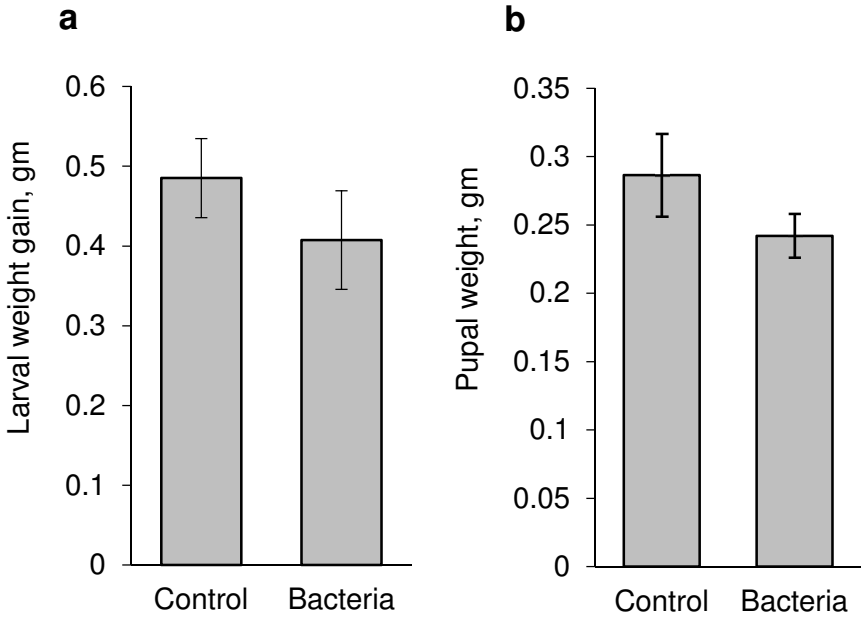
Significant effects are in bold.

These results differed from an earlier study (Van Oosten *et al.*, 2008), in which induced systemic resistance was triggered when larvae of the generalist, *Spodoptera exigua* were fed on intact *Arabidopsis* plants. The bacterial treatment did not have any significant effect on any of the nutritional and growth indices of *M. brassicae* possibly due to feeding of excised leaves from treated plants for a short period during late larval instars. The excision of leaves may have prevented the induction of systemic resistance in calabrese and thus did not produce any significant effect on *M. brassicae* feeding and growth. Furthermore, the effects of bacterial treatments may not have been apparent, because the entire observations on larval indices were recorded in a short span. The variation in the weights of larvae before the start of the experiment (initial larval weight), being the key determinant, significantly affected overall gain in larval weight, relative growth and consumption rates.

M. brassicae was not used in the subsequent experiment, including whole plant bioassays, because of the difficulties in monitoring the weight of food consumed, frass, and weight gain etc. for each caterpillar on each replicate plant. Furthermore, we identified the secondary background factors *viz.* unexpected and variable diapause in the pupal stage, larval weight in earlier instars and potential limitations in

measuring field incidence and damage, associated with *M. brassicae* that may have compromised the use of this insect in subsequent laboratory and field experiments.

Figure 2 Effect of bacterial treatment on larval weight gain and pupal weight of *M. brassicae*



2.2.3 Experiment 3 (Cabbage aphid bioassay)

The changes in the life history characteristics of the cabbage aphid, *Brevicoryne brassicae* in response to the feeding on mixed bacteria inoculum treated calaberese were studied in the laboratory conditions.

2.2.3.1 Experimental setup

The culture of cabbage aphid, *B. brassicae* was obtained from the field and maintained on calabrese plants in an insect rearing cage in the constant environmental conditions specified above. Using a fine paintbrush, three *B. brassicae* adults were introduced on 50 day old differentially treated calabrese plants and allowed to reproduce parthenogenetically. On production of 2-4 nymphs, the original adult aphids were removed and the progeny were allowed to grow. Aphid development on each individual plant was monitored through a series of observations including (1) pre-reproductive period- period in days from larviposition to first reproduction; 2) adult weight on the first day of adulthood; (3) number of pigmented and unpigmented embryos within the adult; (4) the instantaneous rate of increase (r_i), measuring a

population increase ability over specified time (Hall, 1964); (5) the intrinsic rate of increase (r_m), expressed as offspring aphid^{-1} day^{-1} (Wyatt & White, 1977); (6) number of aphid adults and nymphs; and (7) number of leaves infested.

While computing intrinsic rate, numbers of pigmented and unpigmented embryos were used as a measure of effective fecundity. The observations on pre-reproductive period, adult weight and number of pigmented/unpigmented embryos were recorded individually for the first three adults developing from progenies and monitored at every alternate day. The other numerical observations, such as number of adults and nymphs and number of leaves infested were recorded at the interval of 5 days as this period was considered as optimum for development of significant variation in these parameters. Number of aphids were recorded in eight sets of observations (8×5=40 days) and number of leaves infested were recorded over seven sets (7×5=35 days). Since five aphids are optimum to build an aphid colony, the leaf was considered as infested when the numbers of aphids present were five or more. The interplant movement of apterous *B. brassicae* adults and nymphs were limited using sunbags. The 44.0 cm × 20.5 cm transparent sunbags with 24 mm filter were obtained from Sigma Aldrich Co LLC, UK. These bags were then fixed with 15 × 5 cm insect rearing net to avoid excess humidity and to maintain proper aeration for normal aphid colony development.

2.2.3.2 Dissection and microscopy

The earliest maturing first three aphid progenies were isolated from each plant using a fine paintbrush. The span of adult development was highly asynchronous and varied from plant to plant. As a result, the observations on first three progenies turned into adults were recorded, either one by one or concurrently, depending upon colony development. The adults were weighed on the first day of their adulthood using a microbalance and dissected under a compound microscope using 0.15 mm (d) × 15 mm (l) finest stainless steel insect pins. The number of red pigment eyed embryos and unpigmented embryos with soft oval bodies round at both ends were counted under high resolution. The progenies of dissected adults were allowed to grow on respective plants and their growth was monitored using above specified parameters.

2.2.3.3 Results & Discussion

All parameters showed variability in aphid responsiveness to feeding on untreated and treated plants (Table 3).

2.2.3.3.1 Pre-reproductive period

No statistically significant difference was found between pre-reproductive periods of adults fed on untreated and treated plants.

2.2.3.3.2 Adult weight (on the first day of adulthood)

The bacterial treatment reduced the adult weights for first three maturing adults compared to untreated plants. However, these were not statistically different at the 0.05 level.

2.2.3.3.3 Number of embryos

These numbers were comparatively higher in *B. brassicae* adults fed on untreated plants and were reduced significantly in bacterial treated plants (Fig. 3a).

Table 3 Effects of bacterial treatment to calabrese on the life history characteristics of *B. brassicae*

No.	**Parameters**	**Bacteria**	
		t	**P**
1	Pre-reproductive period	-0.05	0.95
2	Adult aphid weight	-1.04	0.31
3	Number of embryos	**-2.76**	**0.01**
4	Instantaneous rate of increase (r_i)	**-3.06**	**0.006**
5	Intrinsic growth rate (r_m)	-1.21	0.24
6	Numbers of aphids		
	Treatment	0.88	0.37
	Time	**4.39**	**<0.001**
	Treatment: time	**-2.54**	**0.01**
7	Number of leaves infested		
	Treatment	0.94	0.34
	Time	**3.87**	**<0.001**
	Treatment: time	**-2.74**	**0.006**

Significant effects are in bold.

2.2.3.3.4 Instantaneous rate of increase (ri)

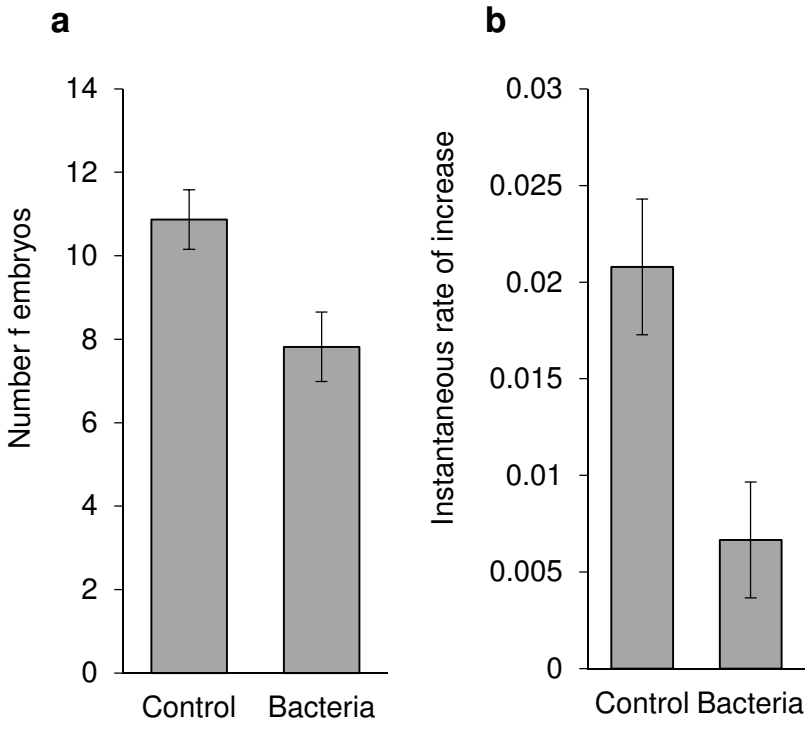
The bacterial treatment showed significant reduction in instantaneous rate of increase (Fig. 3b), which reflected the potential of bacterial addition to calabrese in effective reduction of *B. brassicae* population.

2.2.3.3.5 Intrinsic growth rate (r_m)

The average intrinsic rates were higher on untreated plants as compared to the bacterial treated ones, however, these were not statistically different. The relatively

lower intrinsic growth rates can be attributed to significant reduction in number of embryos in treated plants, typically during initial period.

Figure 3 Mean number of embryos and instantaneous rate of increase of B. brassicae on control and treated plants



2.2.3.3.6 Number of aphids

The number of adults and nymphs measured after every 5 days interval showed considerable variation as time progressed (Fig. 4a). An average aphid count on untreated plants increased periodically over the treated plants. The GLM procedure showed no significant individual effect of bacterial treatment. However, time and interaction terms were significant showing that significant numeric variation occurred in a last few counts. The reductions in number of *B. brassicae* embryos, instantaneous rate of increase, and to some extent, intrinsic rate of increase contributed to reduction in final aphid count.

2.2.3.3.7 Number of leaves infested

The average number of leaves infested were higher in untreated plants as compared to treated ones. This number increased swiftly in untreated plants and gradually in treated ones and followed the same pattern as number of aphids (Fig. 4b). The GLM analyses also showed the similar pattern between these two parameters.

The significant time and treatment: time interactions showed the negative effects of treatments over the experimental duration. A significant variation in number of leaves infested can be attributed to a major decline in aphid population on bacterially treated plants, especially after the sixth set of observations. This suggested that the bacterial treatment was effective, to certain extent, in reducing number of leaves infested.

These results were similar to an earlier study by Pineda *et al.* (2012), in which *Arabidopsis* root colonization with *P. fluorescens* WCS417r showed no effects on weight and intrinsic growth rate of *B. brassicae*. In the present study, however, *Bacillus* spp. mixture showed inter-generational consequences on *B. brassicae* colonization, which included a major drop in number of embryos, aphids and leaves infested, and instantaneous rate of increase. This is likely due to the cumulative effects of induction of systemic resistance, changes in secondary metabolite profiles and reduction in the quality of food allocated, which directly affects the fertility of mother aphids and progenies (Nevo & Coll, 2001; Jahn *et al.*, 2005).

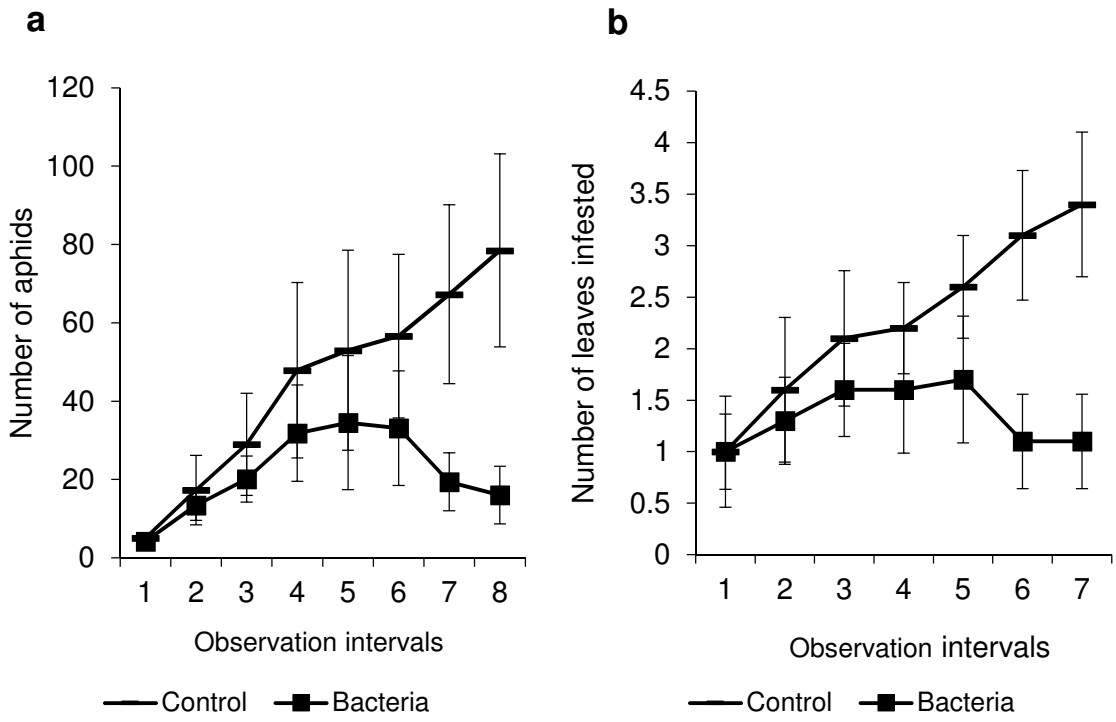
The ultimate effects produced by bacteria on *B. brassicae* were relatively slow in the beginning and subsequently developed gradually over the experimental duration.

Despite the obvious negative effects of *Bacillus* spp. mixture on *B. brassicae*, further studies are needed to explain the effects of individual bacterial species on the *B. brassicae* life history traits. The intrinsic growth rate measured from effective fecundity may have been misleading as the number of embryos were measured on the first day of adulthood, just from three adults, and not during the entire period equivalent to pre-reproductive period.

Conclusions

We chose *B. brassicae* as a model insect in the subsequent studies because (i) unlike *Mamestra*, the above experiment showed promising negative effects on this insect, (ii) the effects of PGP *Bacillus* on this important specialist feeder are unexplored (ii) *B. brassicae* is easy to culture in laboratory conditions (Singh *et al.*, 1994), (iii) intergenerational consequences on this pest can be easily studied (Satar *et al.*, 2005), (iv) the role of glucosinolates against *B. brassicae* is well documented (Newton *et al.*, 2009), (v) this pest is ideally suited to carry out field trials because of natural colonization in the field, cosmopolitan pest status and a range of natural enemies (Duchovskienė *et al.*, 2010).

Figure 4 Changes in (a) aphid and (b) leaves infested in untreated and treated calabrese plants



Thus, continuing with the present plant-insect system, we carried out further experiments to explore the effects of individual and mixed *Bacillus* species on (i) *B. brassicae* life history traits, with alternate system of measurement of effective fecundity (ii) calabrese vegetative and reproductive growth parameters (iii) population dynamics of *B. brassicae* and its important natural enemies, in different environmental conditions (temperate: UK and tropical: India) (iv) changes in calabrese glucosinolate profiles and (v) endophytic bacterial community using high-throughput 16S rRNA sequencing. These experiments constitute the remainder of this thesis.

Chapter 3

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Contents

Chapter 4

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Contents

Chapter 5

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Chapter 6

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Contents

Chapter 7

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Contents

Chapter 8

General Discussion

Through a series of laboratory and field experiments, the effects of plant growth promoting rhizobacteria on the endorhizal bacterial community, plant growth, plant secondary metabolites and plant-insect-natural enemy multi-trophic interactions have been explored.

8.1 Plant growth

Effects of *Bacillus* species on calabrese growth varied in the preliminary laboratory and subsequent field experiments. These effects appeared to be determined by composition and number of bacterial species in the inocula. In the preliminary laboratory experiments, a five species mixture of *Bacillus* (*B. subtilis, B. laterosporus, B. licheniformis, B. megaterium,* and *B. pumilus*) was used. The mixture did not show any significant effects on plant growth, possibly because of potential antagonistic activities between microbial species or due to a lack of careful tailoring of *Bacillus* species in inoculants to those in the John Innes No. 3 compost as the microbial community composition of the compost was unknown. Furthermore, the addition of a non-adapted mixed inocula may also have disrupted the beneficial endorhizal microbes as specified in earlier studies (Conn & Franco, 2004; Roger *et al.*, 2013). Conversely, the three species mixture of *Bacillus* proved to be the most effective in promoting plant growth as these significantly increased reproductive growth, pod, vegetative and total biomass and root weight ratio in the field experiment in UK and vegetative, root, and total biomass in the field experiment in India. These beneficial effects could possibly be attributed to the presence of added species the rhizosphere and minimal disruption of the indigenous microbial community by introduced species, as suggested by Conn & Franco (2004). The discrepancies in the results in preliminary lab experiments and field studies (UK and India) could also be due to interplay of one or more environmental factors that varied in both studies. These include (i) soil microbial community; composition, diversity and abundance, (ii) physical and chemical properties of soil e.g. texture, structure, nutrient status, pH, (iii) temperature and light; constant in the lab conditions and variable in the field, and (iv) precipitation; influence on insect population dynamics in the field.

As suggested by the earlier studies (Arkhipova *et al.*, 2005; Idris *et al.*, 2007; Adesemoye & Kloepper, 2009), the *Bacillus* spp.-mediated increase in nutrient availability and synthesis of plant growth hormones is likely to have contributed towards increase in the onset of calabrese reproductive maturity and biomass in temperate as well as tropical conditions. Despite the variation in the magnitude of plant growth promotion when three species were applied individually, the results from both field studies showed similar trends. *Bacillus cereus* was found to be the least effective of all bacterial treatments in promoting plant growth. This bacterium accelerated calabrese maturity over control plants in UK, however, it did not have any significant effect on plant biomass in both field studies. *Bacillus subtilis* increased the onset of reproductive maturity and pod biomass in UK, and vegetative and total biomass in India. *Bacillus amyloliquefaciens* increased the reproductive growth and vegetative biomass in UK and vegetative, root and total biomass in India. These results show similarity with a number of earlier studies highlighting the plant growth promoting potential of these *Bacillus* species in a variety of crops [e.g. saffron (Sharaf-Eldin *et al.*, 2008), lettuce (Chowdhury *et al.*, 2013), tobacco (Dutta *et al.*, 2013) and soybean (Osburn *et al.*, 1995)].

8.2 Plant secondary metabolites

A significant increase in 4-methoxyglucobrassicin levels in *B. cereus*- and *B. subtilis*-treated calabrese plants, whereas a significant decrease in neoglucobrassicin levels in the mixed treated plants was recorded over the untreated ones. Earlier studies suggest that both these indolic glucosinolates play important roles against herbivory by chewing (van Dam & Oomen, 2008) and phloem-feeding (Kusnierczyk *et al.*, 2008) insects. However, no significant correlation between any of the glucosinolates was found with aphid infestation and natural enemy levels. This is possibly due to differences in feeding styles as phloem feeders do not cause mechanical wounding which could have failed to trigger glucosinolate-associated induced defences in calabrese. These results are in harmony with the earlier study by Brock *et al.* (2013) that showed changes in *Arabidopsis* foliar aliphatic glucosinolates and in contrast with Schreiner *et al.* (2009) that showed no changes in broccoli glucosinolate profiles in response to *E. radicincitans* colonization. Thus, the effects of rhizobacteria on glucosinolates appear to be specific to rhizobacterial and plant species.

8.3 Insect bioassays

The mixed *Bacillus* species inoculant used in the preliminary experiments did not have any significant effect on *M. brassicae* growth parameters, most likely due to lack of induced systemic resistance in excised leaves, feeding of leaves from plants treated with bacteria for a short span, and influence of differences in weights of larvae prior to their use in a bioassay. This initial difference influenced the overall gain in larval weight, and relative growth and consumption rates of *M. brassicae*. On the contrary, the same inoculant significantly suppressed the *B. brassicae* number of embryos, instantaneous rate, and number of aphids and leaves infested. These differences in the effects on herbivores were thought to be due to different feeding style (e.g. Van Oosten *et al.*, 2008), insect species (e.g.Pineda *et al.*, 2012), variation in induction of systemic resistance (Van Oosten *et al.*, 2008), decreased quality of food (Cole, 1997) and duration of feeding. For instance, the effects of *Bacillus* inoculant on *B. brassicae* life history traits and overall development were more prominent in the last few sets of observations. The prolonged duration of feeding of *B. brassicae* (40 days) on *Bacillus* treated plants may have negatively affected its growth and development. The similar effects were not observed on *M. brassicae*, possibly due to short feeding duration (7 days). Furthermore, the differences in feeding styles and degree of specialism between these two insects likely triggered different plant signalling pathways and differentially altered plant secondary defence compounds, as shown by earlier studies (Cole, 1997; Van Oosten *et al.*, 2008; Pineda *et al.*, 2012)

The field experiment in the UK showed that both individual and mixed *Bacillus* spp. treatments significantly reduced *B. brassicae* field infestations. However, all individual species, in varying magnitudes, were more effective in suppressing this pest than the mixed treatment. The earlier studies on generalist phloem feeders showed similar results, with single bacterial species being more effective (Valenzuela-Soto *et al.*, 2010) than multiple species (Boutard-Hunt *et al.*, 2009). The field experiment in India showed contrasting results, in that the same local species of *Bacillus* encouraged the infestation of *B. brassicae*, together with *M. persicae* and *L. erysimi* on broccoli plants. In both field experiments, the effects of *Bacillus* species were achieved through both seed inoculation as well as one additional field application through soil drenching. The 16S rRNA partial sequencing confirmed the successful colonization of all bacteria in the field experiment in UK. Despite this

confirmation, one additional application was done in India and UK experiments to increase the magnitude of plant growth, as reported by an earlier relevant study (Nandakumar *et al.*, 2001).

The average number of *B. brassicae* on control plants during peak infestation time points were much higher on control plants in the UK (>100 aphids plant^{-1}, Week 3) than in India (>60 aphids plant^{-1}, Week 2). Conversely, the average number were far lower on treated plants in the UK (<40 aphids plant^{-1}, Week 3), except mixed treated plants (80 aphids plant^{-1}, week 3) than in India (>50 aphids plant^{-1}, Week 2, 3), except *B. cereus* treated plants (<45 aphids plant^{-1}, Week 3). The interspecies competition between three aphids, as shown by Muller and Godfray (1997), may have reduced overall numbers on control plants in the experiment in India. Conversely, the increase in numbers on treated plants could be due to maximum availability of food through highly vigorous plants, combined with congenial temperature (25-30ºC) for aphid growth (Satar *et al.*, 2005) during the initial period. The increase in temperature to 30-35 ºC in later stages did, however, reduce the overall aphid numbers, similar to that found by (DeLoach, 1974). Thus, a possible trade-off between *Bacillus* mediated plant health promotion (biomass increase) and fitness benefits (aphid resistance) was observed in the experiment in the tropics.

Earlier studies showed that the soil carbon and nitrogen (Dijkstra *et al.*, 2006), plant and soil types (Garbeva *et al.*, 2004), temperature (Zogg *et al.*, 1997), agricultural practices and season (Bossio *et al.*, 1998) play important roles in shaping structure and function of soil microbial communities. Therefore, numerous extraneous factors may have cumulatively influenced the effects of *Bacillus* spp. addition on insect pest infestation in temperate and tropical environments. The soil bacterial diversity and richness are primarily governed by soil variables, ecosystem type and environmental factors (Papke & Ward, 2004; Fierer & Jackson, 2006). For example, different soil types (slightly acidic loamy in UK and black cotton in India) and root zone temperatures (higher in India), in the present study, may have influenced the activity of different bacterial species in rhizosphere (Zhang *et al.*, 1997). The different host plant cultivars (Green sprouting in UK and Imperial in India) may also have responded differentially to rhizobacterial strains, as shown by a similar study (Egamberdieva, 2010) with *Pseudomonas* rhizobacteria. The variations in the diversity, abundance and composition of indigenous soil and endorhizal communities

largely influence the efficacy of seed inoculants (Duineveld *et al.*, 1998). Thus, such variations in native bacterial communities may have contributed to differential effects of *Bacillus* in both studies.

8.4 Natural enemies

The responses of natural enemies (*viz. D. rapae* and syrphid flies) in both studies varied with aphid population density. Thus, *Bacillus* spp. did not have a direct influence on natural enemy population dynamics, which was reported otherwise in a few earlier studies (Commare *et al.*, 2002; Saravanakumar *et al.*, 2008). Instead, these results showed similarity with earlier work by Boutard-Hunt *et al.* (2009), in which *M. persicae* natural enemies (from several families; Anthocoridae, Chrysopidae, Coccinellidae, Hemerobiidae, Cecidomyidae, and Syrphidae) on PGP (*Paenobacillus* and *Bacillus*)-treated bell pepper, *Capsicum annuum* L., responded in a density dependent manner. In the field experiment in the UK, the rate of parasitization of *D. rapae* was higher in observation Weeks 3-5, in response to highest population of *B. brassicae* in Week 2-3, whereas, in India this rate was higher in Week 3-4 and varied in response to the population densities of *B. brassicae* as well as *M. persicae* in Week 2-3. Although *L. erysimi* was abundant in Week 2, its population density was much lower than these two aphids. The magnitude of *D. rapae* parasitization varied in both experiments; the overall rates of parasitization were higher in UK (10 mummified aphids on control plants in week 4) than in India (4.5 mummified aphids on *B. subtilis* treated plant in week 4). The order of preference of parasitization amongst the three available hosts was *B. brassicae*, *M. persicae* and *L. erysimi*, and was principally determined by host choice and population densities. Earlier studies on *D. rapae* host choice reported the similar results, in that *B. brassicae* was reported as a primary host (Ayal, 1987), and *D. rapae* was reported to parasitize *B. brassicae* preferentially over *M. persicae* and *L. erysimi* (Pike *et al.*, 1999).

In both studies (UK and India), syrphid flies were the predominant predators, however, their population densities were far lower than *D. rapae*. In UK, the highest average count was 0.6 fly larvae plant^{-1} on untreated plants in week 4, whereas in India the highest count was 1.5 fly larvae plant^{-1} on *B. subtilis* treated plants. Unlike reported in an earlier study (Nelson *et al.*, 2012), syrphid flies were not effective in regulating *B. brassicae* and other aphid populations, possibly due to differences in (i)

site-specific variation in syrphid species composition, as shown by previous survey (Smith & Chaney, 2007), (ii) consumption rates; inherent variation in consumption rates among different syrphids (Hopper *et al.*, 2011) and (iii) aphid and syrphid population densities.

The environmental factors such as temperature and precipitation also played a key role in governing natural enemy population dynamics. For instance, the extensive rain showers at the end of observation Week 4 and during observation Weeks 5-6 in the field experiment in the UK severely reduced mummified aphid and syrphid fly counts in the last two observation Weeks (5-6). The influence of precipitation on *B. brassicae* and *D. rapae* population dynamics was reported in an earlier study (Hafez, 1961). In India, syrphid fly populations declined in Weeks 3-4 as a result of increase in average daily temperature to 30-35ºC in later-May. Similar results were observed in the recent study by Romo & Tylianakis (2013) that showed the reduced parasitoid effectiveness in response to increased temperature. However, no such effect was observed in mummified aphid counts possibly due to availability of ample host aphid populations and adaption to higher temperature (Rakhshani *et al.*, 2008).

8.5 *Bacillus* spp. inoculation and bacterial endophytes

Successful field inoculation is a necessary attribute for bacteria to exert plant growth promotion (Lugtenberg & Kamilova, 2009). In the first field experiment conducted in UK, the field inoculation of each *Bacillus* species was confirmed (95-99% homology) using the partial sequencing of 16S rRNA amplicons two weeks after sowing. In the second field experiment in UK, the increased relative abundance of *Bacillus* genus in *B. cereus*, *B. subtilis* and mixed treated plants, compared to controls, suggested successful colonization of these species on to broccoli.

The endophytic bacterial community analysed through metagenomic sequencing showed that the bacterial phyla; *Proteobacteria*, *Firmicutes* and *Bacteriodetes* were most abundant across all samples. Kröber *et al.* (2014) also reported the prevalence of *Proteobacteria* and *Bacteriodetes* in lettuce rhizosphere. The higher Shannon (*H'*) and E*var* indices in *B. subtilis*, *B. amyloliquefaciens* and mixed treated plants, compared to the control, were a result of increased overall evenness in treated plants. The relative abundances of different taxa varied in each treatment group, possibly as a cumulative effects of bacterial species specificity (Kröber *et al.*, 2014), numerous

interactions in rhizo- and endospheres (Schmidt *et al*., 2014), and external factors such as root metabolites, plant growth stage and the native rhizosphere microbial community (Marschner *et al.*, 2001; Baudoin *et al.*, 2002). Overall, *Bacillus* species influenced the diversity, evenness and abundance of the endophytic bacterial community to varying extents, as revealed by the recent relevant studies (Kröber *et al.*, 2014; Schmidt *et al.*, 2014).

A large variation in soil microbiota in controlled and field conditions lead to discrepancies associated with the use of microbial inoculants in these two conditions. Microbial inoculants often show promising results in controlled conditions, but produce inconsistent results in the field (Herrmann & Lesueur, 2013), probably because the field soil is more heterogeneous and dynamic in nature. The majority of microbial inoculants used commercially in agriculture contain a mixture of multiple microbial species (Mayer *et al.*, 2010). Even though the microbial species in inoculants are compatible in a formulation, they are more likely to compete with indigenous rhizo- and endo-sphere microflora for roots exudates and space and withstand abiotic conditions (Adesemoye & Kloepper, 2009). As a result of this competition, a decline in the populations of introduced species occur shortly after application (Elsas *et al.*, 1986). The present study showed the similar results, in that the relative abundances of introduced *Bacillus* species were lower compared to those of the other 10 most abundant bacterial genera. Thus, collective biotic and abiotic interactions in the rhizo- and endo-sphere possibly led to variable effects of *Bacillus* species on diversity, evenness and relative abundance of indigenous bacterial species.

8.6 Microbial inoculants

To obtain the intended plant response, the added bacterial species have to build up a sufficiently large population in the rhizosphere, and have a high degree of colonization competitiveness (Bashan, 1998). To attain this, the early seed inoculation coupled with optimised repetitive field applications with a stable carrier based formulation would be of great benefit. Since the indigenous microbial populations vary with plant species and soil (Marschner *et al.*, 2001), a tailoring of microbial species in inoculants according to crop and soil would maximise the plant growth promotion effects in the field conditions. This will also help alleviate the key issue of inconsistency in the results of microbial inoculants use in different crops, soils and

geographical conditions. Although the feasibility of development of 'tailored' inoculants is low, it could be increased through improved understanding of soil and plant associated microbiota via increasingly cheaper metagenomic approaches.

The excessive use of agrochemicals (pesticides and fertilizers) leads to detrimental effects on the environment (Horrigan *et al.*, 2002) as well as the soil microbial community (Marschner *et al.*, 2003). When using the commercial inoculants, the use of chemical pesticides and fertilizers should be carefully considered. This will cause minimal disruption to microbiota, while providing a more suitable microenvironment for added species. The use of 'tailored' microbial inoculants to reduce the agrochemical use and increase the agrochemical use efficiency would be significantly important. For integrated use to be achieved, compatibility testing between inoculants and agrochemicals is recommended to maintain the optimum field efficacy of inoculants. The use of microbial inoculants is becoming increasingly popular, with 10% annual increase every year (Berg, 2009). The increased use of tailored microbial species and carrier technology will not only help reduce the fertilizer application rates (Adesemoye & Kloepper, 2009), but also eliminate the need for their use in coming years. Integrated pest and nutrient management systems, including sustainable components such as tailored microbial inoculants would be a promising and sustainable resource to increase agricultural productivity and protection of the environment.

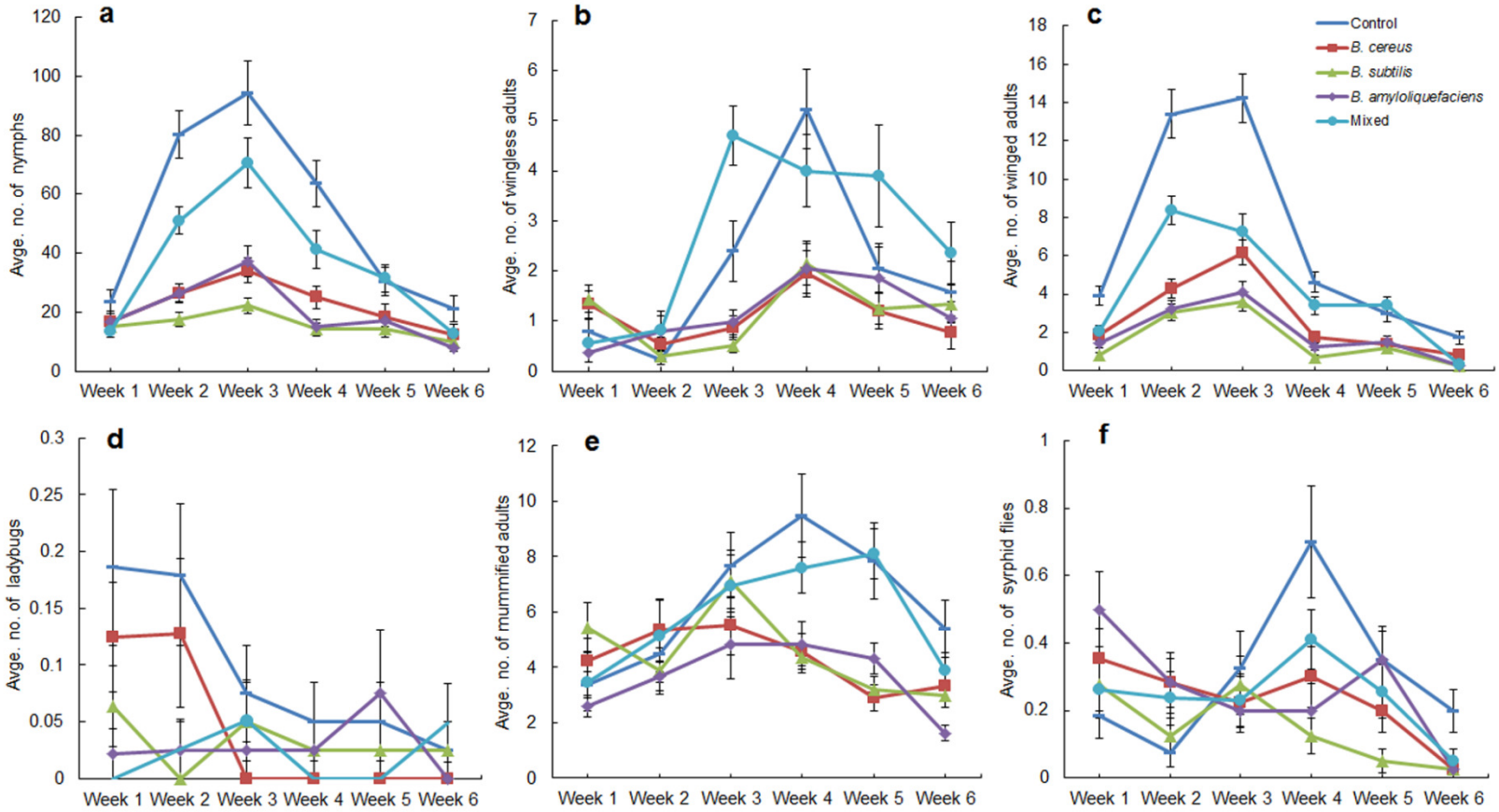
8.7 Concluding remarks

The preliminary experiments showed that the commercial inoculant had variable effects on insect performance and was not as effective as native species in promoting plant growth. The magnitude of plant growth promotion varied between *Bacillus* species. In both field studies, *B. subtilis*, *B. amyloliquefaciens* and mixed treatments increased plant biomass and changed calabrese foliar indole glucosinolate levels. All treatments showed contrasting results on insect herbivores, in which they significantly reduced pest infestation in a temperate climate, but encouraged it in the tropics. The natural enemy population dynamics was governed by host population densities in all experiments. Furthermore, all treatments altered the life history traits of *B. brassicae*, and reduced endophytic bacterial diversity and abundance to varying extents. Thus, it is concluded that PGP *Bacillus* accelerate calabrese maturity, increase biomass, alter

glucosinolate levels and bacterial diversity, evenness and abundance. Furthermore, *Bacillus* spp. shape above ground interactions via significant changes in aphid field infestation levels that vary in temperate and tropical climatic conditions, and indirectly extend up to the natural enemy level in a density dependent manner. Thus, *Bacillus* species have huge plant growth promoting potential, which could be exploited for their commercial use in agriculture by careful tailoring of species in inocula to those in the field soil. Altogether, *Bacillus* mediated increased nutrient availability, phyto-stimulation and pest suppression in the field make them the ideal candidate for integrated pest and nutrient management programmes.

Appendices

Fig. S1 Cabbage aphid and natural enemy population dynamics. (a) nymphs (b) wingless adults (c) winged adults (d) ladybugs (e) mummified aphids and (f) syrphid flies



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