Bio-accumulation and non-target effects of GM derived *Bt* endotoxin in the soil

by

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Summary

Plants may be genetically modified to express an entomopathogenic protein from the bacterium *Bacillus thuringiensis* (Berliner) (*Bt* plants). *Bt* plants are known to affect some above-ground invertebrates, with significant effects on species closely related to target invertebrates and on their natural enemies. *Bt* proteins may enter the soil through root exudates and decomposition of plant material. This study aimed to analyse the effects of *Bt* broccoli (*Brassica oleracea* L. var. *italica* Plenk) on six soil-dwelling invertebrates. No significant differences were detected in nematode (*Panagrellus redivivus* L.) populations living in compost in which *Bt* and non-*Bt* broccoli had grown. The other species were introduced to combinations of *Bt* and non-*Bt* leaves, and compost in which *Bt* and non-*Bt* plants had grown. No differences were detected in Collembola (*Folsomia candida* Willem) populations, but significantly more young woodlice (*Porcellio scaber* Latrielle) survived, and weighed more, in the *Bt* than the non-*Bt* treatments. Slugs (*Deroceras reticulatum* Müller) weighed more in the presence of *Bt* proteins. A higher percentage of earthworm (*Lumbricus terrestris* L.) cocoons hatched in *Bt* than non-*Bt* treatments. In contrast, at a third trophic level, fewer predatory beetles (*Nebria brevicollis* Fabricius) survived when feeding on slugs that had fed on *Bt* leaves than on those fed on non-*Bt* leaves. Leaves from *Bt* plants affected more parameters than compost in which *Bt* plants had grown. Attempts were made to use molecular techniques to analyse the effects of *Bt* broccoli on soil micro-organisms. Individual *Bt* broccoli plants expressed different concentrations of *Bt* protein. The *Bt* plant's control of three Lepidoptera species was tested and only one species was susceptible. These results show that non-target invertebrates, including pest species, can be affected by *Bt* broccoli, sometimes beneficially, and underlines the need for prior testing of GM crops on a range of non-target species.

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1 Introduction

1.1 Overview

Eleven percent of the world's terrestrial surface is used for crop production to feed an ever increasing human population. During the past 50 years 20% of this land has been destroyed by, among other factors, desertification, salination, urban sprawl and erosion (Raven, 2003). The use of agricultural machinery has frequently led to the removal of hedgerows and orchards, which are important areas of habitat that harbour natural predators of pests. Loss of hedgerows and use of heavy machinery has lead to subsequent extensive soil erosion and a decrease in soil fertility. The excessive application of chemical pesticides and herbicides has caused widespread loss of biodiversity within crop systems and pollution of waterways (FAO, 2002). Wisniewski *et al.* (2002) suggest it is time for a further Green Revolution of more sustainable agricultural practices (the first being the development of high yield crops in the 1960s). Several suggestions have been made as to how this may be achieved, including organic farming, integrated pest management (IPM) and the use of genetically modified (GM) crops. It will probably be a combination of these approaches that will eventually provide the required outcome of increased crop productivity without causing further damage to the environment.

IPM is the combination of microbial control, the use of parasites, predators and behaviour-modifying chemicals (e.g. pheromones), with pest-resistant crop varieties (both GM and conventially bred) and agricultural practices including crop rotation, intercropping and field margin management (Dent, 2000). IPM does, however, advocate the use of narrow spectrum and low persistence pesticides, albeit at lower levels and only after pest population monitoring to determine whether crop damage thresholds have been passed. Many growers and consumers object to pesticide application using the negative environmental impacts associated with their use as reason for their objection. Organic farming, on the other hand, involves significantly fewer synthetic chemical inputs into, and onto, the land thus maintaining soil fertility and reducing pollution from pesticides and herbicides. Another method more recently employed for reducing pesticide and herbicide application, is the production of plants genetically modified for pest-resistance.

Much interest has been raised in the possible positive and negative effects on the environment of growing GM plants. Direct effects of growing GM plants include interaction with other species, invasiveness and gene flow into other related plants (Dale *et al.*, 2002). Indirect impacts, caused by changes in agricultural practices, may reduce or increase the efficiency of the control of the pests or weeds that the GM plant is helping to manage, and there may be effects on wildlife, soil and water (Dale *et al.*, 2002).

Herbicide-tolerant plants account for 74% of the GM crops grown worldwide (James, 2000); these plants are tolerant to specific herbicides allowing less frequent spraying of herbicides. Herbicide-tolerant plants also require less tilling of the land (a method of weed control) so help maintain and increase soil fertility and structure (Ammann, 2005). There have been many small scale studies, but more recently, and perhaps more thoroughly, the farm scale evaluations in the UK (The Royal Society, 2003) attempted to answer many of the concerns regarding the use of GM crops. These three-year experiments concluded that, in general, although there were differences in the types and numbers of organisms found in the fields of herbicide-tolerant crops and those containing non-GM plants, the changes could mostly be attributed to differences in crop management regimes rather than to the inherent nature of the herbicide-tolerant plants. There were, however, differences between the effects of the four different GM crops (maize (*Zea mays* L.), beet (*Beta vulgaris* L.) and two varieties of oilseed rape (*Brassica napus* L.)) investigated.

Pest-resistant crops are grown in many countries across the world, although at lower levels than herbicide-tolerant ones (19% of the total GM planting area; Birch & Wheatley, 2005). The majority of these crops produce proteins found in different strains of the bacterium *Bacillus thuringiensis* (Berliner), although some produce lectins, for example from snowdrops (*Galanthus nivalis* L.), or proteinase inhibitors, for example from cowpeas (*Vigna unguiculata* L.) (reviewed in Haq *et al.*, 2004). These compounds are toxic to insects and this has led to concern about the more widespread effects of these GM plants on non-pest insects and thus biodiversity. In this chapter I discuss various aspects related to *Bacillus thuringiensis*, including the reasons its products have been used in biopesticides and, more recently, its genes engineered into GM plants, before discussing the effects of these GM plants on invertebrates and the need for more thorough studies, comparable to the farm scale evaluations for herbicide-tolerant plants.

1.2 *Bacillus thuringiensis*

1.2.1 Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive, spore-forming bacterium with entomopathogenic properties (Höfte & Whiteley, 1989). This action was first recorded in the 19th Century by the Japanese in silk worms (Dent, 2000), then again by Berliner in the 20th Century in diseased flour moth larvae (Van Frankenhuyzen, 1993). It is distinguished from the closely related *B. anthracis* (Ames) and *B. cereus* (Frankland & Frankland) by its ability to produce a crystalline inclusion during sporulation (Whiteley & Schnepf, 1986). *Bt* is found in soils globally but its ecological role remains speculative; the crystalline inclusion confers *Bt* with insecticidal properties against many herbivorous invertebrates so it may be that it is primarily an entomopathogen with the selective advantage of forming a spore, or that it forms symbiotic relationships with plants (Meadows, 1993).

1.2.2 *Bt* as an entomopathogen

Insects ingest the crystalline inclusion and *Bt* spores along with plant material. The toxicity of the protein depends on the organism's capability to not only digest the crystal into its component parts but also to activate these parts. When the organism ingests the crystal the high pH (pH > 9) of the gut dissolves the crystal into its component proteins (protoxins) and gut proteases then cleave the protoxins to form the active toxins. The active toxins have two main regions, the plate and the helix. The plate region binds to specific receptors on the organism's gut epithelial cells, whilst the helix section ruptures the gut cell membranes (Grochulski *et al.*, 1995). This rupturing results in pores developing in the cell membranes through which the epithelial cell contents leak into the gut causing paralysis, cessation of feeding and eventual starvation.

The presence of cell contents in the haemocoel creates an ideal nutrient-rich and high pH environment for spore germination and *Bt* replication (Aronson *et al.*, 1986). The bacterium's other entomopathogenic factors (α-exotoxin, β-exotoxin and phospholipase C) give rise to septicemia (Whitely & Schnepf, 1986). The two-pronged action of starvation and septicemia leads to the prompt death of the organism. The three conditions, the need for a high pH to dissolve the crystal, the appropriate enzymes to convert the protoxin to the toxin,

and the presence of correct gut receptors for the toxin to realise activity, result in a very high specificity of each *Bt* toxin.

There are a number of *Bt* strains attacking a variety of insects across several orders. This variety is related to the combination of *Bt* proteins that the bacteria produce in their crystals. The contrast of the high specificity of the toxin and the wide range of insects affected by it (the host range phenomenon) can be explained by the different ability of insect taxa to process each different *Bt* protein, as well as differences in their gut for processing and binding each toxin (Whitely & Schnepf, 1986).

Originally three *Bt* strains were identified with activity against different orders; *Bt* var. *kurstaki* was Lepidoptera active, *Bt* var. *israelensis* Diptera active and *Bt* var. *tenebrionsis* active against Coleoptera. As interest in *Bt* as a biopesticide grew, more strains were discovered including some that showed nematicidal activity (Mozgovaya *et al.*, 2002; Wei *et al.*, 2003); consequently more crystal proteins were discovered. The initial *Bt* strain classification proved inadequate as some strains were showing activity across more than one order; in response, a nomenclature for the crystal proteins was proposed. The crystal proteins were separated into four groups by structure and insect activity (Table 1.1), and a structurally different protein (Cyt) was also identified exhibiting cytolytic activity (Höfte & Whiteley, 1989). A new proposal by Crickmore *et al.* (1998) for the nomenclature of the crystal proteins was based on the phylogenetics of their gene sequences using a series of numbers and letters (e.g. Cry1Aa). There is some overlap with the original nomenclature, for example, the majority of Cry1 proteins are toxic to Lepidoptera.

Table 1.1 Classification of *Bt* crystalline inclusions into four broad groups by their size, shape and spectrum of activity.

Classification	Crystal Shape	Protein size (kDa)	Activity
Cry1	Bipyramidal	130-138	Lepidoptera
Cry2	Cuboidal	69-71	Lepidoptera and Diptera
Cry3	Flat/irregular	73-74	Coleoptera
Cry4	Bipyramidal	73-134	Diptera

(data from Höfte & Whiteley, 1989)

The genes for the Cry proteins are found within plasmids that are self-transmissible through a conjugation mechanism (Thomas *et al.*, 2002). High levels of recombination probably account for the movement of genes and the formation of many combinations of Cry proteins

within the crystalline inclusion. The acquisition of these Cry proteins explains how *Bt* can colonise such a wide range of insects (Thomas *et al.*, 2002).

1.3 *Bt* as a biopesticide

1.3.1 Introduction

Bt has been used as a biopesticide since the 1930s when it was first produced in France as "Sporeine" (Van Frankenhuyzen, 1993). More recently, there has been a resurgence in *Bt* biopesticide use associated with the increase in organic farming and IPM, where natural control methods are favoured over broad-spectrum chemical insecticides. *Bt* production is a £150 million p.a. business and *Bt* has been used to control pests in fruit, vegetables, soybeans (*Glycine max* L.) and maize, as well as being used in forestry and disease vector management programs (Gelernter & Lomer, 2000). Dipel® is produced by Valent Biosciences (Libertyville, USA); it is the world's main commercial *Bt* product and contains *Bt* var *kurstaki* strain HD-1. The main components of Dipel® are the *Bt* spore and a crystalline inclusion comprising five Cry proteins – Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab. Dipel® is sold by Valent Biosciences specifically for its activity against Lepidoptera pests, although the presence of Cry2 proteins means it is also active against Diptera. The Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), and *Tenebrio* spp. (Coleoptera: Tenebrionidae) larvae are two major pests of potatoes (*Solanum tuberosum* L.) and grain stores, respectively. *Bt* var. *kurstaki* is not effective against either pest species; *Bt* var. *tenebrionsis* can be used against these two pests as it contains Cry proteins specific to Coleoptera.

Other than extensive use against agricultural pests *Bt* has also been used to attack disease vectors. In West Africa, mosquitoes (Diptera: Culicidae) and blackflies (Diptera: Simuliidae) are vectors of the human disease river blindness (Becker & Margalit, 1993). More than 100 strains of *Bt* var *israelensis* have been found locally, some have been safety-tested on representative aquatic organisms. The only organisms affected were the two mosquito and blackfly families that are vectors of the disease. As a result of this testing, *Bt* strain H14 was rapidly exploited and put to use in the field; it reduced river blindness incidence quickly and effectively. No reported cases of resistance in the disease-carrying Diptera were made,

probably due to the complex nature of the crystalline inclusion of strain H14 which has four proteins acting synergistically (Becker & Margalit, 1993).

1.3.2 Advantages and disadvantages of *Bt* as a biopesticide

There are a number of reasons why *Bt* is an effective biopesticide. Firstly, it is a fermentable aerobic bacterium which can be produced using batch fermentation techniques (Dent, 2000; Burges & Jones, 1998). This makes the scale-up from laboratory to industrial production relatively cheap and easy. Secondly, it has high specificity ensuring a limited host range and a reputed lack of non-target organism effects. Thirdly, it is a spore-forming bacterium making it less susceptible to environmental pressures, and thus has a more stable storage and shelf-life than other living pathogens (Gelernter & Lomer, 2000). Finally, the cessation of feeding is immediate so crop damage is limited and yield within a field will remain high.

Bt has poor environmental persistence (Vilas-Bôas *et al.*, 2000). In rainfall the product is washed off leaves (Burges & Jones, 1998) and in sunlight it is quickly inactivated; the half life of the Cry proteins in full sunlight is about 24 h (Burges & Jones, 1998). Formulations have been altered (e.g. addition of stickers and sunscreens) to try and improve persistence but *Bt* biopesticides do not generate sustainable control and must be applied frequently (Gelernter & Lomer, 2000).

As the biopesticide is applied as a spray the amount actually applied to a crop can be variable (Burges & Jones, 1998) and there is a risk of exposure to workers through inhalation and general misuse (Betz *et al.*, 2000). Risk of exposure may not, however, translate to a hazard due to the specific nature of the *Bt* protein. Resistance to *Bt* var. *kurstaki* in *Plutella xylostella L.* (Lepidoptera: Plutellidae) has been reported in Indonesia (Tabashnik *et al.*, 1990), and also to *Bt* var. *aizawi* (Liu *et al.*, 1996). In both examples *Bt* application has been intensive and frequent, this increases the likelihood of resistance spreading rapidly through a population as the insects are under continuous exposure to the toxins. If the exposure is not continuous then the associated fitness costs of resistance would result in it being bred out of the population (Shelton *et al.*, 2002).

Biotechnology has been used to overcome some of these disadvantages (Gelernter & Schwab, 1993). Two examples of such developments are the expression of *Bt* genes in aquatic cyanobacteria and in *Bradyrhizobium* spp. Normally *Bt* biopesticide sprays will result in the

spores and crystals settling to the bottom of pools of water, however, if the proteins are engineered into cyanobacteria that inhabit the water-air interface then the protein is available to surface-active pests such as mosquitoes (Gelernter & Schwab, 1993). The *Bradyrhizobium* spp. colonise root nodules of pigeon pea (*Cajanus cajun* L.) plants. Expressing the *Bt* protein will protect the plant roots from attack by larvae of *Rivellia angulata* (Hendel) (Diptera: Platystomatidae) (Nambiar *et al.*, 1990). The method that has perhaps gained the most interest, however, is the introduction of the *Bt* Cry protein genes into plants. This approach overcomes numerous problems at once, including targeting of internal feeders, continuous supply of *Bt* proteins without repeated spraying, and protection from degradation by micro-organisms (Ely, 1993).

1.4 *Bt* transformed crops

1.4.1 Introduction

Lepidoptera are some of the most destructive pests of domestic crops; the European corn borer, *Ostrinia nubilalis* (Hübner), is one of the most economically devastating pests of maize in North America and Europe (Bourguet *et al.*, 2000). As an internal feeder it is an ideal target for *Bt* plants. Maize has been genetically engineered to express a *Bt* Cry protein to target these Lepidoptera larvae. One maize cultivar, Bollgard® (Monsanto, USA), has been available for commercial planting in the USA since 1996 (Naranjo & Ellsworth, 2002). *Bt* genes encoding Cry1 genes have also been inserted into many other crop species to provide protection against a variety of Lepidoptera larvae. Some examples include rice, *Oryza sativa* L. (e.g. Nayak *et al.*, 1997), cotton, *Gossypium hirsutum* L. (e.g. Liu *et al.*, 2005a) and several brassicas including oil seed rape (e.g. Zhao *et al.*, 2001). Loblolly pine, *Pinus taeda* (L), has also been engineered and varieties exist that are resistant to the Lepidoptera larvae of *Dendrolimus punctatus* (Walker) and *Crypyothelea formosicola* (Staud) (Tang & Tian, 2003). Maize has also been engineered to be resistant to Coleoptera larvae using Coleoptera-specific protein genes (e.g. Cry3A), as have potatoes (*Solanum tuberosum*, L.) to provide resistance to the Colorado potato beetle (e.g. Reed *et al.*, 2001). The gene inserted into the plant is modified such that the protein encoded is not the protoxin described in Section 1.2.2 but the activated toxin. It also has plant rather than bacterial promotors in order to increase expression (e.g. Perlak *et al.*, 1991).

1.4.2 Benefits of, and concerns surrounding *Bt* Crops

There are many benefits of expressing *Bt* toxins in the plant rather than their application as sprays. *Bt* plants provide continuous protection to the plant against internal feeders, such as stem borers, and limit environmental distribution so that only feeding pests are exposed (Ely, 1993). By preventing stem borers causing damage to plants, fungal spores (in particular those of *Fusarium* spp.) cannot enter the plant. Horses and pigs may feed on fungal contaminated plant material, this is a major concern as *Fusarium* spp. produce a mycotoxin (Betz *et al.*, 2000). The *Bt* toxins have greater stability when they are expressed inside the plant as they cannot be washed off as conventional *Bt* sprays may be, they are also protected from UV and microbial degradation (Ely, 1993).

The use of *Bt* crops eliminates the need for broad-spectrum pesticides allowing non-target species to flourish, including, unfortunately, non-target pest species. Bollgard® *Bt* cotton was designed to be resistant to cotton bollworms (*Helicoverpa armigera*) but did not protect the cotton plants from substantial damage caused by the closely related *Helicoverpa zea*. The remedy was to apply at least one application of insecticide (Gore *et al.*, 2003). Subsequent *Bt* cotton developments, for example Bollgard® 2, contain two *Bt* proteins and this GM plant confers resistance to both *Helicoverpa* species (Gore *et al.*, 2003). Increases in leafhopper populations have been recorded in *Bt* cotton fields in China (Men *et al.*, 2005) and there are concerns that this increase could lead to outbreaks of secondary (non-target) pests. Natural enemies could be used to suppress populations of secondary pests (Dale *et al.*, 2002) or the pests may be controlled by more selective pesticides. Spider mites (*Tetranychus* spp., Acari) on *Bt* cotton, for example, can be controlled by acaracides (Ma *et al.*, 2006) although as yet there have been no secondary pest outbreaks requiring significant use of insecticides (Romeis *et al.*, 2006). Continuous monitoring of population dynamics in *Bt* crops are important so that adjustments to the crop management can be made accordingly (Whitehouse *et al.*, 2005).

By using genetically modified crops, fewer insecticide treatments are needed (Betz *et al.*, 2000). This reduction in pesticide use reduces worker exposure (5000 deaths per annum worldwide from pesticide poisoning (Raven, 2003)) and the risk of misuse by inappropriate timing of applications (Ely, 1993). Pesticides poison 70 million birds in USA fields and billions of beneficial insects per annum (Raven, 2003); the use of *Bt* plants may preserve or even enhance populations of beneficial organisms due to the specificity of the toxin (Betz *et*

al., 2000). It is also cheaper to buy *Bt* cotton seeds than the insecticides needed to apply to conventional cotton (Dent, 2000), so the grower has lower production costs. Economic benefits are often the driving factor for adoption of GM crops by farmers and planting pest-resistant crops lead to increased yields (5 to 63%) and cost savings (up to US$ 288 ha^{-1}) (reviewed in Brookes & Barfoot, 2005). Research by Men *et al.* (2005), however, suggested that at least nine insecticides were required to suppress just the secondary non-target sucking herbivore pests on *Bt* cotton in China to levels below the damage threshold; the cost/benefit analysis of planting *Bt* cotton in China may need to be reassessed. On a world-wide scale the change in management of pest-resistant crops (including *Bt*) to reduced or no-till systems (i.e. no ploughing) has resulted in associated reduced machinery use saving 61 million kg of carbon dioxide in fuel emissions in 2004 (Brookes & Barfoot, 2005).

Several recent health scares concerning food in the UK (e.g. salmonella and bovine spongiform encephalopathy) have resulted in a large proportion of the European population losing faith in the agricultural industry. Any proposed new agricultural practices come under considerable scrutiny and GM crops are no exception. Despite the numerous benefits of *Bt* plants and the fact they are grown in many other countries, there are still some concerns in the UK about their use and their effect on the environment. Some of the questions asked include: Will insects develop resistance as they have in some cases with the biopesticide spray? Does the gene involved make a GM plant more invasive than its traditional counterparts, or does gene flow into native species cause them to become weeds? Do GM plant products persist in the environment? Will GM plants be toxic to humans and/or other species?

1.4.3 Resistance and its management

Insects have become resistant to *Bt* biopesticides where spraying has been continuous and at high levels (Tabashnik, 1994). Resistance is also a concern in *Bt* crops where the pests could, after several generations and continuous exposure, become resistant to the in-built plant pesticide. Resistance to *Bt* plants, however, has only developed in selection experiments in the laboratory (Van Rie *et al.*, 1990). A relatively recent review of resistance development to *Bt* crops (Tabashnik *et al.*, 2003) describes how *Bt* resistant insects bred in the laboratory are not necessarily resistant to *Bt* crops in the field. This does not, however, rule out resistance developing in the future. If resistance were to appear then it would be necessary to return to

broad-spectrum chemical pesticides with their adverse effects. Resistance development can be managed using pyramiding of toxins, use of synergistic chemicals, crop mosaics and rotation, ultra-high toxin doses and the use of refugia (Tabashnik, 1994).

The most popular method of preventing resistance developing is the provision of refugia (areas of non-GM crops). This approach is used extensively and it has been shown, for example, that *Plutella xylostella* are attracted equally to *Bt* and non-*Bt* plants (Kumar *et al.*, 2004). The refugia should aim to accommodate 500 *Bt* sensitive insects to every *Bt* resistant individual found in the *Bt* crop (Gould *et al.*, 2002). This ensures that *Bt*-sensitive pests are available to mate with *Bt* resistant pests, and produce *Bt* sensitive offspring (Betz *et al.*, 2000). Farmers are encouraged to plant 20% of total acreage with non-GM varieties (Dent, 2000). There is normally a choice of refugia plants available to farmers: in Australia both sprayed and unsprayed non-*Bt* cotton and three other unsprayed crops (pigeon pea, sorghum and maize) can be used alongside *Bt* cotton (Fitt, 2003). The area of refugia that is planted varies with the type of refugia crop used, for example, 100 ha of sprayed cotton are required to comply with regulations but only 10 ha of unsprayed cotton are needed (Fitt, 2003).

The success of refugia working to reduce pest resistance, however, assumes that the allele coding for resistance is recessive; the first assumption of the refugia theory. Two other main assumptions must be met (Gould *et al.*, 2002). Firstly, resistance must be rare (i.e. few homozygotes present), and secondly, resistant individuals must mate randomly, or preferentially, with susceptible insects. There is one difficulty with this latter assumption and that is the asynchronous development between *Bt* resistant and susceptible larvae which may result in random mating not taking place. For example, asynchronous development has been detected in two Lepidoptera: pink bollworm *Pectinophora gossypiella* (Saunders) (Liu *et al.*, 2001) and European corn borer *Ostrinia nubilalis* (Bourguet *et al.* 2000). Another factor of relevance to the refugia theory is that refugia must be coupled with a high enough *Bt* dose to kill even partially resistant insects (Gould *et al.*, 2002). *Helicoverpa zea* (Boddie) has a much higher tolerance to Cry1Ac than other common maize Lepidoptera pests, so if the dose of *Bt* toxin is expressed at low levels the larvae may develop tolerance (Gould *et al.*, 2002). This difference in susceptibility of Lepidoptera species is further demonstrated by *Bt* cotton being resistant to *Heliothis virescens* in the US but in Australia the main cotton pests (*Helicoverpa armigera* and *H. punctigera*) display a degree of tolerance to the *Bt* crops (Fitt, 2003). In

Australia, therefore, not only have refugia been implemented, but also a threshold for the number of Lepidoptera larvae that are seen on the crop before an additional chemical spray is applied (Fitt, 2003). In this way *Bt* cotton can be used as part of an integrated pest management scheme.

Toxin pyramiding, frequently suggested as a method to prevent resistance emerging, involves introducing the genes for several different Cry proteins into a crop. This practice arises from the theoretical assumption that the probability of an organism assembling the right combination of genes to become resistant to two different proteins is relatively low (Dent, 2000). Cao *et al.* (2002) engineered genes for both Cry1Ac and Cry1C into broccoli (*Brasssica oleracea* L. var. *italica*, Plenk) plants and found that these plants controlled *Plutella xylostella* that had developed resistance to Cry1Ac and Cry1C proteins when expressed separately. There are, however, some concerns that because resistance often evolves through changes to toxin receptors in the Lepidoptera gut (Van Rie *et al.*, 1990) and/or the loss of toxin receptors (Gould *et al.*, 2002), resistance would spread extremely quickly and could possibly confer resistance to several *Bt* proteins. Escriche *et al.* (1997) showed that the Cry1Ab toxin binding site in the guts of three common agricultural Lepidoptera pests, cabbage moth (*Mamestra brassicae*, L.), potato tuber moth (*Phthorimaea opercula*, Zeller) and the beet army worm (*Spodoptera exigua*, Hübner), are shared with the binding sites of the Cry1Aa and Cry1Ac toxins. If the Cry1Ab toxin binding site was altered or lost then these pest species could potentially become resistant to three of the main Lepidoptera-active toxins used in *Bt* crops.

When insects become resistant to *Bt* proteins then that particular *Bt* crop may become nutritionally beneficial to the pest. Sayyed *et al.* (2003) saw an increased rate of development in *Bt* resistant *Plutella xylostella* fed on purified Cry1Ac toxins from bacteria. This increase may not happen with *Bt* toxins expressed through plants; the recombinant protein produced by plants is different to that experienced in the biopesticide. The recombinant protein is not the crystal-bound protoxin found in biopesticides that requires several modes of action to be activated; instead it is already an activated toxin (Section 1.4.1). In the majority of cases, the resistance reported in the laboratory is unstable, probably due to fitness costs associated with the resistance genes (Shelton *et al.*, 2002).

1.4.4 Invasiveness and outcrossing

An invasive plant can invade a local habitat, take over the natural resources to the detriment of the local plants and wildlife, and thus become a potential threat to biodiversity. Some domestic crops are so poorly adapted to the environment that they cannot survive without the intervention of farmers and will disappear rapidly (Dale *et al.*, 2002); they are not invasive. Some crops, however, are invasive. For example, feral oilseed rape plants were found for a period of eight years in road verges near farms after cultivation had stopped (Pessel *et al.*, 2001) but there are no major crops that have become pests. Genetic modification could provide a crop with an advantage (enhanced fitness) over other plants in the locality. Herbicide-tolerance might enhance fitness in areas of agricultural practice (i.e. where herbicides are used, Dale *et al.*, 2002) and *Bt* oilseed rape displayed an increase in fitness under the selective pressure of herbivory in a cultivated plot but not in a natural plot (Stewart *et al.*, 1997). To date, however, evidence indicates that GM plants are no more invasive than their traditional counterparts; GM lines of herbicide-tolerant oilseed rape (Crawley *et al.*, 1993), and maize, and potatoes expressing *Bt* or pea lectins (Crawley *et al.*, 2001), did not persist in the environment any longer than conventional varieties. Other genetic modifications, for example, tolerance to drought, could lead to enhanced fitness for plants growing beyond a farm's boundaries (Dale *et al.*, 2002) leading to invasion by GM crops of other habitats. Alien (non-native) plants are thought to be more of a threat than GM crops. Japanese knotweed (*Polygonum cuspidatum*, Sieb & Zucc) and rhododendron (*Rhododendron ponticum* L.) are notable examples in the UK (Hails, 2002).

There is the possibility of creating invasive species by hybridisation of *Bt* crop plants with wild plants. This is only relevant if there are closely related wild species with which to breed (Raven, 2003). The potato has no wild relatives in the USA with which it is sexually compatible, and the Russet Burbank *Bt* potato cultivar is sterile, which means that the possibility of the gene out-crossing into wild plants is not a real concern (Betz *et al.*, 2000). Hybrids of *Bt* and wild sunflowers (*Helianthus annuus* L., Snow *et al.*, 2003) expressing the *Bt* protein have reduced seed-head damage from herbivores and thus produce a greater number of seeds than hybrids not expressing the *Bt* protein. Snow *et al.* (2003) speculated that the *Bt* transgene could potentially spread rapidly through wild populations of sunflowers in the USA, although these GM sunflowers are not yet commercially available. The risk of

hybridization can be reduced by increasing the distance between *Bt* plants and non-*Bt* related plants. For example, an isolation distance of 6.2 m reduced the number of hybridisation events between *Bt* rice and wild rice to <0.01% (Rong *et al.*, 2007) and 50 m reduce winter oilseed rape crosses with *B. rapa* to 0.04% (Weekes *et al.*, 2005). The establishment of a hybrid relies mostly on the strength of selection (reviewed in Chapman and Burke, 2006), for example in the absence of herbivory *Bt* hybrids of oilseed rape and *B. rapa* produced six times less seed than wild *B. rapa,* and the proportion of hybrids to wild relatives dropped to 16% in one generation but in the presence of herbivory *Bt* hybrids produced 1.4 times more seed and were present at 42% in the next generation (Vacher *et al.*, 2004).

In the UK, the proposed introduction of GM oilseed rape for pest resistance and/or herbicide-tolerance is of concern as the plants could cross with relatives such as wild cabbage *Brassicae oleracea* L., wild turnip *B. rapa* L. and wild mustard *B. campestris* L. If genes for herbicide-tolerance and pest-resistance were to cross over into wild populations there could be a more fundamental problem. These wild populations, adapted to survive away from agriculture, would potentially be more invasive than the GM crop. There may be a need to resort to more environmentally damaging herbicides in order to control their spread which could be detrimental to biodiversity. In the USA the *Bt* and wild sunflower hybrids, however, had no other associated fitness benefits (e.g. water and nutrient stress tolerance) other than reduced herbivory (Snow *et al.*, 2003) and Halfhill *et al.* (2005) showed that the offspring of *Bt* oilseed rape and non-*Bt* wild turnip were less competitive than the parental lines.

1.4.5 Plant material persistence and root exudates

Plant material from GM crops is often incorporated into the soil after harvesting, this leads to the *Bt* protein also entering the soil and being accessible to many soil-dwelling organisms (Tapp & Stotzky, 1995). Flores *et al.* (2005) used carbon dioxide production as a measure of decomposition and showed that several *Bt* plants species decompose more slowly than non-*Bt* ones. A lower lignin content in one *Bt* maize line (Novartis X4334-EPR) than its non-*Bt* maize counterpart has been reported (Escher *et al.*, 2000), but higher lignin content was detected in a further ten *Bt* maize lines (Saxena & Stotzky, 2001a). A higher lignin content was also detected in *Bt* rice, potato, cotton, oilseed rape and tobacco (*Nicotiana tabacum*, L.), although not significantly so for all plant species tested (Flores *et al.*, 2005); this higher lignin could be a contributing factor to their slower decomposition. Potentially, the *Bt* proteins may

be available to soil-dwelling organisms for a longer period than expected in some *Bt* crops. On the other hand, Lachnicht *et al.* (2004) and Cortet *et al.* (2006) showed that there was no difference in the decomposition of *Bt* and non-*Bt* cotton and maize, respectively. *Bt* proteins may also enter the soil via pollen (Losey *et al.*, 1999) in small quantities but the two main avenues are post-harvest incorporation of plant material and root exudates from the *Bt* plants (Saxena *et al.*, 1999).

Saxena and colleagues (Saxena *et al.*, 1999; Saxena & Stotzky, 2000) showed that *Bt* toxins are released from the roots of *Bt* maize into the rhizosphere. Saxena *et al.* (2002a) further showed that this characteristic was not limited to just one *Bt* maize cultivar (NK 4640) but was actually seen in 12 different *Bt* maize cultivars. *Bt* proteins were also found in root exudates from *Bt* potatoes and rice, but not *Bt* oilseed rape, cotton or tobacco (*Nicotiana tabacum* L.) (Saxena *et al.*, 2004) when grown in soil and using a hydroponic system. Saxena *et al.* (2004) speculate how the difference in *Bt* exudation from the plant roots could be due to transformation of the *Bt* genes to make them suitable for expression in plants, somaclonal variation and the position of the endoplasmic reticulum in root cells. In fact the actual protein might be the crucial factor determining exudation (Saxena *et al.*, 2004): those plants that did not exude *Bt* proteins from their roots were all modified to express Cry1Ac proteins whilst *Bt* maize and rice expressed Cry1Ab proteins and the *Bt* potatoes a Cry3 protein.

Proteins are generally unlikely to bio-accumulate in body fat or persist in the environment in the same way as some halogenated chemical pesticides do (Betz *et al.*, 2000). *Bt* proteins are not an exception. They are normally degraded by micro-organisms which use the *Bt* protein as a carbon source (Crecchio & Stotzky, 1998). There are, however, concerns that root exudates lead to continuous introduction of the *Bt* toxin into the soil and that the levels in the soil could exceed the normal consumption, inactivation and degradation levels (Tapp & Stotzky, 1995). This could lead to *Bt* toxin accumulation in soil which could potentially constitute a hazard to non-target organisms or lead to the selection of resistant pests.

Head *et al.* (2002) reported that soils in which Cry1Ac cotton (marketed as Bollgard® by Monsanto) had been grown for up to six years had no detectable levels of *Bt* toxin in the soil, even when plant material had been incorporated into the soil after harvest. Also, Herman *et al.* (2001) showed that the Cry1F protein had a half-life of just one day in soil under laboratory conditions while a binary δ-endotoxin from *Bt* strain PS149B1 had a half-life of

just two days (Herman *et al.*, 2002). Both of Herman *et al.*'s studies centered on a whole protein produced in the bacterium *Pseudomonas fluorescens* (Migula), rather than the recombinant truncated protein produced by modified plants. More realistically, in soil in which *Bt* maize had grown, Sims & Holden (1996) showed that the half-life of the protein was 1.6 days and that the 90% dissipation time was 15 days. This suggests that the protein will disappear quickly from the soil.

Other studies have shown that the persistence of the *Bt* toxin depends on soil characteristics (e.g. pH), humic acid concentration and clay component. For example, microbial activity is high in alkaline soils, toxins are degraded quickly (Tapp & Stotzky, 1998) and *Bt* toxins bind to clay particles (Tapp & Stotzky, 1995). Saxena *et al.* (2002b) showed that in soil with a high clay content, 16% of the introduced toxin leached out of the soil, any remaining toxin had the potential to contaminate the surface waters by erosion and surface run off. Soil with low clay content (i.e. low levels of montmorillonite and kaolinite particles) leached 75% of Cry1Ac protein and this was liable to work its way into the groundwater supply. In another study, *Bt* proteins from *Bt* maize were shown to have a shorter half-life in water than soil (four and nine days, respectively) suggesting that if they were to enter the water supply by either of the routes mentioned by Saxena *et al.* (2002b) the problem would probably not be a long-term one (Douville *et al.*, 2005).

If the *Bt* proteins remain in the soil their activity must be monitored; it may be that it is not retained. *Bt* toxins bind to humic acids and the level of toxicity remains the same as free toxins (Crecchio & Stotzky, 1998). The type of surface active component in clay may also alter *Bt* activity. In the presence of kaolinite, *Bt* activity against insects was retained for more than six months yet in soil with a high level of montmorillonite there was a decrease in toxicity after just 35 days (Tapp & Stotzky, 1998). Plants such as maize, carrot (*Daucus carota* L.), radish (*Raphanus sativus* L.) and turnip do not take up *Bt* proteins from soil (Saxena & Stotzky, 2002). Therefore, if non-*Bt* plants were grown in fields after *Bt* crops then the protein would not become available to herbivores, although it might remain present in the soil.

1.4.6 Toxicity of *Bt* to humans

For the past 40 years *Bt* biopesticides have a human safety record better than any chemical insecticide (Siegel, 2001). Due to the high specificity of the *Bt* toxin, toxicity in mammals and humans is virtually unknown. The few cases where *Bt* bacteria have been isolated from humans it has been in immuno-compromised individuals or where entry to the human system has occurred through damaged tissue. For example, the isolation of *Bt* var. *konkukian* H34 bascteria from war wounds has been reported by Hernandez *et al.* (1998); bacteria also caused infection and killed immuno-suppressed mice when injected sub-cutaneously (Hernandez *et al.*, 1999). These published cases involved *Bt* strains that do not possess proteins with insecticidal activity and, therefore, are not used in biopesticides (Siegel, 2001). The genes from these strains are, therefore, unlikely to be introduced into *Bt* plants and thus enter the food chain.

Betz *et al.* (2000) state that apart from the production of a Cry protein *Bt* plants are equivalent to non-*Bt* plants; Cry proteins are non-toxic to humans and they do not pose a concern as allergens. Combining this information with the strict health and safety regulations imposed on biotechnology companies before the release of GM crops, and the safety record of *Bt* as a biopesticide, it is hardly surprising that Betz *et al.* conclude that *Bt* plants are safe for human consumption. *Bt* crops also prevent infestation by *Fusarium* spp fungi. These fungi have been linked to liver and oesophagal cancers in humans (Betz *et al.*, 2000) and the introduction of *Bt* cotton in India has led to a decrease in the incidence of poisonings by chemical pesticides (Hossain *et al.*, 2004). GM maize and soybeans (including *Bt* varieties) have been eaten for almost 10 years in the USA and, as yet, there have been no reported ill effects.

Codex is a set of guidelines that ensures that GM foods are analysed for direct and indirect/unintentional effects on human health as part of the formal risk assessment prior to commercial use (reviewed in Haslberger, 2003). In GM plants the process of tissue culturing (resulting in somaclonal variation) is one cause of these unintended changes but the actual position of transgene integration into the plant's genome can result in alterations to the plant's normal metabolic pathways and affect the transgene expression and stability (reviewed in Filipecki & Malepszy, 2006). Plants also have different post-translational modification processes (e.g. proteolysis) which can alter the end product (Goldburg &

Tjaden, 1990). For example, a gene for an amylase inhibitor from *Phaseolus vulgaris* L. (bean) when transferred into *Pisum sativum* L. (pea) exhibited a slightly structurally different form which caused allergic reactions in mice (Prescott *et al.*, 2005).

1.4.7 Effects on non-target organisms

If *Bt* plants are to become an integral part of the environment, either through extensive planting or through outcrossing and invasiveness, then it can be expected that many organisms will come into contact with the plants and with soil contaminated with *Bt* proteins through root exudates and post-harvest incorporation of plant residues. The impact of these factors on biodiversity and non-target organisms must be thoroughly investigated. Non-target organisms are defined as any organisms, other than the pest organisms(s), that the *Bt* crops have been engineered to protect the plant against. This includes non-target plant feeders, organisms found in the rhizosphere and phylloplane of the plant, and organisms that predate and parasitise either the pest or non-pest organisms. The term non-target could, in some situations, refer to species of the same order; for example when looking at the effect of *Bt* maize, designed to be active against the European corn borer, on the non-target monarch butterfly (*Danaus plexippus,* L.).

The effect on a non-target species can be described as direct (i.e. caused by the *Bt* protein), indirect or knock-on. Indirect effects are a consequence of trophic interactions whilst knock-on effects are caused by changes in agricultural management of the GM crops, for example, reduced pesticide use (Birch & Wheatley, 2005). There may also be indirect effects on non-target species due to the unintended alterations to the plant's metabolism (Filipecki & Malepszy, 2006). The likelihood of ingestion of the *Bt* protein causing a direct effect may be high if the non-target species is closely related to the target pest, for example in the same genus. This is mainly because it is likely that the correct receptors for the *Bt* protein are present in the gut of the non-target species. It is hypothesised that because of the specific nature of the *Bt* protein (Section 1.2.2) no effects would be seen in non-target species of different orders. Omolo *et al.* (1997), however, showed that a *Bt* isolate toxic to the tsetse fly (*Glossina* spp. (Diptera:Muscidae)) was also toxic to the Lepidoptera stem borer (*Chilo partellus*, Swinhoe). Similarly, Hussein *et al.* (2005) showed that a Coleoptera-specific *Bt* protein inserted into potato plants could also reduce larval growth and reproduction of an unrelated Lepidoptera species, *Spodoptera littoralis* (Boisduval), by reducing food intake.

Sisterson *et al.* (2004) showed that there was a significant difference in total arthropod abundance in *Bt* and non-*Bt* cotton fields; more families were detected on non-*Bt* plants than in fields of *Bt* plants, but within a field using in-field refuge management there was no difference in arthropod abundance. Similarly, in a study of 65 taxa of relevance to pest management no differences were found in ground dwelling arthropod populations between *Bt* and non-*Bt* cotton fields (Torres & Ruberson, 2006). Naranjo & Ellsworth (2002), as part of the Arizona Cotton Report, concluded that the natural enemy abundance and overall arthropod diversity was not directly affected by *Bt* cotton but by the use of pesticides that are used to control additional non-target pests. *Bt* plants are, in fact, thought more likely to lead to an increase in the numbers of non-target insects, and are a general benefit to biodiversity as they will lead to a reduction in use of broad-spectrum chemicals (Betz *et al.*, 2000). These studies, however, only considered the total numbers of arthropods. More specific experiments looking at individual groups or species indicate that significant differences in some fitness parameters are affected by the *Bt* protein (Table 1.2).

1.4.7.1 Herbivores and sap feeders

There were more "chewing herbivore" families collected from non-*Bt* cotton than *Bt* (Cry1Ac) cotton fields, but there were no differences in the number of "sucking herbivore" families found on the two treatments (Sisterson *et al.*, 2004). Two experiments on specific species rather than family groups provide further evidence that *Bt* plants do not have a negative effect on herbivores and sap feeders. Firstly, the herbivorous sawfly (*Athalia rosae*, L. (Hymenoptera: Tenthrediniae)), when feeding on *Bt* (Cry1Ac) oilseed rape, showed no significant differences in mortality, larval development nor weight compared to those feeding on non-*Bt* oilseed rape (Howald *et al.*, 2003). Secondly, a study on a thrip species (*Frankliniella tenuicornis*, Uzel (Thysanoptera: Thripidae)) showed no significant differences in several fitness parameters (egg development, hatching, pre-imaginal development and mortality, pre-ovipostion time, number of eggs and longevity of females) when feeding on *Bt*

Table 1.2 Summary of representative studies that have been undertaken to investigate the effect of different plants engineered to express different *Bt* proteins on a variety of above-ground organisms including target species (also see tables in Lövei & Arpaia, 2005 and Romeis *et al.*, 2006).

Source of *Bt*	Protein	No significant effect	Positive effect	Negative effect	Reference
Purified from *Bt*	Cry1Ac	*Helicoverpa armigera* (Lep.) *Microplitis mediator* (Hymeno.)			Liu *et al.*, 2005a
		Chrysoperla carnea (Neuro.)			Romeis *et al.*, 2004
				Spodoptera litura *Helicoverpa armigera* *Cotesia kazak* *Meteorus pulchricornis*	Walker *et al.*, 2007
	Cry1Ab			*Chrysoperla carnea* (Neuro.)	Hilbeck *et al.*, 1998
	Cry2			*Chrysoperla carnea* (Neuro.)	Hilbeck *et al.*, 1998
Maize	Cry1Ab	*Apis mellifera* (Hymeno.) *Danaus plexippus* (Lep.)		*Chrysoperla carnea* (Neuro.)	Gatehouse *et al.*, 2002
		Frankliniella tenuicornis (Thysano.) *Chrysoperla carnea* (Neuro.)			Obrist *et al.*, 2005
		Coleomegilla maculata (Coleo.) *Orius insidiosus* (Hetero.) *Chrysoperla carnea* (Neuro.)			Pilcher *et al.*, 1997
		Tetrastichus howardii (Hymeno.)		*Cotesia flavipes* (Hymeno.)	Prutz *et al.*, 2004
		Orius majusculus (Hetero.)		*Helicoverpa zea* (Lep.)	Zwahlen *et al.*, 2000
	Cry3Bb	*Coleomegilla maculata* (Coleo.)			Lundgren & Wiedenmann, 2002
	Cry3Bb1	*Coleomegilla maculata* (Coleo.)			Duan *et al.*, 2002
		E. vigintioctopunctata * (Coleo.) *Galerucella vittaticollis* (Coleo.)			Shirai, 2006

* *Epilachna vigintioctopunctata*

Table 1.2 (continued)

Source of *Bt*	Protein	No significant effect	Positive effect	Negative effect	Reference
Maize	Unknown	Spiders			Meissle & Lang, 2005
		Coleomegilla maculata (Coleo.) *Harmonia axyridis* (Coleo.) *Orius insidiosus* (Hetero.)			Musser & Shelton, 2003
		Chrysoperla carnea (Neuro.)		*P. gossypiella* (Lep.) *	Poppy, 2000
Cotton	Cry1Ac	*Microplitis mediator* (Hymen.)		*Helicoverpa armigera* (Lep.)	Liu *et al.*, 2005a
		Aphis gossypii (Hemi.)	*Geocoris pallidipennis* (Hemi.)		Liu *et al.*, 2005b
		Zelus renardii (Hemi.) *Nabis* spp (Hemi.)		*Spodoptera exigua* (Lep.) *Orius tristocolor* (Hemi.) *Geocoris punctipes* (Hemi.)	Ponsard *et al.*, 2002
	Unknown	*Prodenia infecta* (Lep.) *Bemisia tabaci* (Homop.)	Aphids and Aranae Red spider mites (Acari) *Propylaea japonica* (Coleop.)		Deng *et al.*, 2003
		Arthropods			Sisterson *et al.*, 2004
Oilseed rape	Cry1Ac	*Athalia rosae* (Hymen.)			Howald *et al.*, 2003
		Diaeretiella rapae (Hymen.) *Myzus persicae* (Hemi.)			Schuler *et al.*, 2001
				Chrysoperla carnea (Neuro.)	Schuler *et al.*, 2005
		Cotesia plutellae (Hymen.)			Schuler *et al.*, 2003; 2004
Potato	Cry3A			*Aphidius nigripes* (Hymeno.)	Ashouri, 2004
	Cry3Aa	Aranae Carabidae (Coleop.) Staphylinidae (Coleop.)			Duan *et al.*, 2004
				Spodoptera littoralis (Lep.)	Hussein *et al.*, 2005
Rice	Cry1Ab	*Nilaparvata lugens* (Hemi.) *C. lividipennis* (Hemi.) **		*C. medinalis* (Lep.)*	Bernal *et al.*, 2002

* *Pectinophora gossypiella* ** *Cnaphalucrocis medinalis* ***Cytorhinus lividipennis*

(Cry1Ab) and non-*Bt* maize (Obrist *et al.*, 2005).

Significant effects on sap feeders have been reported. A negative effect of *Bt* (Cry1A) cotton on a non-target herbivore, the aphid *Aphis gossypii* (Glover) (Hemiptera: Aphididae), was reported by Liu *et al.* (2005a). In the first generation of these aphids there was a significantly longer reproduction time when feeding on *Bt* cotton than on non-*Bt* cotton, but in the second and third generation this extension was not seen. This suggests that the aphids had overcome the inhibiting factor. Liu *et al.* (2005a) showed that the effects of *Bt* cotton on aphids was accentuated by further genetic modification of the cotton, so that the *Bt* plants also expressed a cowpea trypsin inhibitor which disrupted the aphids' ability to digest proteins.

1.4.7.2 Ingestion of pollen by non-target species

As a natural enemy playing a role in the biocontrol of aphids the ladybird *Coleomegilla maculata* (DeGeer) (Coleoptera: Coccinellidae) is commonly studied when investigating effects of *Bt* crops on non-target organisms. Duan *et al.* (2002) showed that ladybird adult survival rate on pollen from non-*Bt* maize was not significantly different to those feeding on pollen from *Bt* maize designed to target corn rootworm (*Diabrotica* spp. (Coleoptera: Chrysomelidae)). The *Bt* pollen diet also did not affect larval development or survival. Lundgren & Weidemann (2002), on the other hand, reported faster larval development, higher survival and greater pupal weights of ladybirds fed on *Bt* pollen (from maize with Coleoptera-specific *Bt* proteins) mixed with aphids than those fed on only aphids. Both of these studies are not sufficiently realistic to draw any major conclusions from; ladybirds would not feed directly on pollen and would perhaps only ingest very small amounts by accident if it was on their aphid prey. It would be more realistic to look at ladybirds feeding on aphids which have been feeding on *Bt* plants in a tri-trophic study (Section 1.4.7.3).

Losey *et al.* (1999) looked at the effects of pollen from *Bt* plants on monarch butterfly populations. In theory, the larvae of this species could ingest *Bt* pollen that had drifted from *Bt* crops onto their host plant milkweed (*Asclepias syriaca* L.). Losey *et al.* (1999) dusted milkweed leaves with pollen from *Bt* maize (cultivar NK 4640) and introduced monarch butterfly caterpillars to the leaves. Their results suggested that there would be detrimental effects on the monarch butterfly population as the larvae grew more slowly and suffered higher mortality on *Bt* pollen dusted leaves than non-*Bt* pollen dusted ones. There were,

however, weaknesses with this study, including the fact that the dose of *Bt* pollen was not quantified (Gatehouse *et al.*, 2002) and that the exposure level used in the laboratory experiment was probably exceptionally high and not likely to be encountered in the field (Connor *et al.*, 2003).

The likelihood of exposure of the monarch butterfly caterpillars to pollen depends on several factors which, if they did coincide, would be infrequent, and would be limited to just a fraction of the insect population. Sears *et al.* (2001) report that milkweed plants are seen at low densities in agricultural areas in the southern maize belt of the USA, and that only 15-20% of the larvae will be present during the maize pollen season. The hazard to monarchs also depends on the toxicity of the *Bt* maize. In one cultivar, Event 176, the promoter driving *Bt* toxin expression produced higher concentrations of *Bt* than other GM-cultivars, and this cultivar has been proved to be the most likely to have an effect on monarchs (Hellmich *et al.*, 2001) and other non-target Lepidoptera. As a consequence Event 176 was removed from the market in 2003 (Sears *et al.*, 2001). Other cultivars which produce *Bt* proteins toxic to monarch larvae express it at levels below the monarch toxic threshold (Sears *et al.*, 2001).

Losey *et al.*'s (1999) experiment represented a worst-case scenario; the combination of high hazard and high exposure levels. The risk to monarch butterflies is considered negligible when all factors of hazard and exposure are included (Betz *et al.*, 2000), even within a field of *Bt* maize. In fact, monarch butterflies are on the increase in the USA despite further acreage being planted with *Bt* maize. This is probably due to reduced pesticide use (Gatehouse *et al.*, 2002). One solution to reduce fears about effects on the monarch butterfly and other non-target species is the further genetic modification of *Bt* crops so that they do not produce the toxin in the pollen (Pimentel & Raven, 2000). It may also be possible to target the pest species more precisely by choosing a more specific *Bt* protein. Visser *et al.* (1990), for example, discovered a *Bt* protein similar to Cry1C proteins which, when tested, proved to be *Spodoptera exigua* specific with no activity against three other Lepidoptera: *Mamestra brassicae, Heliothis viriscens* (Fabricius) and *Pieris brassicae* L.

1.4.7.3 Organisms at higher trophic levels

Invertebrate species higher in the trophic chain also come into contact with the *Bt* protein by less direct routes. For example, predators and parasitoids of organisms that have come into

contact with the *Bt* protein may be indirectly affected through ingestion of prey/hosts that have fed on GM plants. Marked effects on predators and parasitoids are unlikely due to the specificity of the protein (Section 1.2.2). Predator and parasitoid species are usually members of a different taxonomic order to that of the target pest and, as in most cases the protein has been chosen to target only the pest organisms, no effects would be expected due to differences between the alimentary canal enzymes and protein receptors of the pest and its natural enemies. Even in the presence of appropriate receptors in the natural enemy the protein is unlikely to have an effect, either because the alimentary canal enzymes found in the prey/host and its predator/parasitoid may digest the protein, making it inactive, or higher concentrations of the protein may be required for the *Bt* protein to be effective.

Predators of target species The lacewing, *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), is one predator that has been investigated in the context of the effects of ingesting prey that have been feeding on GM plants. The larvae of this species are known to feed on Lepidoptera larvae. For example, Hilbeck *et al.* (1998) discovered that the Cry1Ab *Bt* protein is toxic to *C. carnea* larvae at 100 $\mu g\ ml^{-1}$. This concentration is relatively high, higher than the expected concentrations *C. carnea* larvae would experience in the field when feeding on Lepidoptera larvae that had fed on *Bt* maize containing 4 $\mu g\ g^{-1}$ *Bt* proteins (Hilbeck *et al.*, 1998). Hilbeck *et al.*'s results were contradicted by Romeis *et al.* (2004) who showed that there was no direct effect of the same *Bt* protein on *C. carnea* larvae even at concentrations 10,000 times higher than found in Lepidoptera larvae. Both Hilbeck *et al.* and Romeis *et al.* used an artificial diet in their studies and a purified *Bt* protein was fed to the *C. carnea* larvae. In the field the larvae would not come into direct contact with the *Bt* protein in this form; instead it would be present inside the prey and may have already been digested or altered. Extrapolation of these results to the field is not straightforward.

Heeding these criticisms, Hilbeck *et al.* (1999) developed and modified their experiments. They fed *Spodoptera littoralis* larvae a diet incorporating a *Bt* protein prior to feeding them to *Chrysoperla carnea* larvae. In this way, any effects were mediated through the prey. Again, on a diet of 100 $\mu g\ ml^{-1}$ of *Bt* protein, the predatory larvae had a higher mortality and development was delayed when ingesting *Bt*-fed larvae compared to those predators consuming larvae not ingesting *Bt* proteins. At lower concentrations, however, the larvae survived and developed normally. Hilbeck *et al.* (1999) concluded that the difference

detected was due to the prey quality being altered at the higher concentration; on ingesting this concentration of protein the *S. littoralis* larvae were showing symptoms of lethargy and flaccidity. There remains one further weakness in their study; by using a purified protein produced either from *Bt* or GM *E. coli,* the *Bt* protein is not being introduced to the Lepidoptera larvae, and thus to the predator, in the same form as it would be found in the crop. Plants use different post-translational packaging in comparison to bacteria so the *Bt* protein produced may be subtly different. It would have been more realistic to feed the *S. littoralis* larvae on a *Bt* plant expressing the protein.

Ponsard *et al.* (2002), in contrast, used Lepidoptera fed on *Bt* plants and showed that two Hemiptera predators (*Orius tristicolor* White and *Geocoris punctipes* Say) had decreased longevity when feeding on *Bt* cotton-fed *Spodoptera exigua* but that two other Hemiptera (*Nabis* sp. and *Zelus renardii*, Kolenati) were not affected by *Bt* proteins in their prey. This again cannot necessarily be extrapolated to the field, as in natural environments these predators would feed on a range of prey and not be limited to *Bt*-affected prey. By incorporating prey choice, Schuler *et al.* (2005) showed that if enough *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) were presented as an alternative to *Plutella xylostella,* then the numbers of *C. carnea* predators were not affected. When, however, few aphids were present, then the numbers of predators on *Bt* plants were lower than on non-*Bt* plants; the Lepidoptera larvae had been killed by the *Bt* protein and thus food supplies were restricted.

The studies mentioned above have been restricted to relatively small-scale laboratory experiments. Pilcher *et al.* (1997) incorporated a two-year field study on the abundance of predators of *Ostrinia nubilalis* in *Bt* and non-*Bt* maize, as well as laboratory work. These authors detected no differences between the numbers of predators including coccinellids, anthocorids and chyrosopids in *Bt* and non-*Bt* fields. In contrast, differences have been detected in GM fields containing herbicide-tolerant plants. For example, there were significantly fewer predators in herbicide-tolerant spring oil seed rape over a whole season than control oil seed rape (Hawes *et al.*, 2003). This was attributed to changes in prey population levels due to different crop regimes, especially weed control, rather than a direct effect caused by the genetic nature of the GM crop.

Parasitoids of target species Liu *et al.* (2005a) allowed *Microplitis mediator* (Haliday) (Hymenoptera: Braconidae) to parasitise *Helicoverpa armigera* (Hübner) Lepidoptera larvae

that had been feeding on a diet incorporating purified Cry1Ac proteins from *Bt* var. *kurstaki* HD-73. Parasitoid egg and larval development were significantly delayed on *Bt* protein-fed *H. armigera* in comparison to those not fed *Bt* proteins. If, however, the adult parasitoids were fed the Cry1Ac protein in a honey solution there was no effect on their longevity nor development of their young. This suggests that it is host fitness, at least in terms of egg hatching, pupal weight, adult weight and adult longevity, rather than a direct effect of the *Bt* protein (both groups received the *Bt* protein in one form or another) that affects the parasitoid. This experiment also used purified protein from *Bt* micro-organisms; in the field the *Bt* proteins would be produced by the plant and would possibly be slightly different due to the truncation of the gene to express the toxin rather than pro-toxin (Section 1.4.1) and post-translational packaging (Goldburg & Tjaden, 1990).

When Schuler *et al.* (2004) studied the effects of *Bt* oilseed rape on *Cotesia plutellae* (Kurdjumov) (Hymenoptera: Braconidae), a parasitoid of *Plutella xylostella*, this packaging (see above) was taken into consideration. In *P. xylostella* that were resistant to the *Bt* plant the parasitoid developed to maturity, but premature mortality of *P. xylostella* larvae susceptible to the *Bt* crop caused parasitoid development to stop. This again indicates that it is the indirect effect of host quality rather than a direct effect of the *Bt* protein that is affecting parasitoid development, as both hosts would have contained the *Bt* protein. Intuitively extrapolation would suggest that numbers of parasitoids would quickly drop in a field of *Bt* oilseed rape. Schuler *et al.* (1999), however, noted that parasitoids would preferentially choose plants that had been damaged by larval feeding compared to those undamaged or damaged by artificial means. Using this extra information, extrapolation of the data to the field suggests no effect on numbers of parasitoids. The behavioural tendency means that the parasitoids were more likely to find fit hosts and develop normally, as only resistant larvae or larvae feeding on non-*Bt* leaves (possibly in a refuge) would cause enough plant damage to attract the adult parasitoid.

It is not only host fitness that can affect parasitoids. Tomov *et al.* (2003) showed that *Bt* protein-induced changes to host behaviour could also affect parasitoids. These authors fed Mexican rice borers, *Eoreuma loftini* (Dyar) (Lepidoptera: Pyralidae), diets supplemented with *Bt* and non-*Bt* sugarcane, and then recorded parasitism by the *Parallorhogas pyralophagus* (Marsh) (Hymenoptera: Braconidae). *E. loftini* was less active, ate less and

suffered lower parasitism on the *Bt* sugarcane diet than when feeding on non-*Bt* sugarcane. This reduction in parasitism could have a long term adverse effect on the parasitoid's population within *Bt* fields. The synergistic interaction of reduced parasitism and reduced parasitoid population could affect the bio-control potential provided by *P. pyralophagus.*

One study, that of Prütz *et al.* (2004), has taken trophic considerations one step further by investigating the effects of *Bt* plants on a fourth trophic level, that of the hyper-parasitoid *Tetrastichus howardii* (Olliff) (Hymenoptera: Eulophidae). The Lepidoptera herbivore *Chilo partellus* (Swinhoe) feeds on maize leaves and is parasitised by *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) and *C. flavipes* cocoons are further parasitised by *T. howardii.* Prütz *et al.*'s study found that *T. howardii* parasitised fewer cocoon clusters, and fewer cocoons per cluster, in the *Bt* maize treatment. Thus there were fewer offspring of the hyper-parasitoid than on the non-*Bt* group and they also weighed less. This effect may be caused by the lower fresh weights of the *C. flavipes* cocoons that had parasitised *C. partellus* feeding on *Bt* maize. It was also noted that *T. howardii* may prefer to be a primary rather than secondary parasite, making extrapolation of these data to the field level more difficult.

Predators and parasitoids of non-target organisms Whether effects of the *Bt* protein are as relevant to the predators and parasitoids of non-target species as they are to predators and parsitoids of target organisms has received little attention; these organisms are just as likely to come into contact with *Bt* proteins and should be considered. The probability of indirect effects may be lower than those observed in predators and parasites of target organisms, as the fitness or abundance of non-target prey/hosts should, in general, not be affected by the action of the *Bt* protein.

Establishing whether the protein reaches the alimentary canal of predators and parasitoids intact is important; it may be that the protein has been digested by the herbivore and is no longer active when it reaches the alimentary canal of the natural enemies. *Bt* protein has been detected in non-target herbivores (e.g. two Coleoptera species, both of the Chrysomelidae family, *Chaetocnema pulicaria* (Melsheimer) and *Diabrotica undecimpunctata howardi* (Barber)), and in common predators found in a *Bt* maize field (e.g. spiders, Harwood *et al.*, 2005) using enzyme linked immunosorbent assays (ELISAs). ELISAs have also been used to detect *Bt* proteins in the honeydew produced by the non-target planthopper *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (Bernal *et al.*, 2002), although the protein presence

did not coincide with any observable effect on either planthopper or predator fitness. Although the ELISA may detect one intact antigen on the *Bt* protein this does not necessarily mean that the protein remains complete, nor that its activity has been retained. In fact, Lutz *et al.* (2005) showed that ELISA-detected Cry1Ab proteins were found throughout a bovine gastrointestinal tract. This may have led to predictions that it may be active, but further immunoblotting tests showed that it had been degraded into its component parts.

Analysis of exposure of predators of non-target species to *Bt* protein is complicated by *Bt* protein levels in the field. Generalist predators can be exposed via multiple routes as they also feed on pollen and leaves. When assessing the levels of *Bt* protein to which predators could be exposed Obrist *et al.* (2006) showed that when *Orius majusculus* larvae were exposed to *Bt* proteins in pollen (3 $\mu g\ g^{-1}$) and spider mites (3.5 $\mu g\ g^{-1}$) they gained significantly more weight than those fed on non-*Bt* food sources. Extrapolation of these laboratory data could lead to assumptions that the *Orius* spp. populations would increase. In the field, however, the concentration of *Bt* protein in *Orius* spp. varied with sampling date (none before pollen shed but some during and after). Obrist *et al.* (2006) suggest that this variation in protein levels in the predator was due to the high levels of protein in pollen (3 $\mu g\ g^{-1}$) and the abundance of spider mites with high levels ($> 12\ \mu g\ g^{-1}$) of protein in them after pollen shed. This highlights the importance of field testing over several sampling dates rather than relying on extrapolation of laboratory data.

Of the studies carried out, it is the predators and parasitoids of non-target sap-feeding insects such as aphids and thrips that are the most common study organisms. This is perhaps because aphids and thrips are economically important pests, and if there was an effect on their predators and parasites this would remove some of the natural bio-control available in the field. Schuler *et al.* (2001) performed a large-scale cage experiment with *Myzus persicae* aphids to investigate the effects of *Bt* oilseed rape on population levels of the parasitoid *Diaeretiella rapae* (Hymenoptera: Braconidae). No differences were observed in the ability of the parasitoid to control the aphid population or in its emergence success between the *Bt* and non-*Bt* plants. In contrast, Ashouri (2004) reports a negative effect on the aphid parasitoid *Aphidius nigripes* (Ashmead) (Hymenoptera: Aphidiidae) when parasitizing aphids feeding on *Bt* potato plants. In this latter study there was an effect on the host aphid, *Macrosiphum euphorbiae* (Thomas) (Homoptera: Aphididae), quality and this resulted in

smaller female parasitoids with lower fecundity. Two quite different *Bt* proteins were used in these studies; the potatoes contained Cry3A and were Coleoptera-active (Ashouri, 2004), whilst the maize was Lepidoptera-active with the Cry1A protein (Schuler *et al.*, 2001). This could potentially explain the contrasting results.

In another laboratory study, Zwahlen *et al.* (2000) showed that there was no significant difference in the mortality or development time of nymphs of the predatory *Orius majusculus* (Reuter) (Heteroptera: Anthocoridae) when fed on *Anaphothrips obscurus* (Müller) (Thysanoptera: Thripidae) feeding on either *Bt* or non-*Bt* maize. The thrips were not affected by the *Bt* protein produced by the plant. At a field level, Deng *et al.* (2003) showed an increase in the numbers of non-target pests such as aphids and red spider mites in *Bt* cotton fields and this led to a substantial increase in the numbers of generalist predators over the course of the growing season. Spider populations, for example, increased by 112%. Meissle & Lang (2005) detected no difference in species richness or abundance of spiders in *Bt* and non-*Bt* maize. Duan *et al.* (2004), similarly, showed no differences between the number of carabids and staphylinids present in *Bt* and non-*Bt* potato fields. Comparing studies of this nature is always difficult. Deng *et al.* (2003), for example, used Lepidoptera-active maize whilst Duan *et al's.* (2004) study centered on potatoes expressing a Coleoptera-active Cry3 protein. Duan *et al.* (2004) and Meissle & Lang (2005) also compared the effect of the *Bt* plant with chemical pesticides. They showed that there was no difference in carabid and staphylinid numbers between *Bt* fields and those treated with three systemic pesticides (Duan *et al.*, 2004) and that pyrethroid-treated fields had a significantly lower spider density than *Bt* and non-*Bt* fields.

Birds Birds will feed on both target and non-target organisms, their predators and parasites, and even seeds of the GM crops; thus, there are several routes through which birds may come into contact with the *Bt* protein. There is anecdotal evidence from the USA that indicating that there are more birds in *Bt* fields possibly due to an increase in invertebrates in *Bt* fields due to the reduction in broad-spectrum pesticide use (EPA, 2001). There, however, seem to be no further peer-reviewed studies to investigate this observation.

Summary of effects on higher trophic levels There is bias in the studies towards investigating beneficial species such as natural enemies of target species. *Chrysoperla carnea,* for example, has been the species of choice in 20% of studies investigating the effect

of *Bt* proteins on predators (Lövei & Arpaia, 2005). The majority of studies also only investigate mortality whilst ignoring many other fitness parameters such as reproduction and prey consumption rates (Lövei & Arpaia., 2003). It is difficult to detect trends and predict patterns.

Effects detected at the third or higher trophic level have generally been attributed to the *Bt* protein acting via an indirect route usually due to changes in prey/host fitness or population levels, although sometimes due to crop management. This trend appears despite the different nature of the studies mentioned with different crops having been engineered to express different proteins having been used, and at very different scales (laboratory through to multi-field). A direct effect of the *Bt* protein would necessitate the protein passing intact from plant, through the prey/host alimentary canal and into the predator/parasite at a high enough concentration to have an effect, and for the correct enzymes and receptors to be present in the alimentary canal of the predator/parasitoid. The probability of this combination is low.

While effects on predator and parasitoid fitness may occur they are only obvious if the host/prey is also affected. This indirect effect of the *Bt* protein on natural enemies could have important implications on the control of pest species; parasitoids and predators act as efficient bio-control agents and may play a role if pest species resistance begins to be recorded in the field. Predators are often generalist and in the field will control non-target as well as target organisms. Predators and parasites may also be a food source for other organisms in the field and thus a change in their numbers or fitness could affect general biodiversity.

1.4.7.4 Soil-dwelling organisms

Many soil-dwelling organisms (e.g. earthworms, Collembola) are important decomposers of organic matter and are essential for maintaining nutrient cycling within the environment. Retaining soil biodiversity is fundamental for maintaining plant growth and above-ground biodiversity (Bardgett, 2005). Soil biota could be susceptible to *Bt* proteins as they will come into contact with *Bt* toxins by feeding on roots and decaying plant material, and also through root exudates (Section 1.4.5). Most research on the effects of *Bt* toxins on soil biota is relatively recent, although it has received increasing interest since the discovery that *Bt* proteins are exuded into the soil from *Bt* plants (Section 1.4.5). In comparison to research

into the effects on above-ground species, however, the studies are restricted to a few species (Table 1.3).

Studies of crops expressing antifungal properties (Bitzer *et al.*, 2002) have detected no differences in Collembola numbers present in the soil of GM and non-GM crops. No significant differences were also detected in the number of Collembola in soil in which *Bt* crops and non-*Bt* crops had grown (e.g. Saxena & Stotzky, 2001b; Duan *et al.*, 2004, Griffiths *et al.*, 2006), and more specifically, no differences in the number of *Folsomia candida* (Willem) (Yu *et al.*, 1997) and *Protophorura armata* (Tullberg) (Heckmann *et al.*, 2006). Yu *et al.* (1997) also investigated the effect of *Bt* cotton on a predator of *F. candida,* the mite *Oppia nitens* (Koch) (Acari: Oribatidae), and detected no differences in their number. Other studies with mites have detected no differences in total number present in the soil surrounding *Bt* and non-*Bt* crops (Al-deeb *et al.*, 2003, Griffiths *et al.*, 2006). Al-deeb *et al.* investigated the effect of *Bt* toxins from maize on nematodes and detected no difference in their numbers, however, in contrast, significantly lower (in the field, Griffiths *et al.* 2005) and higher (in the greenhouse, Griffiths *et al.*, 2006) numbers have been detected in soil surrounding *Bt* maize than non-*Bt* maize. The effects of *Bt* crops on these species are relatively easy to study due to their short life cycles and ease of culture. This has, however, meant that most studies have been short-term and effects on only one or two generations are monitored. An exception was that of Al-deeb *et al.* (2003), who measured Collembola and nematode numbers in the field over two successive years but, as mentioned above, found no significant effects of *Bt* crops.

Saxena & Stotzky (2001b) and Zwahlen *et al.* (2003) measured the effects of *Bt* maize on the earthworm *Lumbricus terrestris* (L.) (Oligochaeta: Lumbricidae). There were no significantly lethal effects or differences in weight up to 45 days (Saxena & Stotzky, 2001b) or 160 days (Zwahlen *et al.*, 2003). After 200 days, the weight of *L. terrestris* feeding in soil from *Bt* maize was 16% lower than the initial weight, whilst *L. terrestris*

Table 1.3 Representative studies of investigations on the effect of plants genetically engineered to express different *Bt* proteins on a variety of surface-dwelling and below-ground organisms. This is not an exhaustive list.

Source of *Bt*	Protein	No significant effect	Positive effect	Negative effect	Reference
Purified from *Bt*	Several	Earthworms Enchytraeids Orbatids *Folsomia quadrioculata* (Collem.)			Beck *et al.*, 2004
	Cry1Ab	*Allajulus latestriatus* (Diplopoda)			Weber & Nentwig, 2006
Corn/Maize	Cry1Ab		*Porcellio scaber* (Isopoda)		Escher *et al.*, 2000
				Nematodes	Griffiths *et al.*, 2005
		Collembola Acari *Delia radicum* larvae (Diptera)	Nematodes		Griffiths *et al.*, 2006
		Nematodes			Griffiths *et al.*, 2007
		Protophorura armata (Collem.)			Heckmann *et al.*, 2006
		Lumbricus terrestris (Oligo.) Collembola			Saxena & Stotzky, 2001b
				A. caliginosa (Oligo.) *	Vercesi *et al.*, 2006
		Allajulus latestriatus (Diplopoda)			Weber & Nentwig, 2006
	Cry3Bb1	Mites Collembola Nematodes			Al-deeb *et al.*, 2003
Cotton	Cry1Ab	*Lumbricus terrestris* (Oligo.)			Zwahlen *et al.*, 2003
		Folsomia candida (Collem.) *Oppia nitens* (Acari)			Yu *et al.*, 1997
Potato	Cry1Ac	*Folsomia candida* (Collem.) *Oppia nitens* (Acari)			Yu *et al.*, 1997
	Cry3Aa	Collembola			Duan *et al.*, 2004

* *Aporrectodea caliginosa*

that had been feeding in soil from non-*Bt* maize weighed 4% more (Zwahlen *et al.*, 2003). In contrast, woodlice (*Porcellio scaber* L., Isopoda: Oniscidea) fed on *Bt* maize leaves were significantly heavier than those fed on non-*Bt* maize leaves (Escher *et al.*, 2000). This increase in weight was attributed to an alteration in the nutritional content of the *Bt* leaves; the *Bt* leaves contained less lignin and more carbohydrates than non-*Bt* leaves (also see Section 1.4.5). The mortality and weight gain of another decomposer, *Allajulus latestriatus* (Curtis) (Diplopoda: Jutidae), was unaffected by feeding on *Bt* maize and purified *Bt* proteins (Weber & Nentwig, 2006).

To confirm whether it is an alteration in nutritional content or the *Bt* protein that was causing the increase in woodlice (Escher *et al.*, 2000) and decrease in earthworm (Zwahlen *et al.*, 2003) weights another treatment, where purified *Bt* proteins were added to non-*Bt* leaves, would be necessary. Purifying proteins from *Bt* plants is difficult so Beck *et al.* (2004) looked at the effect of purified *Bt* proteins from *Bt* bacteria on four groups of soil-dwelling organisms (earthworms, enchytraeids, orbatids and one species of Collembola: *Folsomia quadrioculata* (Tullberg)); no differences were detected in their numbers. Food type may also be a nutritional factor; Romeis *et al.* (2003) showed that Collembola numbers, although not different between GM and non-GM maize treatments, were much lower on the two maize treatments than they were on a yeast treatment.

Most studies have investigated the effect of one *Bt* plant on one species or group of species; it is important to investigate the effects on a wide spectrum of taxa following Beck *et al.* (2004) and Saxena & Stotzky (2001b). Even these kinds of studies, however, remain fairly limited in their range, for example, Saxena & Stotzky (2001b) looked at just one earthworm (*Lumbricus terrestris*, L.) species as representative of that particular taxa - it could be that this particular species is immune to the effects of the *Bt* protein but other earthworm species are not. For example, Vercesi *et al.* (2006) showed that there was a negative effect of *Bt* maize on cocoon hatching rates for the earthworm *Aporrectodea caliginosa* (Savigny). Each group of species has a different functional role in the soil ecosystem and disruption to one function could lead to effects on soil biochemical and nutritional cycling processes (changes to the soil can have widespread consequences, Section 1.1). It is, however, important to note whether the disruption is more or less than that caused by current crop practices. Duan *et al.* (2004), for example, showed that Collembola numbers were higher in permethrin (chemical

pesticide) treated non-*Bt* potato plots than untreated *Bt* and non-*Bt* potato plots, which were not different to each other.

1.4.7.5 Micro-organisms

Micro-organisms are another important component of many soil processes, especially decomposition, and are likely to come into contact with *Bt* proteins in the soil. The effects of *Bt* crops on micro-organisms are varied (Table 1.4) and depend on a variety of factors including the nature of the actual crop. Saxena & Stotzky (2001b) detected no differences in the number of bacteria, fungi and protozoa in soil in which *Bt* and non-*Bt* maize had grown. Griffiths *et al.* (2005), however, detected fewer Protozoa in soil in which *Bt* maize had grown than in soil in which non-*Bt* maize had grown. Donegan *et al.* (1995) investigated three *Bt* cotton cultivars and assessed the soil micro-organisms surrounding their roots. Two of the cultivars had higher numbers of bacteria and fungi than in soil from non-*Bt* cotton plants. Escher *et al.* (2000) investigated bacterial and fungal growth on *Bt* and non-*Bt* maize, and on faeces from woodlice fed on *Bt* and non-*Bt* maize. Fungal growth was equal within both comparisons. While bacterial growth was equal on *Bt* and non-*Bt* maize leaves there was 60% lower bacterial growth on faeces from *Bt* maize fed woodlice than non-*Bt* maize fed woodlice.

The four studies above were restricted in their data to total number of bacteria and/or fungi (or colony forming units). This numerical approach will not detect differences in the many soil micro-organisms that cannot be grown in culture and may overlook changes in the species composition. Methods investigating changes in biomass (e.g. phospholipid fatty acid analysis and community level physiology profiling) are closely related to total number of micro-organisms present but, in general, have detected no differences in the bacteria present in soil from *Bt* and non-*Bt* plants (e.g. Dunfield & Germida, 2003; Griffiths *et al.*, 2005; Griffiths *et al.*, 2006; Griffiths *et al.*, 2007). Phospholipid fatty acid analysis and community level physiology profiling methods may aid understanding if particular groups of micro-organisms are affected, for example, methanogens. Both approaches (numerical and biomass), although popular as they provide a fairly quick indicator of changes, may not pick up on the direct effect of *Bt* crops as the number/biomass of micro-organisms present is indirectly affected by predation, interactions and plant productivity (Lilley *et al.*, 2006).

Table 1.4 Synthesis of some representative studies undertaken to investigate the effect of different plants genetically engineered to express different *Bt* proteins on a variety of micro-organisms, and differences in enzyme activity and processes that may be related to changes in micro-organisms (also see Bruinsma *et al.* 2003).

Source of *Bt*	Protein	No significant effect	Positive effect	Negative effect	Reference
Purified from *Bt*	Several	Microbial carbon content		Increased persistence of two herbicides	Accinelli *et al.*, 2004
Corn or Maize	Cry1Ab	Bacteria			Baumgarte & Tebbe, 2005
		Eubacteria * Heterotrophic bacteria * Mycorrhizal colonization *		Mycorrhizal bacteria Microbial respiration	Castaldini *et al.*, 2005
		Fungi Bacteria on leaves		Bacteria on faeces	Escher *et al.*, 2000
		Fatty acid profiles Community physiology		Protozoa	Griffiths *et al.*, 2005
		Fatty acid profiles Community physiology	Protozoa		Griffiths *et al.*, 2006
		Phospholipid fatty acid analysis			Griffiths *et al.*, 2007
		Protozoa Bacteria Fungi			Saxena & Stotzky, 2001b
Cotton	Cry1Ab		Bacteria Fungi		Donegan *et al.*, 1995
	Cry1Ac		Bacteria Fungi		
		Bacteria Fungi			

* Differences between *Bt* and non-*Bt* detected microbial communities detected but cannot say if positive or negative effect

Table 1.4 (continued)

Source of *Bt*	Protein	No significant effect	Positive effect	Negative effect	Reference
Oilseed rape or Canola	Unknown	Fatty acid methyl ester profiles Community physiology Microbial community			Dunfield & Germida, 2003
Rice	Cry1Ab	Phosphatase	Dehydrogenase ‡	Dehydrogenase ‡	Wei-Xiang *et al.*, 2004a
			Dehydrogenase § Methanogenesis § Anaerobic respiration §	Dehydrogenase § Methanogenesis § Anaerobic respiration § Hydrogen production	Wei-Xiang *et al.*, 2004b
	Unknown	Invertase Arylsulphatase † Dehydrogenase †	Acid phospahatase Arylsulphatase † Dehydrogenase †	Soil urease	Sun *et al.*, 2003

‡ Higher activity in *Bt* treatment up to 14 days but lower after 21 days

§ Lower activity in *Bt* treatment up to 56 days but higher after 56 days

† No difference in activity up to 15 days but higher in *Bt* treatment after 30 days

Microbial biodiversity has also been considered as a method for studying the effects of *Bt* crops on microorganisms and DNA techniques (e.g TGGE, temperature gradient gel electrophoresis) can profile the whole community and easily detect shifts in species present. TGGE was used by Castaldini *et al.* (2005) to detect a difference between eubacteria, heterotrophic bacteria and mycorrhizal fungi present in *Bt* and non-*Bt* maize but it is difficult to determine whether these changes are beneficial or detrimental without a benchmark for natural variations in micro-organism communities (Bruinsma *et al.*, 2003).

Approaches that look at how soil function is affected by growing *Bt* plants may be considered more informative than looking at total numbers and particular species, given that most soils exhibit functional redundancy. General consensus is that species can be lost from soil biota without altering the function of key ecological services (e.g. nutrient cycling and decomposition) (Lilley *et al.*, 2006); there is no direct link between diversity and soil function, and measuring biodiversity may therefore be considered a too sensitive indicator for monitoring the effects of *Bt* crops as soil function can remain apparently unchanged with very few species present.

Changes in soil biochemical processes and enzyme activity can be used as an indicator of functional changes in the soil. Lower urease activity (Sun *et al.*, 2003), microbial respiration (Castaldini *et al.*, 2005) and decomposition of herbicides (Accinelli *et al.*, 2004), have been detected in soil from *Bt* crops compared to soil from non-*Bt* crops. Higher activity of other processes, for example, hydrogen production (Wei-Xiang *et al.*, 2004b) has also been measured. Wei-Xiang *et al.* (2004a, b) and Sun *et al.* (2003) both show that some enzyme activity depends on the sampling time point, for example, dehydrogenase can be higher (up to day 14 and after day 56) or lower in *Bt* rice (between days 21 and 56) than non-*Bt* rice. Wei-Xiang *et al.* (2004b), however, concluded that the effect of *Bt* rice was less than that caused by crop management differences as they compared the two rice treatments with a further control of no plant material which altered the hydrogen production more.

The changes caused by *Bt* plants need to be placed in perspective in a similar manner to Wei-Xiang *et al.* (2004b); this has been impeded by the lack of knowledge of current normal soil functions (Birch & Wheatley, 2005) and the response of soil micro-organisms to variations in other parameters (e.g. temperature, season, crop and nutrient and water stress, Bruinsma *et al.*, 2003). It cannot, therefore, be determined how detrimental the change may be. For

example, phospholipid fatty acid profiling showed that micro-organisms biomass was not affected by the presence of *Bt* crops, but was greater in grass than maize fields (Griffiths *et al.*, 2005) and that *Bt* maize had no greater effect than current pyrethroid insecticide treatments on the microbial community (Griffiths *et al.*, 2006).

Functionally important and vulnerable species (keystone indicators) can be identified in potential sites for *Bt* crop cultivation by looking at the current species present in the soil. If a decrease in these keystone indicators is noted when *Bt* crops are grown then this could lead to the loss of a particular soil function leading to a negative effect on the ecosystem (Lilley *et al.*, 2006). Potential keystone indicators are mycorrhizal and wood decay fungi, along with nitrogen fixing, nitrifying and antagonistic bacteria (Bruinsma *et al.*, 2003). A general analysis of other micro-organism groups should also be implemented possibly combining the techniques described (culturing and biochemical activity) with DNA techniques which can detect shifts in the species present (Bruinsma *et al.*, 2003). Long term, multi-sample studies should also be carried out. Biomass, for example, increased throughout the growing season (Griffiths *et al.*, 2005) indicating the importance of several sampling dates; Lilley *et al.* (2006) recommend the continuous monitoring of soil quality which could indicate slowly accumulating changes in soil function.

1.4.8 Assessing the impact of *Bt* crops

When assessing the environmental impact of GM plants two factors must be taken into consideration: the hazard and the likelihood of organisms being exposed to that hazard (Wilkinson *et al.*, 2003; Poppy & Sutherland, 2004; Birch & Wheatley, 2005). The overall risk to a particular organism can be calculated as the product of these two factors. In Section 1.4.7.2, for example, it was described how the hazard of *Bt* plants to monarch butterflies was high (Losey *et al.*, 1999) but the likelihood of exposure was extremely low (e.g. Sears *et al.*, 2001; Connor *et al.*, 2003) resulting in an overall low risk to monarch butterfly populations. Terms, however, are defined differently by different risk assessment frameworks (Hill, 2005). Once a risk has been identified and assessed there are often methods for managing and possibly minimising that risk (risk mitigation; Hill, 2005), for example, minimising the risk of pest species developing resistance to *Bt* proteins involves persuading farmers to implement and maintain refugia (Section 1.4.3).

Laboratory tests exposing organisms to *Bt* proteins can assess the hazard (e.g. the effect on various fitness parameters). Laboratory tests, however, are not ecologically realistic, they ignore the function of ecosystems as a whole (Jepson *et al.*, 1994) and only describe the acute, rather than the chronic or long term effects (Birch & Wheatley, 2005). There is also a limit to the number of toxicology tests that can be carried out in the laboratory making the selection of representative test species necessary but complex and somewhat arbitrary (reviewed in Jepson *et al.*, 1994).

The probability of exposure is more difficult to measure and depends on a myriad of issues. In the case of the *Bt* plants used in this study determining the probability of exposure depends on the level of proteins in the plant, the level of protein exudation into the soil, the persistence of plant material in the soil and the decomposition rates of the protein (Section 1.4.5). In addition the role of species interactions must also be taken into consideration (Wilkinson *et al.*, 2003). In general, it is recommended that to establish the actual risk laboratory tests are combined with longer term semi-field and field studies (i.e. a tiered risk assessment approach; Wilkinson *et al.*, 2003; Poppy, 2000; Poppy & Sutherland, 2004). Sometimes, only monitoring the effects post-release of GM plants will provide the complete picture (Birch & Wheatley, 2005).

Risk assessment can be divided into analysis of direct and indirect effects (Hails, 2002). In the case of *Bt* plants direct effects are due to toxicity of the *Bt* plants, and hybrid plants resulting from crosses with the *Bt* plants (Section 1.4.4), to target species (Poppy, 2000). There may also be a direct effect caused by the *Bt* protein on non-target species (e.g. mortality of monarch butterflies, Losey *et al.*, 1999). Indirect effects could be the plants' effect on non-target species caused by changes to plant physiology (e.g. changes in lignin content, Escher *et al.*, 2000), trophic interactions (e.g. prey quality, Hilbeck *et al.*, 1999) or the results of agricultural management changes (e.g. reduction of pesticide resulting in improved farmer health (Huang *et al.*, 2005)) and introduction of refugia to reduce resistance development (Section 1.4.3). There are also knock-on effects caused by changes in agricultural management (Birch & Wheatley, 2005) for example reduction in fossil fuel consumption due to reduced pesticide application by heavy machinery (Hails, 2002) and financial benefits (Hunter, 2000). These ethical/social effects are not discussed in this present study which concentrates on the effect of *Bt* plants on non-target organisms. It is,

though, important to distinguish between these three types of risk in order to determine whether the inserted gene and the plant itself, or the management of the GM plant, is the cause of effects on species.

It must be remembered that the overall performance of GM plants is the result of the plants' entire genetic background and the crop management practices and not one single introduced gene (Ammann, 2005). The calculated risk of growing *Bt* plants must, therefore, be compared with current conventional pest control measures (Poppy, 2000; Poppy & Sutherland, 2004); if the risk falls within the same range as, or lower than, conventional practices then it may be deemed an acceptable method of farming. Filipecki & Malepszy (2006) describe how most *Bt* crop effects fall within the range of changes seen caused by different cultivars of the same crop. Biodiversity, in particular, is no more likely to be affected by GM plants than by any other change in agriculture and an appropriate reference point for acceptable levels of change caused by any agricultural development is required (Connor *et al.*, 2003). In the farm-scale evaluations of herbicide-tolerant plants, for example, it was shown that the effect of different crop regimes, especially weed control, was greater than the effect of the GM plants themselves (Hawes *et al.*, 2003). The risks calculated, if any, must be weighed against the benefits (Section 1.4.2) of growing these crops, especially in developing countries (Connor *et al.*, 2003).

1.5 Current status of studies

Various studies have investigated the consequences of direct and indirect contact of above-ground invertebrates and their natural enemies with *Bt* proteins through feeding on GM plant foliage, sap and pollen (Section 1.4.7.1, 1.4.7.2, and 1.4.7.3), and on below-ground invertebrates (Section 1.4.7.4) and micro-organisms (Section 1.4.7.5) encountering the protein through plant roots and protein exudates secreted into the soil. Results vary, but, in general, non-target species closely related to the target organism are sometimes affected while species not closely related seem to suffer no ill-effects and are sometimes even affected beneficially (Tables 1.2 and 1.3). There is still a wide variety of species that have not been investigated, notably those in the mollusc taxa. Many effects are indirect, resulting in changes in population sizes due to changes in food resources, rather than direct effects such as changes in mortality, longevity or developmental rates. There are also very few integrated

studies which investigate the effects of *Bt* crops on a range of taxa, the work of Saxena & Stotzky (2001b) being one notable example.

Many studies that showed a negative effect used a *Bt* protein purified from either *Bt* or another bacterium. This protein is not the same as the one produced in *Bt* plants (e.g. Hilbeck *et al.*, 1999). The gene placed in *Bt* plants is altered such that the protein produces the active toxin, not the crystal bound protoxin that requires several modes of action to be activated (Section 1.4.1). Plants also use different post-translational packaging of proteins which may lead to the *Bt* protein being, for example, glycosylated. This may alter the *Bt* protein's properties and toxicity (Goldburg & Tjaden, 1990). Insertion of the gene and plant culturing methods may also disrupt other pathways (reviewed in Filipecki & Malepszy, 2006) leading to the changes in nutritional status of the plant as noted by, for example, Escher *et al.* (2000). It is therefore, important to only use the *Bt* protein produced by plants for studies investigating the effects on non-target organisms and preferably the *Bt* plants themselves.

Many of the studies have investigated the effects of *Bt* plants on a single species in the laboratory. Though valuable, they are probably an indicator of worst-case scenarios where the species has no food choice and does not take into account all the other variables that could alter the effects of the *Bt* protein (e.g. decomposition of the protein). Field-scale tests are probably the best way to gain an indicator of the effects that would be seen after widespread planting. Few such studies have been conducted (e.g. Al-deeb *et al.*, 2003); the planting of GM plants in the UK is currently tightly regulated so experiments must be kept at a small scale.

When effects were seen using the *Bt* protein produced in *Bt* plants several studies (e.g. Duan *et al*, 2004; Hawes *et al.*, 2003) noted that the effect was less than that caused by differences in crop variety and management. Whenever a new agricultural practice is reviewed for general use the impact of it, and its management practices, should be compared with the effects caused by current crop practices (Poppy, 2000; Poppy & Sutherland, 2004, Section 1.4.8). This is not always the case and it is not easy to establish a threshold for acceptable effects (Dale *et al.*, 2002).

1.5.1 Project aims

Brassicas are major crops in the U.K. with 1.9 million tonnes of oilseed rape alone being grown in 2006 (Defra, 2006a) and other brassicas accounting for a further 527,000 tonnes (Defra, 2006b). Oilseed rape prices were £160 per tonne in 2006 (Defra, 2006a) so any developments in the control of brassica pests are therefore of direct relevance to the agricultural industry and economy. Brassicas are affected by a wide range of pests, from at least seven insect orders, that cause damage to all parts of the plant (Earle *et al.*, 2004); control tactics must take into account their different feeding strategies. A promising development, therefore, is the production of *Bt* plants; plants genetically modified such that the *Bt* toxin is integral to the plant structure and is protected from degradation. Oilseed rape (*Brassica napus* L.) with its commercially important oils became the first commercially available *Bt* brassica, thanks to industry-backed research. The development of other *Bt* brassicas has lagged behind that of *Bt* maize (*Zea mays* L.) and oilseed rape, but is increasing especially in the Far East; production and uses are reviewed in Earle *et al.* (2004). Broccoli (*Brassica oleracea* L. var. *italica,* Plenk) is one such crop that has been modified.

Bt plants excrete *Bt* proteins into the soil in root exudates (Section 1.4.5; Saxena *et al.*, 1999, 2002a, 2004; Saxena & Stotzky, 2000). There is contradictory evidence for the persistence of these excreted *Bt* toxins in the soil environment but, in general, persistence appears to depend on which *Bt* Cry protein is being expressed and the soil characteristics (Tapp & Stotzky, 1995; 1998). It would also appear that however long the toxins spend in the soil, they retain their activity to target organisms. Most available evidence (Table 1.3) suggests that the *Bt* toxins do not affect soil organisms. A few effects have, however, been detected and as only a few species, or groups, have been considered it is important that more species from a broader range of taxa are studied before a conclusion can be made as to the general effect of *Bt* crops on non-target soil organisms.

Bt broccoli has not yet been deployed agriculturally and before it can be released into the environment its efficacy against pests and its effect on non-target organisms must be thoroughly investigated. This present study sets out to explore the direct effects of root exudates from *Bt*-engineered broccoli plants on a range of non-target soil-dwelling species. This is one of the few studies investigating the effect of one specific *Bt* plant on a range of taxa. The range of organisms chosen is representative of the various functional groups

present in horticultural soil ecosystems. The effects of *Bt* plant root exudates on the complexity of the soil-microorganism community is also investigated. Developing on studies carried out above-ground (e.g. Schuler *et al.*, 2004) the study also aims to determine trophic consequences – do *Bt* toxins move through the food chain, and what, if any, are the consequences of *Bt* toxin exudation on higher trophic levels?

2 Molecular techniques to determine the presence of *Bt* genes and to investigate the variability of *Bt* proteins in genetically modified broccoli plants

2.1 Introduction

2.1.1 *Bt* broccoli plants

The *Bt* broccoli (*Brassica oleracea* L. var. *italica*, Plenk) seeds used in this study were obtained from Elizabeth Earle, Department of Plant Breeding and Genetics at Cornell University (NY, USA). At Cornell University sections of flowering Green Comet broccoli stalk were placed in medium and transformed using a modified bacterium *Agrobacterium tumefaciens* (Smith & Townsend) carrying a construct coding for the Cry1Ac protein from *Bacillus thuringiensis* (Berliner) and kanamycin resistance. The kanamycin resistance gene allows the selection of transformed from untransformed plant cells when placed on selective agar containing kanamycin. Those cells that survived were determined to be carrying the *Bt* gene and were used to regenerate transformed plants (Earle *et al.*, 1996). Plants containing a single insertion of the gene (checked by southern hybridisation) were then crossed with the original parent line (Green Comet) to produce seeds (Earle *et al.*, 1996) in a hemizygous mix for the *Bt* gene segregating 1:1 for resistance to *Plutella xylostella* L. (Table 2.1). Although these plants were specifically developed to be active against *P. xylostella*, a major crucifer pest in the USA, as Cry1Ac is a Lepidoptera-active *Bt* protein the plants should also provide resistance to other Lepidoptera pests.

Table 2.1 Expected ratio of seeds received after crossing a transformed plant with a single insertion of the *Bt* gene with the original parent line Green Comet.

Parents	Bt – (transformed plant)		- - (Green Comet)	
Genes	Bt	-	-	-
Progeny (seeds received)	Bt -	Bt -	- -	- -
Insect resistance	Resistant (50 %)		Susceptible (50 %)	

Green Comet plants are, however, themselves F1 hybrids; they have been produced by crossing two separate lines to create plants with an improved broccoli phenotype which are heterozygous at all alleles (Table 2.2a). Breeding these F1 plants with each other produces a

second generation (F2) with a mix of genotypes displaying the range of genes possessed by the parents in different combinations (Table 2.2b). The *Bt* seeds supplied for this project by Cornell University, as mentioned previously, were produced by crossing the *Bt* plants (Green Comet with a *Bt* gene inserted) with the Green Comet; these will grow into plants not only mixed for resistance and susceptibility to Lepidoptera but will also have the same background variation as the F2 generation.

Table 2.2 The expected progeny of breeding a) two genetically diverse plants to produce an F1 generation, and b) two F1 plants with introduced variation in the F2 progeny.

	a				b			
Parents	**AA**		**aa**		**Aa**		**Aa**	
Genes	**A**	**A**	**a**	**a**	**A**	**a**	**A**	**a**
Progeny	**Aa**	**Aa**	**Aa**	**Aa**	**AA**	**Aa**	**Aa**	**aa**
	Green Comet (F1)				**F2**			

2.1.2 Selecting *Bt* broccoli plants

As the batch of *Bt* seeds from Cornell University was expected to be a segregating F2 generation 1:1 for *Bt* to non-*Bt* (Table 2.1) it was necessary to screen the seeds for the *Bt* gene to select the correct plants for experimental use. As the *Bt* gene does not confer a morphologically distinct phenotypic trait the presence of the *Bt* gene has to be detected using one of a range of methods. An insect bioassay (e.g. Cao *et al.*, 1999) using the target species is one method that could be used to select the *Bt* plants. By exposing *P. xylostella* larvae (the target species) to leaves from the plants and assessing mortality, the plants expressing the gene could be determined. Such bioassays are made more complex by *P. xylostella* being a strictly controlled insect in the UK due to its potential pest status.

Kanamycin was used to select the transformed cells (Section 2.1.1) so spraying the explants (plants growing from the callus) with this antibiotic could also be used to select those plants with the *Bt* gene (Weide *et al.*, 1989). Older plants without kanamycin resistance do not die in the same way as the plant cells in culture but some discolouration is seen on their leaves.

Determining whether older plants are kanamycin resistant and thus contain the *Bt* gene depends on the researcher's subjective decision as to whether there has been any leaf discolouration.

The two methods described above are fraught with confounding difficulties. It was, therefore, decided to use two molecular techniques to determine the presence of the *Bt* gene. The polymerase chain reaction (PCR) tests directly for the presence of the gene, while the ELISA (enzyme linked immunosorbent assay) tests for the protein coded by the gene. The use of PCR and sequencing for screening for new *Bt* genes and thus new *Bt* proteins that could potentially have insecticidal activity has been reviewed by Porcar & Juárez-Pérez (2003), whilst Cho *et al.* (2001), Cao & Earle (2003) and Grace *et al.* (2005) have all used PCR techniques to confirm the transformation of *Bt* brassicas. Monoclonal antibodies and an ELISA to quantify Cry1Ac proteins extracted from *Bt* cotton were developed by Palm *et al.* (1994) whilst Tapp & Stotzky (1995) developed a dot-blot ELISA which detected both soil-bound and free *Bt* proteins in the soil. With the increased use of ELISAs several commercial kits have now been developed to detect *Bt* proteins in plant material. These kits are not only used to confirm transformation but also to check for contamination of supposedly non-GM agricultural products.

In this chapter studies using both PCR and ELISA techniques to select *Bt* plants are described. Neither approach indicated that the expected ratio of 1:1 (*Bt* to non-*Bt*, Table 2.1) was present in the mixed bag of seed from Cornell University. The ELISA also indicated variability in the concentration of protein present in the *Bt* plants. This approach was also used to determine whether *Bt* protein could be detected in compost in which transformed plants were grown, and thus become available to decomposers and other soil organisms.

2.2 Methods

2.2.1 Plant culturing

For the PCR studies *Bt* and non-*Bt* broccoli (Green Comet) seeds were planted, two to a pot, 1 cm below the compost (John Innes No. 2) surface in plant pots (10 cm deep, 7 cm diameter) and grown for the first month in a controlled environment room (16:8 h L:D cycle, 19°C). The plants were then transplanted into individual plant pots (20 cm deep, 15 cm diameter)

before being transferred to a greenhouse where the temperature did not drop below 16°C with a L:D cycle of 16:8 h.

For the ELISA work *Bt* and non-*Bt* broccoli seeds (Green Comet) were placed in separate seed trays (30 x 20 x 10 cm; John Innes No. 2 compost) 0.5 cm below the surface. The trays were placed in a greenhouse (see above). The seedlings germinated within five days and were watered every three days for two weeks before being transplanted into individual plant pots (15 cm diameter, 20 cm tall), again with John Innes No. 2 compost.

All the plants were watered every one to three days as required. Plants were kept for up to six months, removing flowering heads as necessary to prevent seeding.

2.2.2 Sample preparation

Leaf discs were sampled from each plant by snapping shut the lid of a micro-centrifuge tube (approx. 1 cm diameter) at the appropriate location on the leaf tissue. The samples were always taken at three months old, unless stated, from the most recently unfurled leaf nearest the top of the stem (Figure 2.1) to standardise sampling. Discs were also taken from leaves that had fallen from the plants and been dried in the greenhouse for four weeks.

Compost samples, on the other hand, were collected by turning out plants from their pots and using a 1 cm diameter soil corer to take a 0.5 g sample 3 cm into the root ball (Figure 2.1). Compost samples were homogenised with a pestle and then a sub-sample (approx 20 mg) measured into a new micro-centrifuge tube. Roots were sampled by gently shaking compost away prior to washing to remove any remaining compost. Roots were weighed before being placed in clean micro-centrifuge tubes.

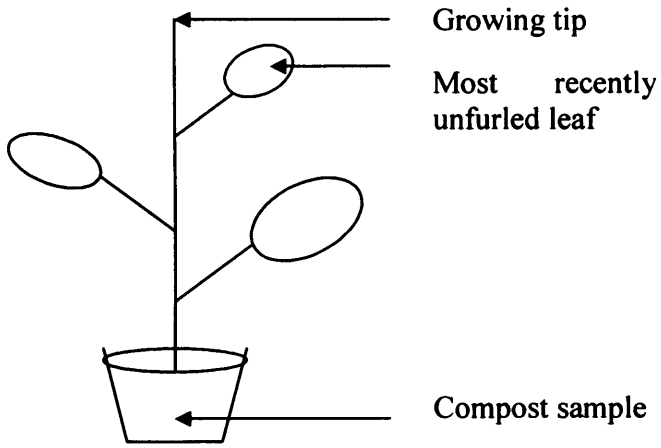

Figure 2.1 Location of leaf disc and compost sampling.

2.2.3 Plant DNA extractions

DNA extractions were based on Edwards *et al.'s* (1991) protocol for extracting DNA from *Arabidopsis*. One leaf disc (Section 2.2.2) from each plant was ground in 400 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) using a pestle for 30 seconds before being centrifuged in a MSE Microcentaur centrifuge (Sanyo, Loughborough, UK) for one minute at 13000 rpm. 300 µl of supernatant was transferred to a clean micro-centrifuge tube and 300 µl of 100 % isopropanol added. The samples were vortexed and allowed to stand for two minutes before being centrifuged as above for a further five minutes at 13000 rpm. The supernatant was decanted and the pellet air-dried for one hour. The pellet was re-suspended in 100 µl TE buffer (10 mM Tris-HCl pH 7.6 and 1 mM EDTA).

2.2.4 Polymerase chain reactions

PCR amplifications were carried out in 0.5 ml microstrip tubes (Abgene, Epsom, UK) on an Applied Biosystems Geneamp PCR system 9700 (Warrington, UK); reagents were from an Invitrogen (Paisley, UK) Taq polymerase kit and primers ordered from Operon (Cologne, Germany). A negative control with the DNA template replaced by RNAse-free water was run with each PCR.

To observe whether DNA had been amplified, 5 µl PCR product was mixed with 2 µl 6x loading dye before loading on a 1.5 % agarose gel along with 4 µl 1 Kb pair ladder (Invitrogen). The agarose gel (15 x 15 x 0.5 cm) was prepared by mixing the agarose with 100 ml 1x TAE buffer (40 mM Tris-acetate pH 8, 1mM EDTA), 0.25 µg ml^{-1} ethidium bromide and then allowing to set for 20 minutes. The gel was placed in a horizontal electrophoresis tank filled with 1x TAE buffer and an electric current was provided by an EC250.90 Thermo PowerPac (Thermo Electron Corporation, MA, USA). The electric current was set at 120 V for 45-60 minutes. The gel was then placed under an ultraviolet light transluminator (312 nm, Genegenius Bioimaging System (Syngene, Cambridge, UK)) and an image captured using Genesnap 4.00.00 Software (Syngene, Cambridge, UK).

2.2.4.1 General primers

To confirm that DNA extraction had been successful the extractions were screened with primers PUV2 and PUV4 (Orchard *et al.*, 2005) that amplified a section of 18S rDNA (Table 2.3). This screening ensured that samples that tested negative with Cry1Ac primers were not false negatives due to poor extraction. The reactions were carried out with 2 µl extracted DNA sample in 25 µl volumes (1x PCR buffer, 3 µM magnesium chloride, 0.2 mM dNTPs, 2 µM each primer and 1 U taq polymerase). The thermal profile consisted of an initial denaturing step at 95°C (3 min), then 40 cycles consisting of denaturing at 95°C (1 min), annealing at 60°C (1 min), and extension at 72°C (1 min) before a final extension step at 72°C (5 min).

Table 2.3 Primer pair details including sequence, length of DNA amplicons and primer references.

Primer	Sequence of primer	Length of DNA strand amplified	Source
PUV 2 F	TTCATGCTAATGTATTCAGAG	429 bp	Orchard *et al.*, 2005
PUV 4 R	ATGGTGGTGACGGGTGAC		
Cao F	CAACTAGGTCAGGGTGTC	1.35 Kbp	Cao & Earle, 2003
Cao R	AGCGCATCTGTTAGGCTC		
Cry F	CAACTAGGTCAGGGCGTG	1.35 Kbp	Adapted from Cao & Earle, 2003
Cry R	CGCGCATCGATTCGGCTC		

2.2.4.2 Cry1Ac primers

Once extractions had been confirmed to contain DNA using the 18S rDNA primers (Section 2.2.4.1) the extractions were screened for the Cry1Ac gene. Two primers from Cao & Earle

(2003, Table 2.3) were chosen as a suitable pair; Cao F and Cao R were aligned with the sequence of the Cry1Ac gene (Genbank accession number M11068) using the program BioEdit (Mahidol University, Thailand). As it was observed that the primers were not a perfect match a second pair of primers (Cry F and Cry R, Table 2.3) was also designed to amplify the same 1.35 Kb pair fragment.

Before screening the whole set of samples preliminarily testing of the two primer pairs was carried out with seven samples (five *Bt* from Cornell University and two Green Comet plants). The reactions were carried out with 2 µl extracted DNA sample in 25 µl volumes (1x PCR buffer, 2 µM magnesium chloride, 0.08 mM dNTPs, 0.5 µM each primer and 1 U taq polymerase). The thermal cycle consisted of an initial denaturing step at 94°C (3 min), then 30 cycles consisting of denaturing at 94°C (1 min), annealing at 48°C (1 min), and extension at 72°C (1 min), before a final extension step at 72°C (10 min).

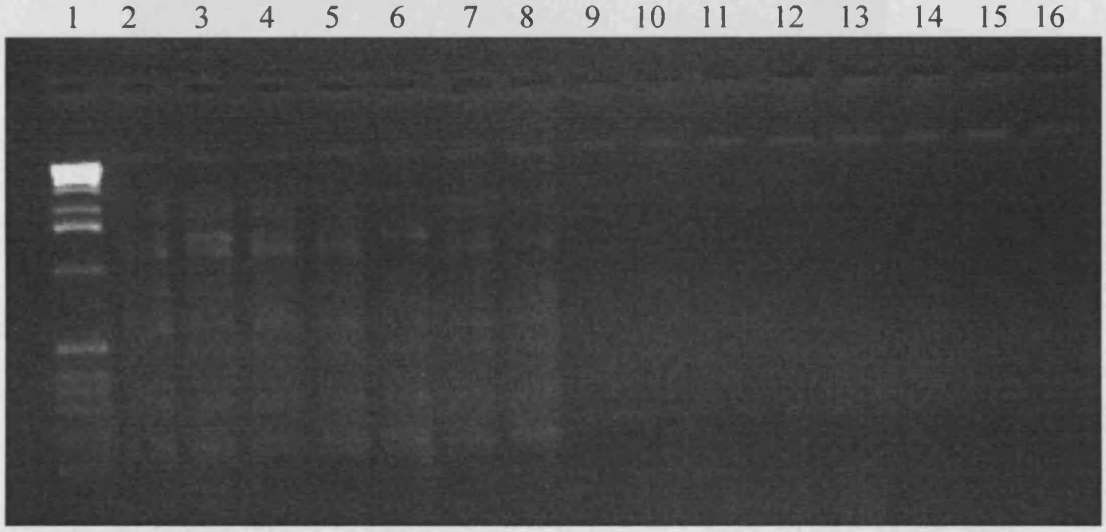

Figure 2.2 Agarose gel from the preliminary testing of the two sets of primers. Lane 1 is a DNA ladder, Lanes 2-8 samples amplified by the Cao F and R (Cao & Earle, 2003) primers and Lanes 9-15 by the adapted primers (Cry F and R). Lanes 5, 8, 12 and 15 contain non-*Bt* plant samples, Lane 16 a negative control and the rest are *Bt* plant samples.

No bands were seen on the agarose gel for the adapted primers (Cry F and Cry R) but the Cao & Earle (2003) primers amplified multiple products (Figure 2.2, Lanes 2-8). A gradient PCR was carried out to deduce a better annealing temperature; the same *Bt* sample was placed in

12 wells of a microstrip (with the same reaction mix as before) in a Biorad DNA engine peltier thermal cycler (Hemel Hempsted, UK) that enabled the establishment of a gradient of 12 different annealing temperatures from 46 to 58°C. This provided the information that the multiple bands were seen below 50.3°C and above 55.8°C (Figure 2.3). The annealing temperature for the Cao & Earle (2003) primers was therefore set at 55°C for screening the remainder of the samples.

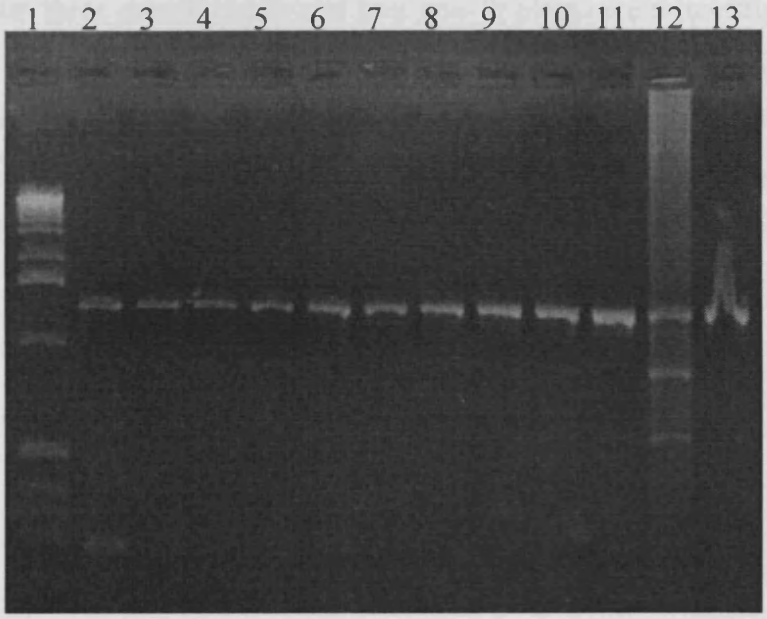

Figure 2.3 Agarose gel from gradient PCR. Lane 1 is DNA ladder, Lanes 2-13 have the annealing temperature gradient: 46, 46.3, 46.9, 47.7, 48.8, 50.3, 52.0, 53.4, 54.5, 55.3, 55.8, 56.0 °C respectively. Multiple bands can be seen in Lanes 2-7, 12 and 13. Optimum temperature for annealing is between Lanes 10 and 11 i.e. 55°C.

2.2.5 Enzyme linked immunosorbant assay (ELISA)

The ELISA kit used was produced by Abraxis (Warminster, PA, USA) and can detect both Cry1Ab and Cry1Ac proteins. The protocol for use (provided by Abraxis) is given in Appendix 1. After the final ELISA step (Step 16) the stronger the yellow colouration in the wells, the more *Bt* protein is present. An Emax® precision microplate reader (Molecular Devices, Wokingham, UK) was used to read the optical density of the wells at 450 nm within 20 minutes of adding the stop solution. The optical densities of *Bt* protein standards were used to construct calibration curves and these in turn used to calculate the concentration of *Bt*

protein present in the samples (per gram fresh weight, see Appendix 1). The level of positive detection was set for each ELISA as the mean plus three standard deviations of the optical density of non-*Bt* plant samples.

Leaf discs (Section 2.2.2) were taken from 183 plants grown from the mixed bag of seed and processed using the ELISA. Nineteen *Bt* plants had leaf disc samples taken at each of three, six and nine months to investigate if there was temporal variation in the production of *Bt* over time. Four, three month old *Bt* and four non-*Bt* plants were investigated more thoroughly for variation of *Bt* protein concentration between samples from different plant origins. Leaf discs were taken from four different areas of the leaf (Figure 2.4a) and from two different age leaves on each plant, the most recently unfurled and the one nearest the base of the stem (Figure 2.4b).

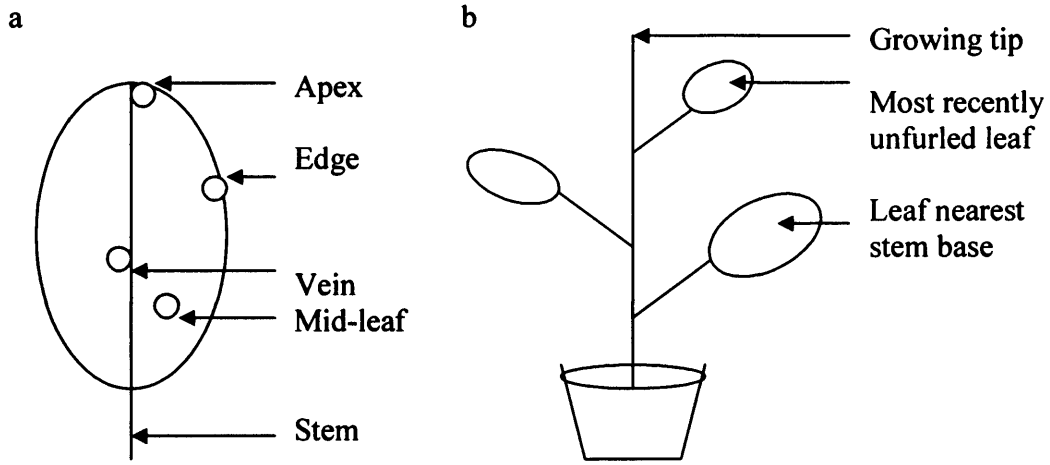

Figure 2.4 Origin of a) leaf disc samples taken from each leaf, and b) two different age leaves.

Compost samples were taken from plant pots of 139 plants to determine whether *Bt* protein is capable of infiltrating the compost thus becoming available to soil organisms. Root samples (n=48) and discs from leaves that had fallen from plants (n=28) were also tested to determine if decomposer organisms would come into contact with the *Bt* protein by this means. These samples all came from plants that already tested positive for the *Bt* protein in their leaves using the three standard deviation of the mean threshold described above.

2.2.6 Statistics

PCR

The data were analysed in Minitab. Chi-square tests were used to determine whether the observed number of plants testing positive for the *Bt* gene was different from the expected number defined by the ratio of 1:1 provided by Cornell University (Earle *et al.*, 1996).

ELISA

The data collected to explore the variation within plant tissue were not normally distributed, although various attempts were made it proved not possible to transform the data to meet the assumptions of parametric tests (normality of data and residuals, and homogeneity of variances). Non-parametric statistics were therefore applied to look for differences; a Kruskal-Wallis test for differences between the four areas of the leaf (Figure 2.2a) and a Mann-Whitney test for differences between the two different age leaves (Figure 2.2b). Pearson's correlation was used to determine if there was a correlation between the concentration of *Bt* in the compost and roots, and the concentration in the leaves.

2.3 Results

2.3.1 PCR results

Sixty *Bt* and 20 non-*Bt* plants germinated and were tested using PCR. The DNA extractions all tested positive for DNA using the 18S rDNA primers (Figure 2.5) and were screened for the Cry1Ac gene and production of the Cry1Ac protein. Forty-two of the *Bt* plants tested positive for the Cry1Ac gene, the other 18 and all of the 20 non-*Bt* plants tested negative for the Cry1Ac gene (Figure 2.6). This was significantly different to the expected ratio of 1:1 ($X^2 = 9.6$, $P<0.05$; Table 2.1) but not from a 3:1 ratio ($X^2 = 0.8$, $P<0.05$). All of the plants that tested positive for the Cry1Ac gene also tested positive in the ELISA (threshold calculated from non-*Bt* samples was 1.2 ng g^{-1}). None of the plants that tested negative in the PCR tested positive in the ELISA.

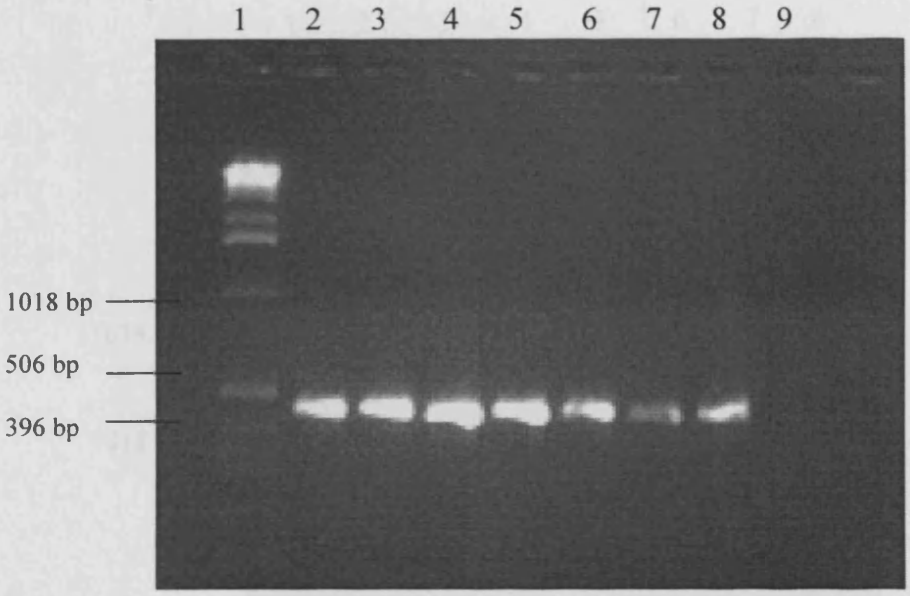

Figure 2.5 Agarose gel with ethidium bromide staining to show bands of correct size (429 bp) after a PCR with 18S rDNA primers PUV2 and PUV 4. Lane 1 DNA ladder, Lanes 1, 2, 3, 5 and 6 samples from *Bt* and Lanes 4 and 7 non-*Bt* plants, Lane 9 negative control.

2.3.2 ELISA results

120 of the 183 plants grown from the seeds provided by Cornell University tested positive (greater than the threshold) for the presence of *Bt* protein using the ELISA; this includes those also tested with PCR. This is significantly different to both the expected ratio of 1:1 (X^2=19.02, P<0.05) and to a 3:1 (X^2=8.67, P<0.05). *Bt* protein concentration ranged from 0.01 to 8200 ng g^{-1}; only eight plants however tested for more than 1000 ng g^{-1}, the majority of plants were in the range of 0.1-100 ng g^{-1} (Figure 2.7).

Root samples were taken from 48 *Bt* plants from Cornell University and, of these, 26 tested positive (83.9 ± 19.0 ng g^{-1}, threshold calculated from non-*Bt* plant samples = 28 ng g^{-1}). Compost samples were taken from 139 plant pots and 30 tested positive, albeit at low levels (0.05 ± 0.02 ng g^{-1}, threshold calculated from non-*Bt* plant samples = 0.01 ng g^{-1}).

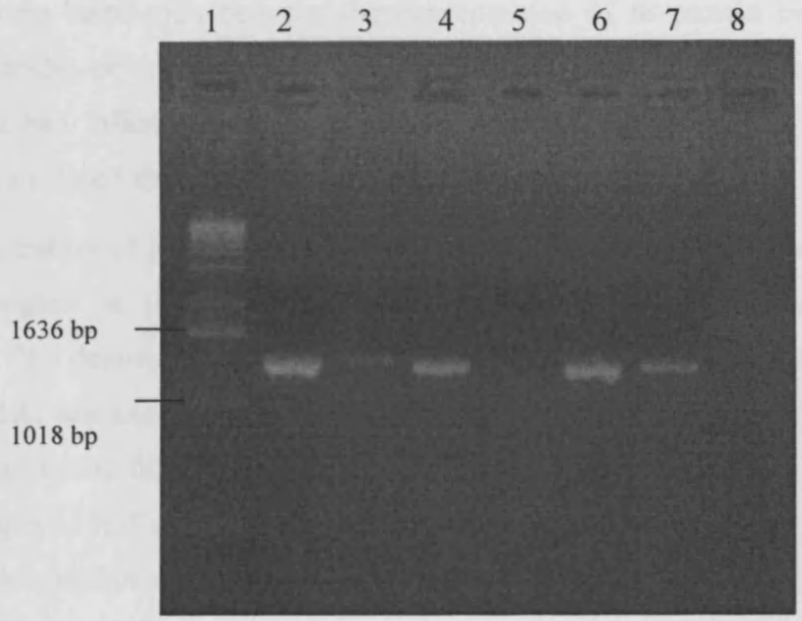

Figure 2.6 Agarose gel with ethidium bromide staining to show bands of correct size (1.35 Kbp) after a PCR with Cao F and Cao R primers (Cao *et al.*, 2003). Lane 1 DNA ladder, Lane 2-7 samples from *Bt* plants, Lane 8 negative control. In Lane 5 is a *Bt* plant without the *Bt* gene.

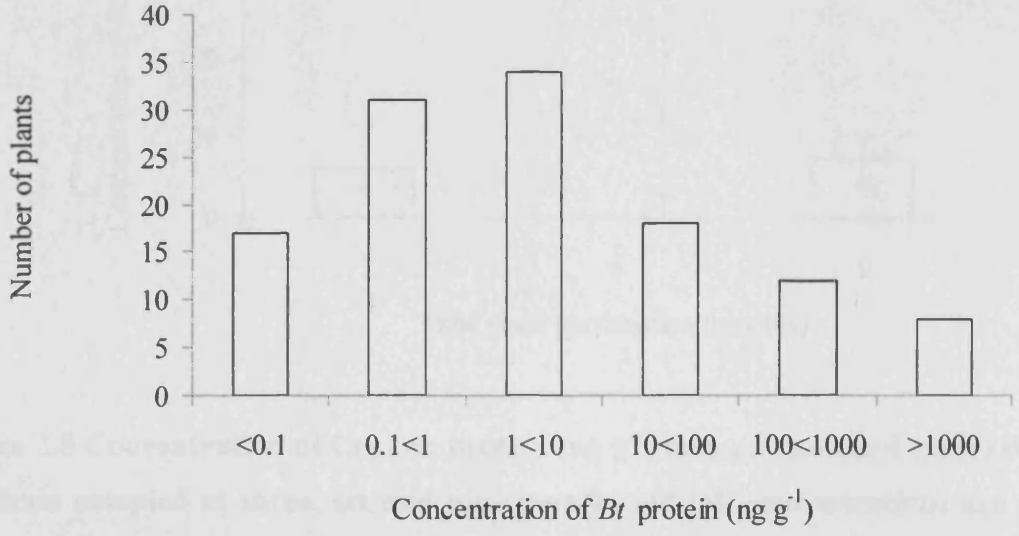

Figure 2.7 Number of *Bt* plants expressing various concentrations of *Bt* proteins above the ELISA threshold (n=120, 63 plants did not test positive). Note the uneven spacing of the concentration categories.

There was no correlation between the concentration of *Bt* protein in leaf and either root (R^2=0.05, n=26) or compost (R^2=0.07, n=30). *Bt* protein was also found to be present in leaves that had fallen and begun to decompose from 28 *Bt* plants (1.51 ± 0.39 ng g^{-1}, threshold calculated from non-*Bt* plant samples = 0.07 ng g^{-1}).

The concentration of *Bt* protein in the leaves of 19 *Bt* plants tested at various time intervals, although highest at six months (Figure 2.8), was not significantly different ($H_{2,56}$=2.09, P=0.352). The detailed study of four plants grown from the *Bt* seeds indicates that one ("*Bt* 1", Table 2.4) was expressing the *Bt* protein at levels much lower than the other three. There was no significant difference between the concentration of Cry1Ac detected in the two different ages of leaf sampled on each plant (W_7=18.5, P>0.99, Table 2.4) nor between the different areas within a leaf ($H_{3,31}$=0.24, P=0.971, Table 2.5).

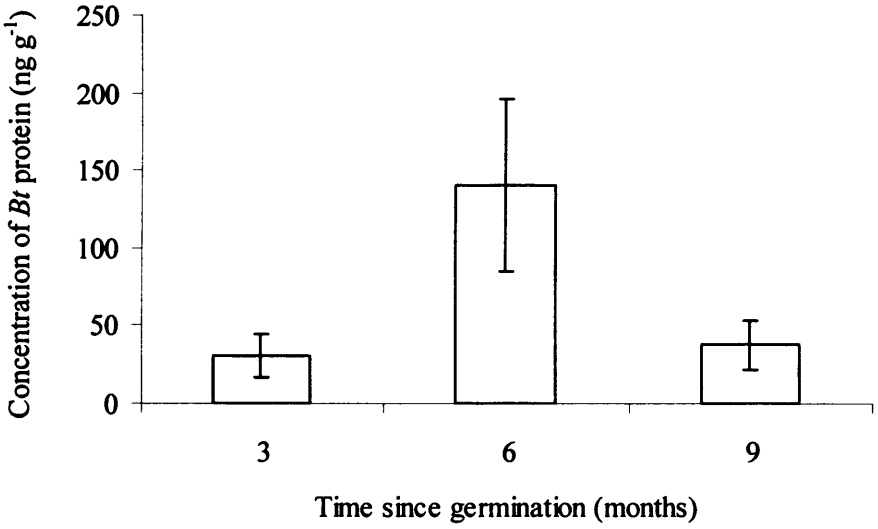

Figure 2.8 Concentration of Cry1Ac protein (ng g^{-1}, mean ± standard error) detected in 19 plants sampled at three, six and nine months old (all concentrations are above the ELISA threshold).

Table 2.4 Cry1Ac concentration (ng g^{-1}, mean ± standard error) in two different age leaves from each of four *Bt* brassicas (all concentrations are above the ELISA threshold).

Bt plant	Leaf at top of stem	Leaf near base of stem
1	4.42 ± 1.55	15.12 ± 3.77
2	257.53 ± 0.001	257.53 ± 0.001
3	206.63 ± 33.74	243.74 ± 13.80
4	256.51 ± 0.71	255.51 ± 2.03

Table 2.5 Cry1Ac concentration (ng g^{-1}, mean ± standard error) in four different areas of leaves from each of four *Bt* plants (all concentrations are above the ELISA threshold).

Leaf part	Concentration
Apex	176.14 ± 41.45
Edge	188.28 ± 39.91
Mid-leaf	185.71 ± 40.63
Vein	192.86 ± 41.66

2.4 Discussion

PCR and ELISA were used to determine which plants, grown from the seed obtained from Cornell University contained the *Bt* gene. PCR techniques enabled determination of the gene presence while the ELISA not only showed the gene was being transcribed and translated into a protein but also allowed quantification of the amount of protein produced. The expected ratio of 1:1 *Bt* to non-*Bt* plants grown from the mixed bag of seed is not supported by the chi-square statistical results from the PCR analysis; a 3:1 ratio is more likely (Section 2.3.1). Such a ratio could have occurred if two *Bt* plants were crossed; although the initial transformed *Bt* broccoli had a single gene insertion (Earle *et al.*, 1996), crossing two of these plants would produce 25 % of seed with two copies of the gene, 50 % with one copy and 25 % with none (Table 2.6). The differences observed in protein production with the ELISA could be related to the number of copies of the gene. Multiple gene copies can result in higher expression, for example, β-glucuronidase expression in oilseed rape (*Brassica napus* L. (Bavage *et al.*, 2002)) and it seems possible that those broccoli plants with two *Bt* gene copies would produce more *Bt* protein than those with one gene copy.

Table 2.6 Expected ratio of progeny if two transformed plant with a single insertion of the *Bt* gene were crossed to produced seed.

	Transformed plant		Transformed Plant	
Parents	Bt -		Bt -	
Genes	Bt	-	Bt	-
Progeny (seeds received)	Bt Bt	Bt -	Bt -	- -
	↘	↓	↙	↓
		Resistant		Susceptible

Multiple gene copies can also result in unusual gene expression patterns in further generations e.g. luciferase expression in backcrosses of one GM wheat (*Triticum aestivum* L.) line (of three tested) did not obey Mendelian genetics (Bourdon *et al.*, 2002) and nor did *Bt* protein expression in hybrids between *B. napa* and some GM events of *Bt* oilseed rape (Zhu *et al.*, 2004). The chi-square results from the ELISA (Section 2.3.2), which included a larger sample number than the PCR, did not conform to either the expected Cornell ratio 1:1 nor the 3:1 ratio suggested in Table 2.4. It is unclear therefore from these data whether the seeds received match the information provided with them. For this present study though, quantification of the protein that the test organism was being exposed to was more important than the gene copy number.

There was no variation in the protein levels within each broccoli plant. In GM oilseed rape Cry1Ac protein production did vary both between leaves on plants and over time, with the highest levels of *Bt* protein recorded at leaf emergence (Halfhill *et al.*, 2003) and at flowering time (Le *et al.*, 2007). This present ELISA study, however, did show a large variation in the concentration of protein between conspecific broccoli plants (Section 2.3.2). Environmental factors such as temperature (Olsen *et al.*, 2005; Le *et al.*, 2007) and drought (Traore *et al.*, 2000) have been shown to alter the concentration of *Bt* produced by *Bt* cotton (*Gossypium hirsutum* L.), oilseed rape and maize (*Zea mays* L.) respectively (also see review by Dong & Li, 2007) but the broccoli in this present study were all grown in the same environmental conditions.

The variation in *Bt* protein levels between individual plants raises a number of issues regarding the commercial viability of the seeds; primarily if the level of protein produced in some plants is not high enough to kill even partially resistant pests then resistance may

develop (Gould *et al.*, 2002). Cao *et al.* (2005) reported variability in the concentration of *Bt* protein (0-2000 ng g^{-1}) produced by individual *Bt* kale (*Brassica oleracea* var. *acephala* L.) plants and showed that the *Bt* kale with undetectable levels of Cry1Ac proteins suffered similar levels of damage as non-*Bt* kale. In their study the level of protein detected in the kale leaves (with ELISA) affected the level of resistance to a pest species; plants with the *Bt* gene (tested using PCR) but producing undetectable levels of protein (tested with an ELISA) suffered more damage from pests than those with a high level of protein. The efficacy of the *Bt* broccoli plants used in the present study, and their toxicity to three Lepidoptera species, is reported in Chapter 3.

Care should be taken in comparing Cao *et al.*'s study with the present investigation as in the latter the *Bt* broccoli plants are a back cross whilst Cao *et al.*'s study looked at protein levels in the initial transformed *Bt* kale plants and the *Bt* kale plants could also contain multiple copies of the gene. Multiple gene copies can result in altered expression in the next generation; crossing wheat plants that were homozygous for a luciferase gene and expressing luciferase at a high level resulted in GM plants expressing luciferase at levels between 0.22 and 0.5 times lower than the initial transformed wheat plants (Bourdon *et al.*, 2002). Gene expression can also be affected by genetic background; activity of β-glucurinidase was more varied (up to four fold) in F2 and backcross generations than in the original transformed line of GM white clover (*Trifolium alba* L. (Scott *et al*, 1998)) but *Bt* oilseed rape and *B rapa* hybrids had similar levels of *Bt* protein (0.9-3.1 μg g^{-1}) as the initial transformed plants (0.8-1.7 μg g^{-1} (Zhu *et al.*, 2004)).

This variation in expression seen in second generations (e.g. Scott *et al.*, 1998) is probably why single insertion events are often chosen for further breeding as described for the broccoli plants (Earle *et al.*, 1996). These broccoli plants are the result of investigations into brassica transformation techniques and *Bt* gene inheritance rather than commercial application. It is recommended that for a commercial GM broccoli one of the parent plants of the F1 Green Comet is transformed with the *Bt* gene. A GM plant could be selected with a single insertion and high expression. This transformed plant could then be crossed with the other parent plant resulting in a batch of seeds that are both *Bt* and Green Comet resulting in no background genetic variation and consistent *Bt* protein expression.

Bt proteins were detectable in *Bt* broccoli leaves throughout the plant (young and mature leaves, and roots) and over the plant's life (up to nine months and in senescent leaves). This suggests that the *Bt* protein is available to plant feeding organisms above and below ground, and to decomposers. Just over half (54%) of the plants that tested positive for *Bt* protein in their leaves also tested positive for the protein in their roots. It could be expected that 100% would test positive as the gene is under the control of the 35S constitutive plant promoter (Earle *et al.*, 1996), however, a high threshold was employed to confirm positives in the *Bt* samples calculated from the change of colour detected in non-*Bt* sample wells on the ELISA plate. This change of colour is probably due to cross reactivity of the *Bt* antibody with a substance in the root tissue, other than the *Bt* protein, that is not present in the leaves. As a consequence the threshold may have been sufficiently high to obscure positives in the *Bt* root samples.

Bt protein was detected at in compost in which a few of the *Bt* plants had been growing in very low quantities but above the threshold as defined in Section 2.2.5. These ELISA kits are designed for testing for the presence of *Bt* protein in seed and leaves and thus have not been tested for cross-reactivity with root and compost samples; this has been accounted for by calculating a threshold from the non-*Bt* samples but may be partly responsible for the low detection rates in the compost samples. To test more accurately the concentration of *Bt* protein released from Bt broccoli roots they could be grown hydroponically (e.g. Saxena *et al.*, 1999). Head *et al.* (2002) did not detect *Bt* protein in soil in which *Bt* cotton had been grown for three years, although there are many more environmental processes in action under field conditions which could remove the proteins from the soil than occur in plant pots. *Bt* proteins may have been present in Head *et al*'s. (2002) samples but at concentrations below their threshold which was higher (3.68 ng g^{-1}) than the one used here (0.01 ng g^{-1}). They analysed their samples having developed their own antibodies and ELISA so it may have been more suitable for detecting proteins in soil than the commercially available kit used in this study.

Bt proteins from root exudates of *Bt* maize were shown by Saxena *et al.* (1999) to enter the soil. Levels of 95 μg g^{-1} were calculated from larvicidal bioassays although SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) did not detect the protein in the same soil. Later, Saxena *et al.* (2004) showed through larvicidal assays and ELISA

(Envirologix, Portland, ME) that *Bt* proteins were not released from oilseed rape roots into the soil. This difference between maize and oilseed rape is, perhaps, unsurprising as the two species are not closely related. Interestingly, *Bt* protein was detected in some samples from around the roots of a different brassica in this present study.

The availability and toxicity of *Bt* proteins in substrate can vary with the type of soil (e.g. Tapp & Stotzky, 1995; 1998) which could explain the discrepancies observed between studies, along with the different monitoring methods. Albeit at low levels, it is likely that decomposers and other soil inhabiting organisms are exposed to *Bt* proteins, either through the presence of roots and fallen leaves of *Bt* plants or through incorporation of plant material post-harvest. The effects of the *Bt* protein on some of these organisms are investigated further in Chapter 4.

As a consequence of the studies reported in this chapter throughout the rest of study (and thesis) plants derived from the Cornell University seeds were tested for the presence of the *Bt* protein. Only those plants testing positive in the ELISA (standardised as described in Section 2.2.5) were used in tests with target and non-target species. As this thesis focuses on the possible effects of *Bt* protein from GM plants the Green Comet plants were used as the control. This choice of control could potentially be criticised as the F1 Green Comet plants are all similar whilst the *Bt* plants are genetically mixed and comparable to a F2 generation (Table 2.2). It could be argued that it would be preferable to cross Green Comet with the *Bt* seeds, test them all for *Bt* protein production and then compare the effects of the *Bt* and non-*Bt* plants in this third generation (R. Collier, pers. comm.). These F3 broccoli plants could certainly be considered more equivalent (i.e. contain a similar amount of background variability other than *Bt* protein production) for comparison as a control but would have taken to long to produce. It was deemed more appropriate for this study to monitor the level of protein produced by these plants and, therefore, the potential levels of exposure to organisms. In this way it was hoped that a clearer picture would be produced of what would happen in the field if *Bt* broccoli plants, and their potential offspring from crosses with non-GM species, were grown.

3 Investigating the effects of *Bt* broccoli plants on three target Lepidoptera species

3.1 Introduction

The larval stage of *Plutella xylostella* L., the diamond back moth (Lepidoptera), is a very destructive pest of crucifer plant species throughout the world. Control of *P. xylostella* larvae is difficult. The species has developed resistance to many pesticides including *Bacillus thuringiensis* Berliner (*Bt*) biopesticide sprays, and the control costs are estimated to be US $1 billion annually (Talekar, 1992). *Bt* resistance was first reported in Indonesia (Tabashnik *et al.*, 1990) and was attributed to intensive insecticide application. Potentially, resistance to *Bt* plants could emerge in the future (Tabashnik *et al.*, 2003) and various strategies have been developed and deployed to delay *P. xylostella,* and other insects, acquiring resistance (Section 1.4.3). To date these strategies appear to have been successful with resistance only being reported in laboratory selection experiments (e.g. Ferre & Van Rie, 2002). The lack of reported resistance in the field is just one of the many reasons which makes the planting of *Bt* crops very attractive in comparison to the use of more toxic chemical pesticides (further reasons are discussed in Section 1.4.2); many *Bt* plant species are either already being planted commercially or are at the development stage.

The *Bt* broccoli (*Brassica olearcea* L. var. *italica* Plenk) plants used in this study were specifically genetically engineered to be active against *P. xylostella* by production of the Cry1Ac *Bt* protein (Section 2.1.1). The Cry1Ac protein is reportedly active against a wide spectrum of Lepidoptera and the *cry1Ac* gene has also been inserted into other plant species to defend against attack by other Lepidoptera pests. For example, the gene was used in the creation of *Bt* rice (*Oryza sativa* subsp. *indica* L.) to confer resistance against *Scirpophaga incertulas* Walker (Lepidoptera) (Nayak *et al.*, 1997) and *Bt* loblolly pine (*Pinus taeda*, L.) for resistance to *Dendrolimus punctatus* Walker (Lepidoptera) and *Crypyothelea formosicola* Staud (Lepidoptera) (Tang and Tian, 2003).

Different species of Lepidoptera require different concentrations of *Bt* proteins to cause death (Table 3.1), and to prevent insects developing resistance the *Bt* protein dose within a plant must be sufficient to kill even partially resistant insects (Gould *et al.*, 2002). When targeting

more than one pest species, the concentration of protein present within the plant must be carefully considered. Other factors and mechanisms for the development and prevention of resistance to *Bt* proteins are described in Section 1.4.3.

Table 3.1 The 50% lethal concentration values for three different Lepidoptera feeding on three different Cry1 *Bt* proteins.

Cry Protein	*Manduca sexta*	*Heliothis virescens* (ng cm^{-2})	*Mamestra brassicae*
Cry1Aa	5.2	90	165
Cry1Ab	8.6	10	162
Cry1Ac	5.3	1.6	2000

(adapted from Höfte and Whitley, 1989)

Individual *Bt* broccoli plants used in this study expressed different concentrations of *Bt* protein (Section 2.3.2). This is likely to result in differing degrees of control, for example, different concentration of *Bt* protein in kale (*Brassica oleracea* var. *acephela* L) resulted in different levels of pest control (Cao *et al.*, 2005). If all the plants in a field do not express a consistently high enough level of protein to kill the partially resistant pests, resistance could evolve throughout the population (Gould *et al.*, 2002). To explore the *Bt* concentration variability further, in this current study, the effectiveness of the *Bt* broccoli plants against various Lepidoptera species was investigated.

Two Lepidoptera species were initially chosen for investigation, *Mamestra brassicae* L. and *Pieris brassicae* L. The larvae of these species cause extensive damage to the leaves of brassicas and are major agricultural pests. As this study is centred around the effects on soil-dwelling organisms caused by the exudation of Cry1Ac proteins from *Bt* plant roots, a third Lepidoptera species was also chosen: *Agrotis segetum* (Denis & Schiff). The larvae of this species live in the soil and cause extensive damage to the root system of many plants. It was hypothesised that feeding on *Bt* broccoli plants would cause the larvae of all three species to die.

3.2 Methods

3.2.1 *Agrotis segetum* culturing

Agrotis segetum eggs and an artificial food block (H. Bathon, Germany) were placed in a plastic container (12 x 12 x 7 cm) for two weeks. Once large enough to handle, the larvae were separated into ten larvae per container and fed on 5 cm^2 portions of swede (*Brassica napus* L.). A sheet of tissue paper (Kimwipe) was also added to maintain moisture levels. Every two weeks larvae were placed in a clean container, and swede sections added as necessary. Pupae were placed in larger plastic containers (20 x 15 x 15 cm) and once the adults hatched they were provided with cotton wool soaked in 10% honey solution. Eggs were generally laid around the container lids which were then placed in a new container to raise the larvae. All culturing was performed at 20°C in a controlled environment room (16:8 h light:dark cycle).

3.2.2 *Agrotis segetum* experimental design

Leaf discs were taken from *Bt* plants (Section 2.2.2) and were processed using an ELISA kit (Section 2.2.5) to determine the concentration of *Bt* protein to which the larvae were exposed, only plants with concentration higher than three standard deviations of the mean of non-*Bt* plant samples were used. This threshold of three standard deviations of the mean was chosen as those below the threshold were shown, using PCR, not to contain the *Bt* gene (Section 2.3.1). Five second instar larvae were placed on the surface of the compost at the base of ten three month old broccoli plants, five *Bt* and five non-*Bt,* grown as described in Section 2.2.1. Leaves from the plant were placed on the soil surface covering the larvae to provide some initial food and shade. The plants were watered every two days (by filling plant pot saucers to avoid drowning the larvae as they moved into the compost). After seven days the larvae were removed from the plants and compost, and weighed. All the larvae from each treatment were placed in two plastic containers (12 x 12 x 5 cm) and continued to be fed leaves from the respective treatment till pupation; adult hatching rates were then monitored.

3.2.3 *Mamestra brassicae* culturing

Mamestra brassicae eggs derived from a culture at the Centre for Ecology and Hydrology (Oxford, UK) were placed in plastic containers (12 x 12 x 7 cm) and, when the larvae

hatched, cabbage leaves added. When the larvae reached their second instar, tissue paper (Kimwipe) was also added to absorb excess moisture and the larvae were separated, ten per container. Once pupae formed they were transferred to a larger rearing cage (30 x 30 x 100 cm). This contained cotton wool soaked in 10% honey solution for the adults to feed on, and a cabbage plant for the females to lay eggs on. The culture was then maintained in these rearing cages, occasionally removing excess frass, replenishing the cotton wool and changing the cabbage plant when the leaves were depleted. As the culture grew pupae were moved into new rearing cages. All culturing was performed at 20°C in a controlled environment room (16:8 hr light:dark cycle). The cabbage leaves came from "Advantage" spring cabbage plants (HDRA, Coventry, U.K.) that had been grown under the same conditions as the broccoli plants (Section 2.2.1).

3.2.4 *Mamestra brassicae* experimental design

0.75 g and 15 g Dipel® were added to two bottles of 1 litre of deionised water. This gave a concentration of Dipel® at the manufacturer's recommended dose (Dipel® recommended), and 20 times the recommended dose (Dipel® x 20), respectively. Non-*Bt* leaf discs were soaked in these two suspensions for 20 minutes before air drying for 30 minutes. Twenty small (5 cm diameter) Petri dishes, each containing two, seven day old *M. brassicae* larvae, were placed in a controlled environment room (20°C, 16:8 h light:dark cycle) for 48 h. Two leaf discs (Section 2.2.2) were added to each of the dishes; five of the replicates contained non-*Bt* leaf discs and five contained *Bt* leaf discs from five different plants with concentrations already determined to be above the ELISA threshold (Section 3.2.2). A further five dishes contained Dipel® recommended and Dipel® x 20 leaf discs. After 24 h two further leaf discs of the appropriate treatment were added, and after 48 h the percentage of leaf discs eaten was estimated and the larvae weighed. The larvae and remaining Dipel® leaf discs were frozen at -20°C and processed using the ELISA (Section 2.2.5).

3.2.5 *Pieris brassicae* culturing

Eggs were obtained from Blades Biologicals (Edenbridge, Kent, U.K.) and maintained in plastic containers. When the first larva emerged cabbage leaves were added to the container. Once three days old, the larvae were divided with ten larvae per container (12 x 12 x 7 cm), and fed cabbage (*Brassica oleracea* var. *capitata* L.) leaves. A sheet of tissue paper

(Kimwipe) was added to maintain moisture levels. When the larvae pupated they were transferred to a large rearing cage and raised as described for *M. brassicae* (Section 3.2.3). All culturing was performed at 20°C in a controlled environment room (16:8 h light:dark cycle) and similar cabbage leaves to those used for the *M. brassicae* culturing were used (Section 3.2.3).

3.2.6 *Pieris brassicae* experimental design

3.2.6.1 *Pieris brassicae* and *Bt* broccoli leaf discs

15 g Dipel® was added to 1 l of deionised water. This gave a concentration of Dipel® at 20 times the manufacturer's recommended dose (Dipel® x 20). The Dipel® recommended dose was not used in this test on the basis of no mortality being observed with *M. brassicae* (Section 3.3.2). Non-*Bt* leaf discs were soaked in this suspension for 20 minutes before air drying for 30 minutes. Five, three day old larvae were placed in 18 small (5 cm diameter) Petri dishes with two leaf discs (Section 2.2.2). Six of the dishes contained *Bt* leaf discs (already determined by ELISA to have a concentration of *Bt* protein above the threshold, Section 3.2.2), six non-*Bt* leaf discs and six Dipel® x 20 treated leaf discs. The larvae were allowed to feed for five days adding new leaf discs as necessary; the number of surviving larvae were counted each day.

3.2.6.2 *Pieris brassicae* and *Bt* broccoli plants

Four month old broccoli plants grown from the mixed bag of seed were tested for the concentration of Cry1Ac protein produced in their leaves using an ELISA (Section 2.2.5). Four *Bt* plants with *Bt* protein in their leaves at a higher concentration than three standard deviations of the mean of non-*Bt* plant samples (*Bt* +) were selected for experimental purposes, as were four with concentrations below this level (*Bt* -). Four non-*Bt* Green Comet broccoli plants were also selected (non-*Bt*). Note that in all considerations henceforth for *P. brassicae* experiments non-*Bt* refers to Green Comet and *Bt* + and *Bt* – to the two categories of plants grown from the mixed bag of seed provided by Cornell University. Seven, three day old, *P. brassicae* larvae were then placed on each plant and allowed to feed for seven days. Recovered larvae were weighed and processed individually using the ELISA kit (Section 2.2.5). The low weight of the larvae recovered led to the first extraction step being adjusted so that the samples were extracted in 250 µl rather than 500 µl of extraction buffer.

3.2.7 Data analysis

The ELISA kit also provided *Bt* solutions of standard concentrations and these were loaded onto the ELISA plate with the samples. The optical densities of these wells were used to construct calibration curves (Section 2.2.5). The equations of the calibration curves were used to calculate the concentration of *Bt* proteins in leaves and Lepidoptera samples, adjusting for differences in sample weights (as described in Appendix 1). Samples were only designated as positive if the optical density was greater than the mean plus three standard deviations of the optical density of non-*Bt* samples. Analysis of variance (ANOVA) tests were used to determine whether there were any significant differences between the treatments in the number of larvae recovered, their weights, the optical density of ELISA processed samples and the concentration of protein in samples (Minitab v14.1, Minitab Inc., PA, USA). If the data did not conform to the assumptions of normality and equal variances, they were transformed appropriately. Kruskal-Wallis tests were used where the data could not be transformed to conform to the assumptions of ANOVA. *Post-hoc* tests were used to detect where differences existed; a least significant difference (LSD) value was calculated after ANOVA and Mann-Whitney tests after a Kruskal–Wallis using Bonferroni's approximation. Pearson's correlation co-efficient was used to see if there was a relationship between the optical density and/or concentration of *Bt* protein in the leaves and larvae.

3.3 Results

3.3.1 *Agrotis segetum* results

All larvae survived on both *Bt* and non-*Bt* plants. Many larvae had burrowed into the soil and it is assumed they were feeding on the plant roots as well as the leaves supplied. The mean weight of the five larvae from each plant was calculated and these data analysed using each plant as a replicate to avoid pseudoreplication. There was no significant difference in the weights of the larvae (T_8=1.59, P=0.150) across treatments although the mean weight of the larvae feeding on the *Bt* plants were numerically heavier (811.4 ± 133.6 mg) than those feeding on the non-*Bt* plants (581.4 ± 54.5 mg). All the *Bt* plants tested positive for *Bt* protein (i.e. greater than three standard deviations of the mean of non-*Bt* samples) but contained different concentrations of the Cry1Ac protein, ranging from 2 to 86 ng g^{-1}. A second ANOVA on the weights of the larvae feeding on just the *Bt* plants showed a

marginally non-significant difference between the weights of the larvae on each plant ($F_{4,24}$=2.84, P=0.055, Figure 3.1). There was no correlation between the weights of the larvae feeding on each *Bt* plant and the concentration of *Bt* in the plant (R^2= -0.219, P=0.316). There was also no significant difference between treatments for the number of larvae pupating or the number of adults hatching (X^2_1=1.125 and 0.153, respectively, P>0.05 in both cases).

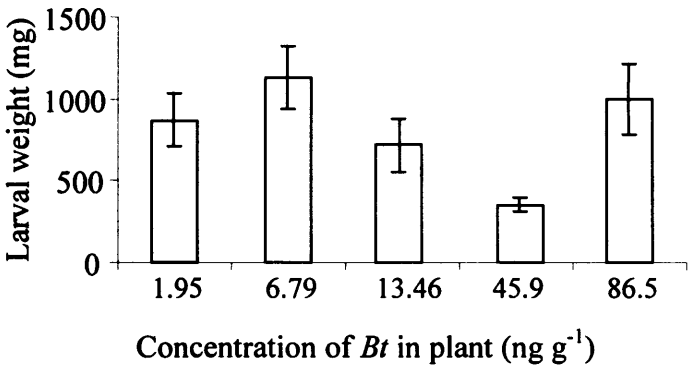

Figure 3.1 Weight of *Agrotis segetum* larvae (mean ± standard error) feeding on five *Bt* plants with different concentrations of *Bt* protein.

3.3.2 *Mamestra brassicae* results

After 24 h the larvae feeding on the Dipel® x 20 treated leaves were lethargic and the anterior halves of their bodies were a darker green than the posterior halves. The larvae in the other treatments were active and were a consistent colour along their length. After 48 h, five of the larvae feeding on the Dipel® x 20 treated leaf discs had died but all of those larvae feeding on the Dipel® recommended, the *Bt* and the non-*Bt* leaf discs survived (Figure 3.2).

There was a significant difference in the percentage of leaf discs eaten between the treatments ($F_{3,19}$=14.67, P<0.001). The arcsine transformed data set did not conform to the assumptions of ANOVA. Larvae feeding on the Dipel® x 20 treated leaf discs had eaten significantly less of the leaf discs than larvae from the other three treatments (LSD=0.0096, P<0.05, Figure 3.3). There was also a significant difference between the weights of the larvae from the four treatments ($F_{3,19}$=5.13, P=0.008). The LSD tests show that larvae feeding on the *Bt* leaf discs weighed significantly more than those feeding on the non-*Bt* and Dipel® x 20 treated leaf

discs, but not more than those on the Dipel® recommended treated leaf discs (LSD=0.076, P<0.05, Figure 3.4).

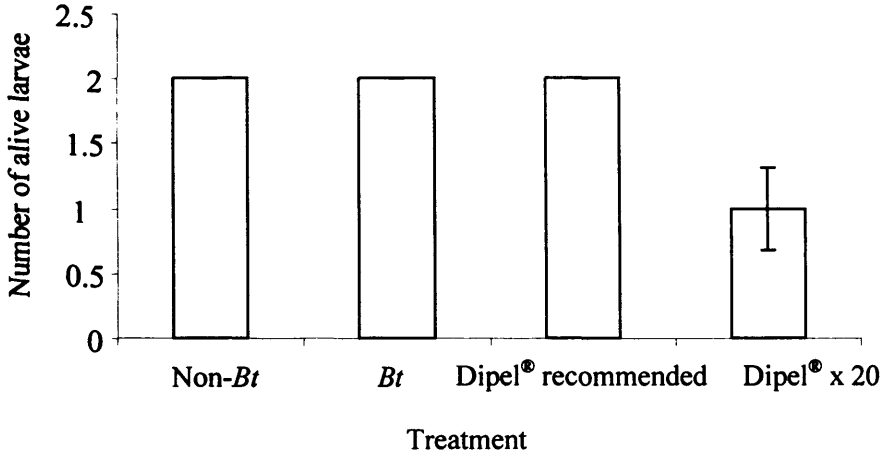

Figure 3.2 Number of *Mamestra brassicae* larvae alive (mean ± standard error) after 48 h of feeding. All larvae were alive in all replicates for non-*Bt*, *Bt* and Dipel® recommended treatments.

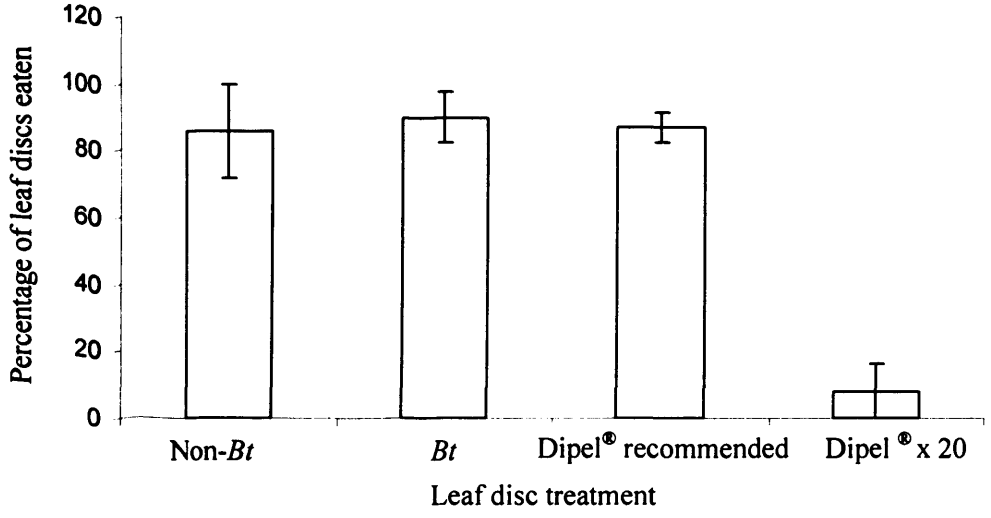

Figure 3.3 Percentage of leaf discs eaten (mean ± standard error) by two *Mamestra brassicae* larvae. Lowercase letters indicate where significant differences lie between the treatments (P<0.05).

Leaf discs from each plant were found to be positive for *Bt* proteins but again contained different concentrations of protein (110.6 ± 66.7 ng g^{-1}). The concentration of *Bt* proteins for the Dipel® treatments were 1.46 (Dipel® recommended) and 80.3 (Dipel® x 20) ng g^{-1}.

All of the larvae feeding on the Dipel® treatments tested positive for *Bt* proteins (i.e. greater than three standard deviations of the mean optical density of larvae feeding on non-*Bt* samples) but three of the ten larvae feeding on *Bt* plants proved negative. The ELISA results show that there was a difference between the optical densities of the wells which contained samples from the larvae in the different treatments ($H_{3,39}$=16.59, P=0.002). When the concentrations of *Bt* protein are calculated, adjusting for the difference in weights between the larvae, a large variation (55.06 ± 33.49 ng g^{-1}) in the concentration of protein is detected in the larvae feeding on the *Bt* leaves (Figure 3.5). A significant difference is found between the treatments ($H_{3,39}$=27.86, P<0.001); those larvae feeding on the non-*Bt* leaves have a significantly lower concentration of protein than those feeding on the two Dipel® treatments (P<0.05) but not the *Bt* plant treatment ($W_{1,19}$=1083.5, P=0.121, Figure 3.5). There was no correlation between the concentration of *Bt* protein detected in the *Bt* leaf discs and the amount detected in the larvae (R^2=0.484, P=0.271, with log transformations).

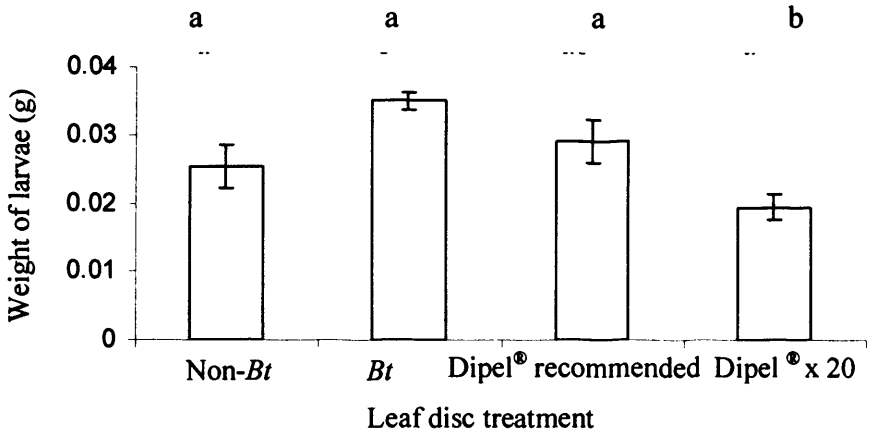

Figure 3.4 Weight of *Mamestra brassicae* larvae (mean ± standard error) feeding on different leaf disc treatments. Lowercase letters indicate where significant differences lie between the treatments (P<0.05).

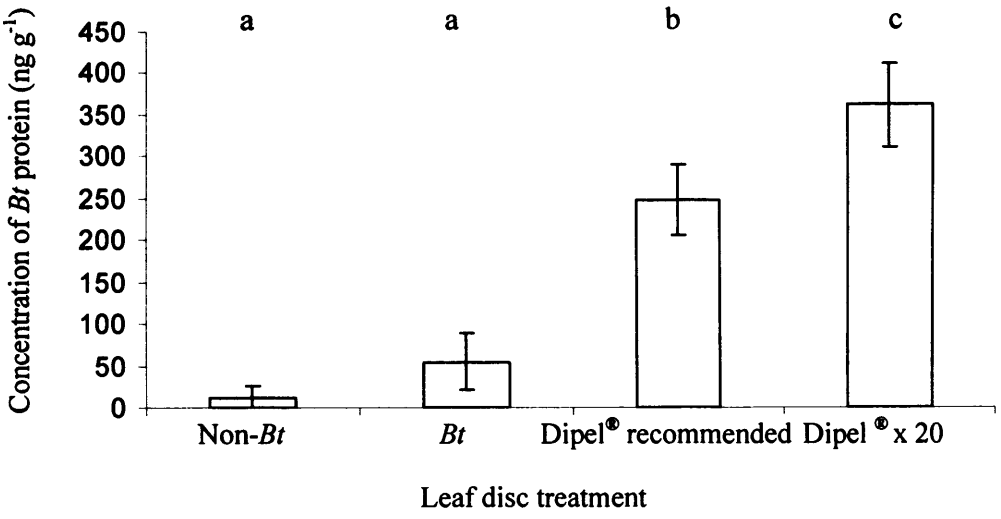

Figure 3.5 Concentration of *Bt* protein (mean ± standard error) in *Mamestra brassicae* larvae. Lowercase letters indicate where significant differences lie between the treatments (P<0.05).

3.3.3 *Pieris brassicae* results

3.3.3.1 *Pieris brassicae* and *Bt* broccoli leaf discs

All larvae had died in the Dipel® x 20 treatment and in the *Bt* leaf treatment by the third and fifth day, respectively; some of the larvae that were feeding on the non-*Bt* leaves survived (Figure 3.6). Dipel® x 20 treated leaf discs contained 80.3 ± 6.5 ng g^{-1} *Bt* proteins and the *Bt* leaf discs 14 ± 5.3 ng g^{-1}.

3.3.3.2 *Pieris brassicae* and *Bt* broccoli plants

There were many cadavers in the pot saucers but these deaths cannot be attributed with certainty to the *Bt* protein as the larvae may have fallen from the plant and drowned. Of the larvae that were found on the soil or still on the plant, those on the non-*Bt* plants and the *Bt* - plants (plants from the mixed bag of seed that tested negative for *Bt* proteins with the ELISA) were recovered alive. All of those feeding on the *Bt* + plants (plants from the mixed bag that tested positive for *Bt* proteins with the ELISA) were dead. There was a significant difference between the treatments in the number of surviving larvae (square root transformation,

$F_{2,11}$=8.68, P=0.008) with fewer larvae alive on the *Bt* + plants than on the *Bt* - and non-*Bt* plants (LSD=1.82, P<0.05, Figure 3.7).

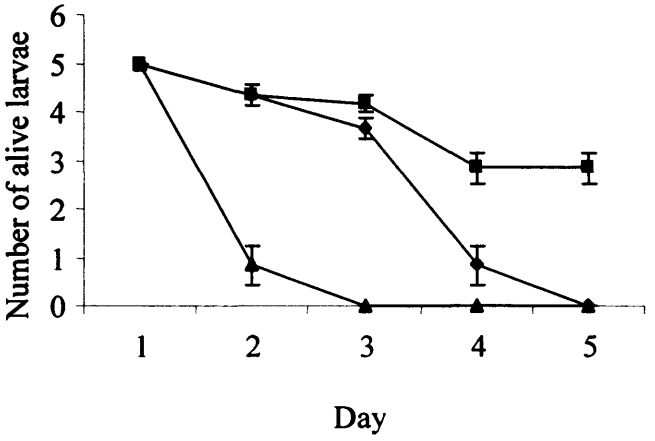

Figure 3.6 Number of *Pieris brassicae* larvae (mean ± standard error) counted alive over five days whilst feeding on *Bt* (♦), Dipel® treated (▲) and non-*Bt* (■) leaf discs.

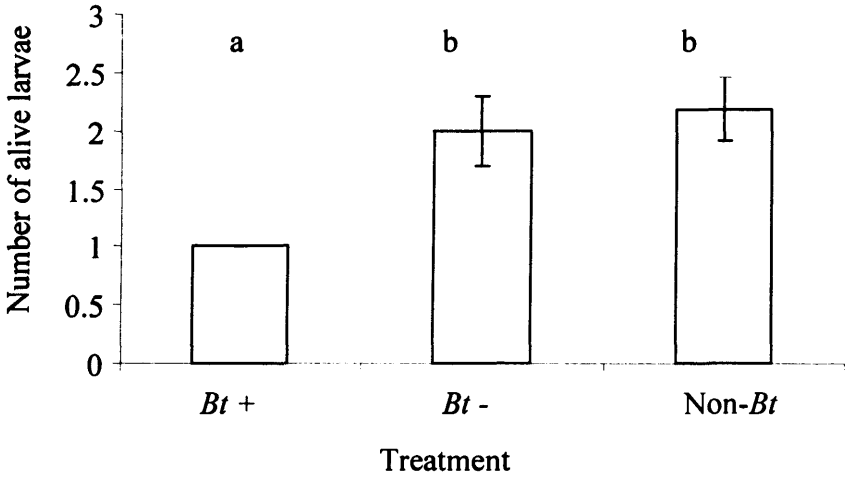

Figure 3.7 Number of *Pieris brassicae* larvae alive (square root transformation) after five days of feeding on plants (mean ± standard error). Lowercase letters indicate where significant differences lie between the treatments (P<0.05). *Bt* + and *Bt* – are plants grown from the mixed bag of seed that tested positive and negative, respectively, for *Bt* proteins with the ELISA, non-*Bt* plants are Green Comet.

There was also a significant difference in the weights of the larvae recovered from the treatments ($F_{2,31}$=4.90, P=0.014). The *post-hoc* tests showed that the larvae feeding on non-*Bt* leaves were significantly heavier than the dead larvae that had been feeding on the *Bt* + plants (LSD=2.50, P<0.05), but the larvae feeding on *Bt* - plants were not significantly different from the larvae in the other two treatments (Figure 3.8). The mean weight of larvae collected from each plant was used as a replicate in each treatment to avoid pseudoreplication.

Statistical analysis of the ELISA results showed that there was a significant difference between the optical density of wells containing samples from the three treatments ($F_{2,31}$=61.6, P<0.001). *Post-hoc* tests shows that the three treatments were all different to each other (LSD=10.84, P<0.05, Figure 3.9); the larvae found dead on the *Bt* + leaves had a higher optical density than the larvae feeding on the *Bt* - leaves which also had significantly higher optical density than the larvae feeding on the non-*Bt* leaves. Calculation of the concentration of protein in the larvae using the ELISA kit standards (Section 3.2.7) controlled for the difference in weights detected between the treatments and it was found that there is also a significant difference in the concentration of protein found in the larvae ($H_{2,31}$=17.54, P<0.001). Those larvae feeding on *Bt* + plants had a considerably higher concentration of *Bt* proteins in them (85.72 ± 38.32 ng g^{-1}) than the other two treatments (all larvae calculated as containing 0 ng g^{-1}).

The ELISA also showed that there was a difference in the concentration of protein in each of the four *Bt* + plants (range 2.8 to 27 ng g^{-1}). There was no statistically significant correlation between the concentration of the protein detected in the *Bt* + plants and that detected in the larvae (R^2=-0.405, P=0.596).

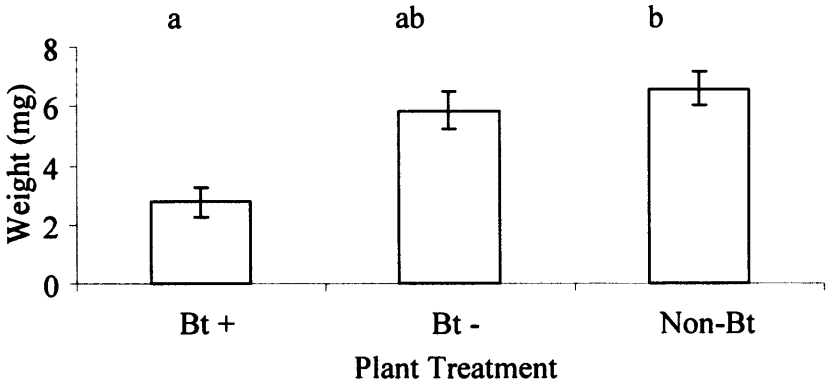

Figure 3.8 Weight of *Pieris brassicae* larvae (mean ± standard error) recovered from plants. Lowercase letters indicate where significant differences lie between the treatments (P<0.05). *Bt* + and *Bt* - are plants grown from the mixed bag of seed that tested positive and negative for *Bt* proteins with the ELISA, non-*Bt* plants are Green Comet.

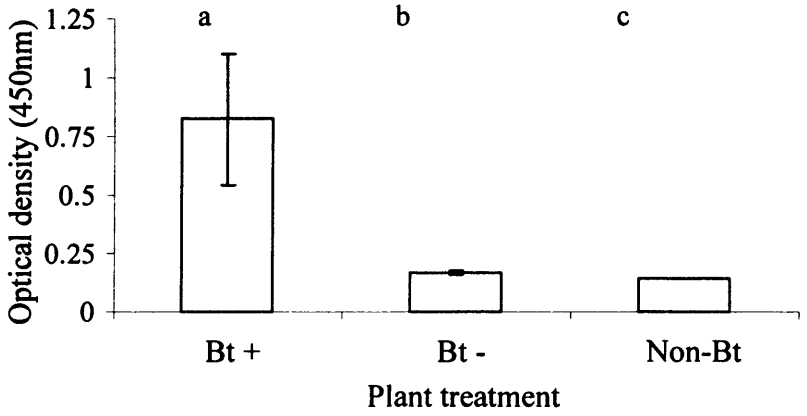

Figure 3.9 Optical density (mean ± standard error) of *Pieris brassicae* larvae processed using the ELISA kit. Lowercase letters indicate where significant differences lie between the treatments (P<0.05). *Bt* + and *Bt* – are plants grown from the mixed bag of seed that tested positive and negative for *Bt* proteins with the ELISA, non-*Bt* plants are Green Comet. The optical densities were not converted into concentrations as all larvae feeding on both *Bt*- and non-*Bt* leaf discs were calculated to have no protein in them making statistical analysis difficult.

3.4 Discussion

These three target species experiments were preliminary to aid the investigations into the plant *Bt* levels in Chapter 2 and, because the main aim of this study was not to investigate the control of target species but to look at the effect of *Bt* plants on non-target species, they were not repeated. Only one of the three Lepidoptera species tested in these experiments died after feeding on the leaves of *Bt* broccoli plants. *Pieris brassicae* larvae were killed by *Bt* plants expressing *Bt* proteins at 2.8 - 27 ng g^{-1} but *M. brassicae* and *A. segetum* larvae were not killed by *Bt* plants containing higher concentrations of *Bt* protein. Some of the surviving *M. brassicae* larvae in the Dipel® recommended (1.46 ng g^{-1}) and Dipel® x 20 (80.3 ng g^{-1}) treatments did, however, appear limp and lethargic as though they were beginning to suffer the effects of the *Bt* protein (i.e. gut paralysis and cessation of feeding) as indicated by the significantly lower percentage of leaf discs eaten on the Dipel® x 20 treated leaves (Figure 3.3). *Pieris brassicae* larvae died after between two and five days of exposure to *Bt* protein; a longer or higher exposure to the Dipel® recommended treatment or *Bt* leaves may also have killed the *M. brassicae* larvae (although two and five days have been recommended as a suitable length of exposure for other target Lepidoptera species e.g. *P. xylostella* (Mohan & Gujar, 2001) and *Scirpophaga incertulas* (Nayak *et al.*, 1997)). A review of *Bt* brassicas (Earle *et al.*, 2004) suggests that the modified plants are capable of controlling *Brassica* specialists such as *P. brassicae* but not more generalist Lepidoptera species (including *M. brassicae* and *A. segetum*), and that the control of generalists depends on the *Bt* protein levels in the plant. In this study, where concentrations of *Bt* protein vary it is perhaps not surprising that control of the three different species also varies.

Higher concentrations of Cry1 *Bt* proteins are required to cause mortalities in *M. brassicae* than two other Lepidoptera (Table 3.1; Höfte and Whiteley, 1989) and different *Bt* proteins have different lethal doses for *Helicoverpa armigera* Hübner (Liao *et al.*, 2002). If different species require different concentrations of protein before death ensues this could explain why the Dipel® x 20 leaf discs caused mortality in *M. brassicae* larvae, but the *Bt* and Dipel® recommended leaf discs did not. Dipel® does, however, contain a combination of toxic compounds (including other Cry proteins and exotoxins produced by bacteria that have regenerated from spores, Section 1.2.2) and it could have been one of these, and not just the Cry1Ac proteins, that caused the mortality of *M. brassicae*. This would explain why no

negative effects were observed in the larvae feeding on the *Bt* leaf discs. To test for this, the components of Dipel® would have to be separated using protocols such as sucrose density centrifugation (e.g. Liao *et al.*, 2002). It would also be beneficial to investigate the effects of these two concentrations of Dipel® on the third species tested, *A. segetum*.

Mamestra brassicae larvae feeding on *Bt* plants were heavier after just two days than those feeding on non-*Bt* plants. This is a relatively short period in which to find a significant result. Larvae feeding on *Bt* leaves may have been heavier because their digestive system had become paralysed due to the effects of the protein (Section 1.2.2) resulting in their guts being more full than those feeding on non-*Bt* leaves which would have egested frass. Yet, unlike those larvae feeding on Dipel® x20 treated leaves, there was no observed lethargy (Section 3.3.2). This increase in weight was also detected in slugs (*Deroceras reticulatum* Müller, Gastropod) and woodlice (*Porcellio scaber* Latrielle, Isopoda) feeding on *Bt* broccoli leaves (Chapter 4) and has been detected in woodlice feeding on *Bt* maize (*Zea mays*, L.) (Escher *et al.*, 2000). The results for recommended Dipel® treated leaf discs and *Bt* leaf discs were not significantly different for any of the *M. brassicae* parameters measured. This suggests a direct effect of the *Bt* protein on larval weights and it is only high concentrations of *Bt* protein (Dipel® x 20) that cause mortality to *M. brassicae*

Escher *et al.* (2000) hypothesised that differences in woodlice (*P. scaber*), a non-target species, weight were related to the lower lignin content of *Bt* maize leaves than non-*Bt* maize. It would be beneficial to investigate the nutritional value of the *Bt* broccoli to see if this could also be the cause of the increase in *M. brassicae* weights. The change in nutritional value could be related to the transformation process, insertion of the *Bt* gene could disrupt other plant metabolic pathways (reviewed in Filipecki & Malepszy, 2006), or the method of breeding of the *Bt* broccoli plants with the Green Comet plants to create seeds (Section 2.1.1). Breeding would result in differences other than the presence of the *Bt* gene between the plants (Table 2.2). If the *Bt* gene segregates with a gene of nutritional value then there would be a link between *Bt* content and, for example, lignin content.

In a longer term experiment (through to pupation, Sayyed *et al.*, 2003) showed that *P. xylostella* larvae feeding on cabbage leaf discs treated with purified Cry1Ac had a significantly higher pupal weight than those feeding on leaves without the added Cry1Ac protein. Sayyed *et al.*'s *P. xylostella* populations had been selected to be resistant and the

authors speculate that resistant larvae may actually use the protein as an additional food source. The *M. brassicae* culture used in this study had not, as far as I am aware, been previously exposed to *Bt* proteins and/or Dipel®. The long term effect of *Bt* proteins was not monitored for *M. brassicae* but it was shown that there was no effect of the *Bt* plants on the long term development of the *A. segetum* larvae (pupation and adult hatching, Section 3.3.1). The use of a purified *Bt* protein by Sayyed *et al.* (2003) rather than a transgenic plant could be the critical point and a review (Tabashnik & Carriere, 2004) of the effects of *Bt* plants shows that all other scientific studies point to there being only negative effects on pest species rather than the apparent positive increase in weight seen in this study.

The three Lepidoptera species tested in these experiments are pests of broccoli and other brassicas. If they are not killed by the *Bt* protein when present in low concentrations (i.e. are resistant to a low dose) it is possible that the populations would develop resistance; Gould *et al.* (2002) suggested that a plant had to control even partially resistant individuals. The emergence of resistant populations is a potential problem especially when it is considered that a large proportion of the plants from the mixed bag of seed were expressing a low concentration of *Bt* proteins (Section 2.3.2). Some of these plants would not provide control of *P. brassicae,* which would appear to be the most susceptible of the three species tested to *Bt* proteins: mortality was seen on plants expressing *Bt* proteins at 2.8 ng g^{-1}.

Resistance to Cry1Ac proteins in GM plants could have wider implications. Cry proteins often share common receptors in the larval gut, for example, the Cry1Ab toxin binding site in the guts of three common Lepidoptera pests (*M. brassicae*, *Phthorimaea operculella* Zeller and *Spodoptera exigua* Hübner) are shared with the binding site of the Cry1Aa and Cry1Ac toxins (Escriche *et al.*, 1997). As resistance can evolve by an alteration in the structure of the receptors (Van Rie *et al.*, 1990), resistance to one protein could potentially result in resistance to several others. This has been shown to be true for *Ostrinia nubilalis* (Hübner) and *P. xylostella* in laboratory experiments. *Ostrinia nubilalis* resistant to Cry1Ab proteins showed cross resistance to Cry1Ac and CryF proteins (Siqueira *et al.*, 2004), whilst *P. xylostella* with resistance to Cry1C proteins also displayed resistance to Cry1Ac proteins (Zhao *et al.*, 2001). In this latter study, however, another population of *P. xylostella* did not display cross resistance, so cross resistance development may depend on how the resistance is brought about.

The ELISA kit used in this present study can not only be used to detect *Bt* proteins in plant material but also in Lepidoptera species that have been feeding on *Bt* plants. Although no correlation was detected between the concentration of *Bt* protein in the larvae and the plants they had been feeding on, with a larger data set regression analysis may be possible and provide information about a link between the concentrations in plants and Lepidoptera larvae. ELISA could also be useful when analysing exposure of non-target Lepidoptera species but other studies have only used ELISA to detect *Bt* proteins in groups other than Lepidoptera (e.g. predators; Harwood *et al.*, 2005). *Agrotis segetum* larvae were observed to burrow into the compost and may be exposed to proteins in the root tissue and compost from root exudates (Saxena & Stotzky, 1999). No measurements were, however, taken to determine the level of exposure; in future studies this would be an important piece of additional information. All samples tested were wet weights, which is generally discouraged as differences in water content of the three species could compromise results. The use of dry weights, however, could also be criticised as the Cry1Ac antigen, to which the ELISA antibody binds, could be altered during this process making the ELISA inefficient. Leaf disc removal from the plants could also result in the toxin degrading during the experimental period; Cry1Ab proteins in rice were found to decline by 80% over a five month storage period at 37 °C with most of the decrease in concentration occurring in the first month (Zaidi *et al.*, 2005). In these experiments the leaf discs were tested at most 48 h after removal from the *Bt* broccoli plants.

It would be interesting to test other Lepidoptera species, especially the target species *P. xylostella,* to determine if further differences in mortality can be detected. Control of *P. xylostella* by 50% of the *Bt* plants has already been reported in the laboratory (Earle *et al.*, 1996, Section 2.1.1). If the target species is suppressed by the *Bt* plants in the field, the apparent tolerance of *M. brassicae* and *A. segetum* to *Bt* plants seen in this study could lead to concerns about secondary pest outbreaks that would require application of pesticides (Section 1.4.2). A more in-depth study investigating the effects of *Bt* proteins, from both GM plants and Dipel® at various concentrations, would also be beneficial, not only over the short term (i.e. mortality and weight), but also over the long term (i.e. pupation, adult hatching rates and future generations). Effects would need to be compared with those of different broccoli cultivars and the *Bt* – plants (Section 3.2.6.2) to determine whether it was an effect of the *Bt* protein itself or differences in metabolites caused by the GM process (reviewed in Filipecki

& Malepszy, 2006), or by the breeding to produce the mixed bag of seeds (Table 2.2). Metabolites to pay particular attention to in brassicas are secondary defence chemicals such as glucosinolates and alkaloids (Karban & Baldwin, 1997), but also lignin due to Escher *et al.*'s (2000) findings. *Bt* maize has been found to have significantly higher levels of some defence volatiles than non-*Bt* maize but this increase was due to insect damage rather than a change in biochemical pathways caused by the GM process (Dean & DeMoraes, 2006).

Refugia, comprising non-*Bt* plants, are often planted between fields and are an important component of resistance control strategy (Section 1.4.3). Farmers, however, consider these between-field refugia as an area of land which is an economic waste. Trap crops (plant species that are more attractive than the main crop to pests) may also be used as a method of limiting damage to the main crop. Cao *et al.* (2005) postulate that *Bt* kale (*B. oleracea* var. *acephala*) can be used as both a trap crop and a cash crop for planting around non-transgenic fields of cabbage (*B. oleracea* var. *capitata*). The mixed bag of seeds used in this study could also be put forward for this purpose. The *Bt* broccoli plants used in this study are actually representative of *Bt* plants cross-pollinating in the field, either inbreeding or crossing with wild relatives, resulting in what has been described in this chapter as *Bt* + and *Bt* - plants. By planting these mixed seeds as trap crops the *Bt* – seeds would act as refugia for the survival of target species reducing the probability of resistance appearing in the field. A mixed field of *Bt* and non-*Bt* plants (4:1) controlled eggplant (*Solanum melongena* L.) pests to the same level as a whole field of *Bt* crops and damage caused by the target pest, *Leptinotarsa decemlineata* Say (Coleoptera), remained below the economic damage threshold (Mennella *et al.*, 2005). *Bt* maize has also been suggested for the use as a trap crop to control *Eldana saccharine* Walker (Lepidoptera) on sugar cane (*Saccharum* spp., Keeping *et al.*, 2007) although this study did not use a mix of *Bt* and non-*Bt* seed.

These mixed seeds must also be considered for their potential for invasiveness (Section 1.4.4). These seeds may not have a selective advantage as the *Bt* gene does not resist attack by at least two Lepidoptera species so they would suffer similar levels of damage as non-*Bt* plants; with no fitness advantage the risk assessment (Section 1.5) for invasion by these *Bt*/non-*Bt* hybrids plants might be calculated as low. In the presence of herbivory, though *Bt* hybrids of *Bt* oilseed rape (*B. napa* L.) and wild *B. rapa* L. produced 1.4 times as much seed as *B. rapa* and represented 42% of the population in next generation (Vacher *et al.*, 2004).

The invasive potential of these seeds should be investigated. A reservoir of *Bt* protein in invasive hybrid plants could also affect resistance strategies as the hybrid plants would alter the *Bt* plant to refugia ratio (Le *et al.*, 2007).

These tests were preliminary pilot studies, hence the low number of replicates; the three species were also tested in different ways for preliminary exploration making it impossible to compare them directly. In many ways each experimental design was a progression of the previous. For example, a Dipel® treatment was introduced with *M. brassicae* experiment when no death was observed with the *A. segetum* larvae feeding on the *Bt* plants. When no death was observed with the manufacturer's dosage (Dipel® recommended), the Dipel® x 20 dose was introduced in the *P. brassicae* experiment. To investigate whether the higher weights of *M. brassicae* on *Bt* plants was caused by an indirect effect of the GM process (Filipecki & Malepszy, 2006) rather than the *Bt* protein itself on *Bt* plants the *Bt* - plants (progeny from the cross between a *Bt* and non-*Bt* plant not expressing *Bt* proteins) were introduced to the *P. brassicae* experiment. In hindsight all three species should have been investigated with both concentrations of Dipel®, both *Bt* + and *Bt* - plants and, like the *A. segetum,* through to pupation and maybe into a further generation.

4 The effects of Cry1Ac proteins produced by genetically modified broccoli on non-target organisms

4.1 Introduction

Bt toxins are active against a range of organisms across a range of invertebrate orders (Section 1.2.2). *Bt* plants can be genetically engineered to be effective against a single pest, or possibly a closely related group of pest species, by choosing a *Bt* protein specifically active against the target (Table 1.1). Taxa other than the target species are described as non-target organisms. Before *Bt* plants and other innovative agricultural developments are made commercially available, laboratory tests are performed to explore the potential impact on natural biodiversity. These studies generally begin with species that are related to the target species, but are not of pest status, before progressing to investigate effects on organisms that are not related to the target species, pest or otherwise. Species that have an important ecosystem role, such as natural enemies, decomposers and pollinators are often focussed on the most (Lovei *et al.*, 2005). Non-target organisms can come into contact with the *Bt* protein in a number of different ways, for example, by consumption of the *Bt* plant, contact with *Bt* proteins in the soil from root exudates, or predation and parasitism of organisms that have been in contact with *Bt* proteins. This chapter considers the first two scenarios; the latter scenario is discussed in Chapter 5.

The Cry1Ac protein produced in *Bt* broccoli (*Brassica oleracea* L. var. *italica* Plenk), and used in this study, was designed to protect the plant from species in the order Lepidoptera. The term non-target, therefore specifically refers to non-Lepidoptera species. In some studies, however, the term non-target may refer to species of the same order; for example when looking at the effect of *Bt* corn designed to be active against the European corn borer (*Ostrinia nubilalis,* Hübner) on the non-target monarch butterfly (*Danaus plexippus,* L.) (e.g. Gatehouse *et al.*, 2002). The likelihood of a direct effect resulting from ingestion of the *Bt* protein is high if the non-target species is closely related to the target organism; in this case *Bt* protein receptors in the gut of the non-target species are more likely to be similar to those of the target species.

Studies on the effects of *Bt* proteins on closely-related species were discussed in Chapter 3. The specific nature of *Bt* proteins (Section 1.2.2) allows us to hypothesise that, in general, no effects would be seen in non-target species of different orders, although there are some exceptions (Section 1.4.7). As the effects of *Bt* proteins on above-ground non-target herbivores and sap feeders have been extensively studied (Section 1.4.7.1 and Table 1.2) these species are not considered in this chapter. Research into the effects of *Bt* plants, especially maize (*Zea mays*, L.), on decomposers and soil-dwelling organisms (Section 1.4.7.4 and Table 1.3) has, more recently, gained priority with the discovery that some *Bt* plants exude *Bt* proteins into the soil (Saxena & Stotzky, 2000; Saxena *et al.*, 1999, 2002a, 2004).

Many soil-dwelling organisms are pests, causing considerable damage to the underground components of crops (e.g. cabbage root fly, *Delia radicum* L., and turnip moth, *Agrotis segetum,* Denis & Schiff). Plants can be, and have been genetically engineered to target specifically these species (e.g. *Bt* potatoes (*Solanum tuberosum* L.) target ground-dwelling potato tuberworms, *Phthorimaea operculella*, Zeller, Douches *et al.*, 2004). In these cases other non-damaging soil fauna should be considered as non-targets. Many surface and soil-dwelling species are decomposers and may feed directly on decomposing plant material, as well as coming into contact with root exudates and decomposing plant material. Determining whether the *Bt* protein is still active after plant senescence, in the decomposing tissue, is therefore essential.

In Chapter 2 *Bt* protein produced by the *Bt* broccoli plants used in this study was found to be detectable in senescent leaves and the compost surrounding the plant roots. In this case, soil dwelling organisms could, potentially, come into direct contact with the protein. The effect of the *Bt* protein on micro-organisms is discussed in detail in Chapter 6. In this chapter, the effects of *Bt* proteins on five non-target species are considered. These species included slugs as herbivorous pests, woodlice and Collembola as numerically significant surface-dwelling decomposers, and earthworms and nematodes as underground decomposers. To avoid results being confounded by the variation in the concentration of *Bt* protein detected in the plants and in compost (Chapter 2) only *Bt* plants that were expressing *Bt* protein above the threshold of mean plus three standard deviations of the non-*Bt* plants (Section 2.2.5) and thus contained the *Bt* gene (Section 2.3.1), and the compost from around their roots, were used.

4.1.1 Nematodes

Nematodes are a ubiquitous group of organisms inhabiting many niches and playing many different roles in the environment (e.g. parasites and decomposers). *Panagrellus redivivus* L. a free living, plant feeding soil nematode was chosen as the test species for this study. *P. redivivus* could come into contact with the Cry1Ac protein by either feeding on *Bt* broccoli plant material or by contact with root exudates. Some *Bt* isolates have been found to produce proteins that are toxic to nematodes, reducing their fecundity and multiplication around plant roots (Mozgovaya *et al.*, 2002). Wei *et al.* (2003) explored the effects of seven *Bt* toxins on five different nematode species, including *P. redivivus*. Some *Bt* toxins were found to be toxic to one or more nematode species. None of the toxins tested were in the Cry1 group. In other studies, Cry1Ab from *Bt* maize has been found non-toxic to nematodes (Saxena & Stotzky, 2001b) and specifically Cry1Ac was not toxic to *Caenorhabditis elegans* (Say) (Wei *et al.*, 2003).

In both studies (Saxena & Stotzky, 2001b; Wei *et al.*, 2003) the Cry1A protein was purified from *Bt* bacteria and fed directly to the nematodes in the laboratory. The protein produced by *Bt* plants differs from that produced by the bacteria as it does not require activation from the protoxin (Section 1.4.1); these experiments (Saxena & Stotzky, 2001b; Wei *et al.*, 2003), by using purified proteins, did not take into account changes to the protein that may have occurred by placing it under the control of plant packaging methods compared to the micro-organism (Goldburg & Tjaden, 1990). More realistically, and at a field level, Cowgill *et al.* (2002) investigated the effect of ingesting tissue from a potato genetically engineered to produce a cysteine proteinase inhibitor on free-living nematodes. As the inhibitor conferred partial resistance to potato cyst nematodes closely related free-living nematodes could also have been at risk. The results, however, showed no significant effect on nematode abundance.

Donegan *et al.* (1997), on the other hand, found the number of nematodes surrounding a litterbag containing tobacco (*Nicotiana tabacum* L.) plants, engineered to produce a proteinase inhibitor with insecticidal properties, increased when compared to non-transgenic litterbags. Two further studies investigating the effects of *Bt* protein on nematode numbers showed no significant differences; in these cases in *Bt* maize fields that were engineered to be active against Coleoptera using the Cry3Bb1 protein (Al-deeb *et al.*, 2003) and Lepidoptera

using the Cry1Ab protein (Saxena & Stotzky, 2001b). One study has reported a significantly lower number of nematodes in Cry1Ab producing *Bt* maize fields than in non-*Bt* maize fields (Griffiths *et al.*, 2005); this difference, however, was not as large as the differences revealed between sites and crops. Similarly differences in nematode numbers between *Bt* and non-*Bt* fields for 8 different GM events were not related to *Bt* content but growth stage of the maize (Griffiths *et al.*, 2007).

4.1.2 Collembola

Collembola are important consumers of fungi and plant residues (Hopkins, 1997). The parthogenic *Folsomia candida* (Willem) was chosen as the test species for this study, mainly for the ease with which it may be raised and cultured in the laboratory. There appears to be no published reference to a *Bt* protein that is directly toxic to Collembola so it seemed unlikely that *Bt* brassicas would have an effect on this species.

Studies involving genetically modified antifungal wheat (*Triticum aestivum* L.) (Romeis *et al.*, 2003) and herbicide-tolerant soybeans (*Glycine max* L.) (Bitzer *et al.*, 2002) detected no effect on Collembola species. These particular genetic modifications, however, do not confer an insecticidal property. Proteinase inhibitors have a general insecticidal activity and Donegan *et al.* (1997) found fewer Collembola surrounding litterbags containing proteinase inhibitor tobacco leaves than those containing non-transgenic tobacco. *Bt* plants, in contrast, have a more specific spectrum of activity; Duan *et al.* (2004) showed that there was no difference in the total number of Collembola collected from a *Bt* (Coleoptera-active) potato field in comparison to a control field. Similarly, Al-deeb *et al.* (2003) detected similar numbers of Collembola in *Bt* (Lepidoptera-active) and non-*Bt* maize fields. While these experiments considered the total number of Collembola across several groups, more specifically Heckman *et al.* (2006) showed that there was no effect on *Protaphorura armata* (Tullberg) after four weeks of exposure to two different *Bt* maize varieties whilst *F. candida* had lower reproduction on one of four *Bt* maize varieties tested than the non-*Bt* equivalent (Clark & Coats, 2006).

4.1.3 Woodlice

Woodlice play an essential decomposer role in many ecosystems. They are often considered as secondary decomposers as they may feed on leaf litter already colonised by micro-

organisms (Sutton, 1972). *Porcellio scaber* (Latreille) was chosen as it is an easily-cultured species, found throughout the world but native to the UK. Although arable field ploughing may disturb populations and cause dispersal problems, woodlice are likely to enter such fields and forage from their hedgerow habitats (Sutton, 1972). Previously used in GM crop studies, Escher *et al.* (2000), for example, showed that juvenile *P. scaber* had lower mortality and adults did not show any ill-effects from feeding on Cry1Ab producing *Bt* maize. In fact, the adults were heavier when feeding on *Bt* maize in comparison to those feeding on non-*Bt* leaves (Escher *et al.*, 2000).

4.1.4 Earthworms

Earthworms are often used as test organisms for assessing toxicity in the environment (reviewed in Reinecke & Reinecke, 1994) and are another important group of soil decomposers. *Dendrobaena rubidus* (Savigny), chosen as a taxon representative, is a very efficient decomposer and is often sold for home composting by organic companies who report that it will eat half its body weight every day. As this level of ingestion would provide an efficient turnover of the *Bt* plant material the likelihood of observing *Bt* protein effects were considered plausible. As *D. rubidus* are found near the soil surface they are more suited to small scale microcosm studies than, for example, *Lumbricus terrestris* L., another common test species, which prefers deep soil. *L. terrestris* living in soil in which *Bt* maize was growing, or had grown, were not affected by the *Bt* toxin (Zwahlen *et al.*, 2003 and Saxena & Stotzky, 2001b, respectively). In contrast, a greater increase in weight gain of another earthworm species, *Eisenia fetida,* was reported on two different *Bt* maize varieties when compared to their non-*Bt* equivalent but not on two further varieties (Clark & Coats, 2006). Vercesi *et al.* (2006) report just one statistically significant effect of *Bt* maize on *Aporrectodea calignosa* (Savigny) fitness: cocoon hatching rates were reduced from 95 to 75% but only when adding ground maize powder to soil at 5 g kg^{-1}. In a more realistic set-up where *A. caliginosa* were in the presence of growing *Bt* plants earthworm weights were not affected, but reproduction and cocoon hatching rates were not measured (Vercesi *et al.*, 2006).

4.1.5 Slugs

Deroceras reticulatum (Müller) is a herbivorous, major agricultural, mollusc pest (Runham & Hunter, 1970). A negative (higher mortality) effect of *Bt* proteins on slugs could be a "hidden bonus" to the grower, in this case, as it would be reducing the detrimental effects of two pests, the Lepidoptera and the slug. A positive effect on the slugs could, however, mean an increase in damage to the plants by the slugs. This would nullify the reduction of damage caused by the target Lepidoptera. There is one study that shows that *Bt* proteins from GM maize are detectable in slugs using ELISA (Harwood & Obrycki, 2006) but, as far as I am aware, there have been no studies looking at the biological effects of *Bt* plants on slugs, or indeed snails.

4.1.6 Summary

Significant negative effects of *Bt* crops have been seen on nematode numbers (Griffiths *et al.*, 2005), *F. candida* reproduction (Clark & Coats, 2006) and *A. caliginosa* cocoon hatching rates (Vercesi *et al.*, 2006) but significant positive effects have been seen in adult woodlice weights and juvenile survival (Escher *et al.*, 2000) and *E. fetida* weights (Clark & Coats, 2006). Studies investigating the effects of *Bt* plants which have explored the effects on total numbers of a group or range of species (e.g. Al-deeb *et al.*, 2003) have found no significant direct effect; such studies may have overlooked the effects on individual species. *Bt* plants producing Cry1Ac proteins have also tended to be overlooked as Cry1Ab genes are more commonly inserted into plants. In some cases the result was protocol specific, for example, the effect on *E. fetida* hatching rates was seen with soil amended with ground *Bt* maize but not with soil in which *Bt* maize plants were growing (Vercesi *et al.*, 2006).

Although, worms, woodlice, nematodes and Collembola have all been investigated previously, albeit in a handful of individual studies, this has not been previously done in an integrated study where several species are tested against one specific *Bt* plant line. This study is among the first to take such an integrated approach. In addition, no studies of the effect on non-target species have been carried out using *Bt* broccoli and I believe this to be the first study into the effects of *Bt* plants on any mollusc. On the basis of the assumed specificity of the *Bt* protein I will test the hypothesis that there will be no effect on the five non-target species investigated. With, however, a small number of effects on juvenile numbers and

reproduction reported (e.g. Escher *et al.*, 2000; Clark & Coats, 2006) specific attention was paid to these parameters.

4.2 Methods and results

4.2.1 Nematodes

4.2.1.1 Nematode Methods

4.2.1.1.1 Nematode culturing and preparation

Panagrellus redivivus were obtained from the Centre of Ecology and Hydrology (Lancaster, UK). Frozen peas were boiled up in a small amount of water and homogenised before pouring to a depth of 2 cm in plastic containers (8 x 12 x 12 cm). A sub-sample of the original culture was then stirred into the cool pea mixture before a lid pierced with five air holes was placed on top. The pea/nematode culture was kept at approximately 25°C. Additional deionised water was misted onto the surface every two days to maintain moisture.

Nematodes were removed from the pea culture using a wet extraction method (adapted from Whitehead and Hemming, 1965). The pea culture mix was spooned into a tapped funnel, lined with one layer of blue-roll (Kimwipe) paper over a 2 mm mesh. Water was poured into the funnel until just above the pea-mix level and then left for 2 - 3 h at 20°C. During this period the nematodes could be seen moving into the water and gravitating to the bottom of the funnel. The nematodes were allowed to flow into a collection beaker by opening the tap (Figure 4.1).

1 ml sub-samples were taken from the collection beaker and the number of nematodes per millilitre counted using a cell counter. The mean value of three sub-samples was then used to calculate the dilution rate to create inoculates of 7,500 nematodes ml^{-1}. This provided a final density of 500 nematodes g $compost^{-1}$; higher than that generally found in agricultural fields (100 g dry $soil^{-1}$; Ingham, 2000).

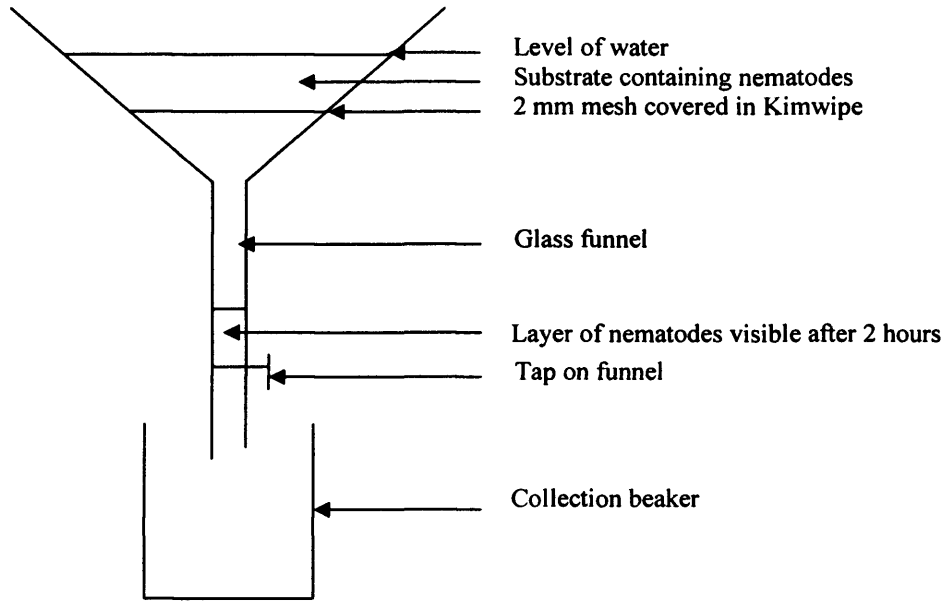

Figure 4.1 Apparatus used to extract nematodes from pea and soil substrates.

4.2.1.1.2 Experimental design

Bt and non-*Bt* broccoli plants were grown for three months in a greenhouse in John Innes No. 2 compost (Section 2.2.1). One day before the compost was required the plants were removed from their pots and as much compost as possible shaken from the root system. The compost was spread out on trays (10 x 80 x 80 cm), allowed to dry overnight at 20°C, and then passed through a 2 mm sieve and mixed thoroughly. Compost from the roots of non-*Bt* brassicas was referred to as "non-*Bt* compost" and compost from *Bt* broccoli as "*Bt* compost". Some John Innes No. 2 compost which had not had plants growing in it was dried and sieved in the same way.

15 g of each compost type was weighed out into 30 ml plastic medicine cups (4 cm diameter, 3 cm deep). There were four compost treatments, ten replicates of each: *Bt* compost and non-*Bt* compost to which 9 ml water was added, John Innes No. 2 compost with 9 ml water (control) and finally John Innes No. 2 compost to which 9 ml Dipel® had been added at 20 times the manufacturer's recommended concentration (15 g l^{-1}) (Dipel® compost). 1 ml nematodes were added to each medicine beaker and a lid fitted before the containers were placed in a controlled environment room at 16°C (16:8 h L:D cycle). Leaf discs and compost samples were analysed using ELISA (Section 2.2.5).

4.2.1.1.3 Harvesting extraction method

The wet extraction method (Section 4.2.1.1.1, Figure 4.1) was used to remove the nematodes from the experimental compost. Samples were, however, left for 24 rather than 3 h. On Days 7 and 8, four replicates of each treatment were removed for extraction. On Day 9 the final two replicates were extracted. Three 1 ml sub-samples were taken from the collecting beaker to allow determination of the number of nematodes; the mean of these three counts was used to estimate the total number of nematodes recovered.

4.2.1.1.4 Analysis

Where the data conformed to its assumptions (homogeneity of variances and normality of residuals) ANOVA was used, followed by calculation of a least significant difference value to determine where the differences between the treatments occurred. If the data set did not conform, even after transformations, a non-parametric Kruskal-Wallis test followed by Mann-Whitney tests was used. These statistical tests were also used throughout the following sections for the other four species. All statistical analysis was performed using the program Minitab v14.1 (Minitab Inc., PA, USA).

4.2.1.2 Nematode Results

The nematodes were exposed to *Bt* compost from *Bt* broccoli plants expressing 107 ± 94 ng g^{-1} *Bt* protein. The *Bt* compost did not test positive for *Bt* proteins but the Dipel® treated compost contained 15.54 ng g^{-1}. There was a low nematode recovery rate across the treatments (<20%) suggesting this method is not the most efficient for extracting nematodes. Some enchytraeids were observed to be present when nematodes were being counted and they may have predated on the nematodes reducing their numbers. There was a significant difference ($F_{3,39}$=5.14, P=0.006) between the number of nematodes (square root transformed) recovered from the various treatments (Figure 4.2). The control treatment was significantly higher than the Dipel® treatment and the *Bt* compost treatment (LSD=10.47, P<0.05); the *Bt* compost treatment, however, was not significantly different to the Dipel® and non-*Bt* treatments.

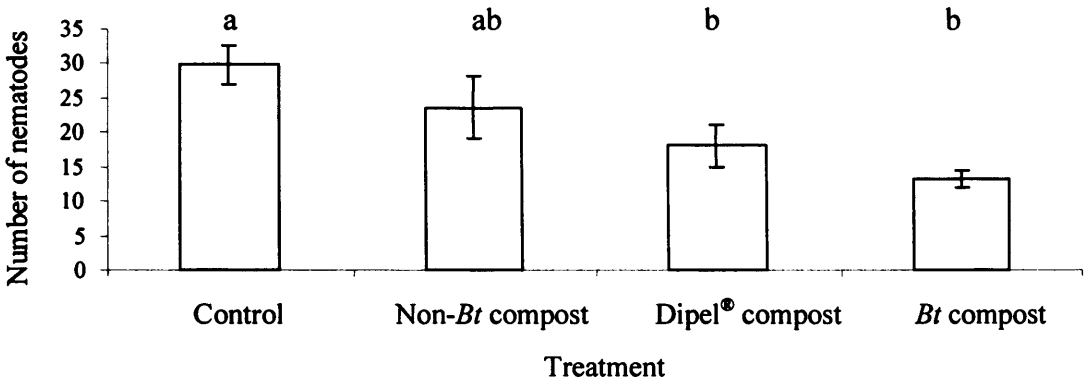

Figure 4.2 Number of nematodes (square root transformed) recovered after one week in contact with four different compost treatments (mean ± standard error). Different lowercase letters indicate significant differences between treatments. The control is John Innes No. 2 compost in which no plants had grown.

4.2.2 Collembola

4.2.2.1 Collembola Methods

4.2.2.1.1 Collembola culturing

5.4 g activated charcoal was carefully mixed into 540 g plaster of Paris before quickly stirring in 360 ml water. This mix was immediately poured into the base of four plastic containers (8 x 14 x 14 cm) to approx. 2 cm depth. After 2 h, deionised water was added to cover completely the plaster, and the pots were allowed to soak for 24 h. A paintbrush was then used to wipe the excess charcoal from the surface of the plaster before the water was poured away.

20 Collembola (*F. candida*) were added to each pot from Cardiff University cultures. A small amount (<20 mg) of dried baker's yeast (*Sacchromyces* spp.) was also added and a lid with air holes placed on top. Every two weeks extra yeast and water were added, and every six months new cultures were established from the older ones.

4.2.2.1.2 Exposure to *Bt* in food

Plaster of Paris (Section 4.2.2.1.1) was poured to 1 cm depth into 40 round plastic containers (10 cm diameter and 5 cm deep), and a glass tube was pressed slightly into the centre. After the plaster had set, the tube was removed leaving a well. The pots were then soaked for 24 h with deionised water before the food source was placed in the well. 10 of the pots contained a *Bt* leaf disc (Section 2.2.2), 10 pots non-*Bt* leaf discs, 10 non-*Bt* leaf discs that had been soaked in Dipel® (15 g l^{-1} for 10 minutes and air dried for 30 minutes) and the final 10 pots, 20 mg bakers yeast (i.e. approximately the same weight as a leaf disc). The 1 cm diameter leaf discs came from plants that had been grown in a greenhouse as described in Section 2.2.1, and extra leaf discs were kept for ELISA analysis (Section 2.2.5).

Collembola that had been feeding on yeast (Section 4.2.2.1.1) were placed in a soil sieve with mesh diameter of 250 µm (Nickel-Electro Ltd, Weston-Super-Mere, UK). Of those Collembola that did not pass through the sieve 10 individuals were placed in each pot. After one week the number of adults in each pot was counted and the food source refreshed and replenished. 1 ml water was also added to keep the plaster damp. Any eggs present were collected, counted and placed in a clean plaster of Paris pot containing the same food source as the adults and denoted as Cohort 1. This continued for four weeks, with Cohorts 2, 3 and 4 being collected in Weeks 2, 3 and 4, respectively, resulting in 200 pots in total.

Each cohort was raised for four weeks after collection (i.e. Cohort 1, 2, 3, and 4 were harvested on Week 5, 6, 7, and 8); at this time the hatched juveniles were shaken out and placed in the top of a set of stacking soil sieves. Damp blue roll (Kimwipe) was placed in the base of the sieve column to maintain moisture. The lid was replaced, and the stack left for 15 minutes. Juveniles in each layer were placed in a micro-centrifuge tube and frozen before being counted. Each layer indicated a difference size range of juveniles (<125, 125-140, 140-200, 200-250, >250 µm). Cohort data were collected to look for adaptation to the food source over time. If the original adults adapted to the new food source, resulting in better quality eggs, then this would be detected in hatching rates and in juvenile growth rates.

4.2.2.1.3 Exposure to *Bt* in compost and food

45 g *Bt* compost was placed in 10 round plastic containers (10 cm diameter and 5 cm deep) and compressed with fingertips; 45 g non-*Bt* compost was placed in a further 15 containers.

The compost was prepared as in Section 4.2.1.1.2. *Bt* leaf discs were placed in five of the *Bt* compost and five of the non-*Bt* compost pots; non-*Bt* leaf discs were placed in five of the *Bt* and five of the non-*Bt* compost pots; non-*Bt* leaf discs soaked in Dipel® (Section 4.2.2.1.2) were placed in the remaining five non-*Bt* compost pots. All leaf discs were of 1 cm diameter and collected as described in Section 2.2.3. Some leaf discs were kept for ELISA analysis (Section 2.2.5).

Collembola cultured on plaster and fed on yeast (Section 4.2.2.1.1) were sieved (Section 4.2.2.1.2), and 15 individuals (< 250 μm) were placed in each container. The containers were then all placed in a controlled environment room at 16°C (16:8 h L:D cycle). Each week the food source was removed and replenished; a light misting of water was provided to maintain moisture levels. After four weeks the soil was placed in a Tullgren funnel (Burkard Agronomics, Middlesex, UK) and the Collembola extracted over 24 h in three concurrent runs. Numbers collected were counted under a binocular microscope.

4.2.2.2 Collembola Results

4.2.2.2.1 Exposure to *Bt* in food

Some mortality was observed in the original 40 pots during the four weeks but the number recovered was ≥60% across all treatments. The *Bt* leaf disc treatment had the lowest mean number of adults recovered after four weeks but it was not significantly different from the other treatments ($H_{3,39}$=0.44, P=0.933). The *Bt* leaf discs came from one plant containing 95 ng g^{-1} *Bt* protein whilst the Dipel® treated leaf discs had 80.3 ± 6.5 ng g^{-1}.

The total number of eggs recovered from the yeast treatment pots over the four weeks was significantly higher than the other three treatments ($H_{3,39}$=23.88, P<0.001; Figure 4.3). There was, however, no significant difference between the three leaf treatments. By removing the yeast treatment data set from the analysis, the data were normalised. A second comparison of just the three leaf treatments showed that there was no significant difference between the total number of eggs produced ($F_{2,29}$=1.67, P=0.206).

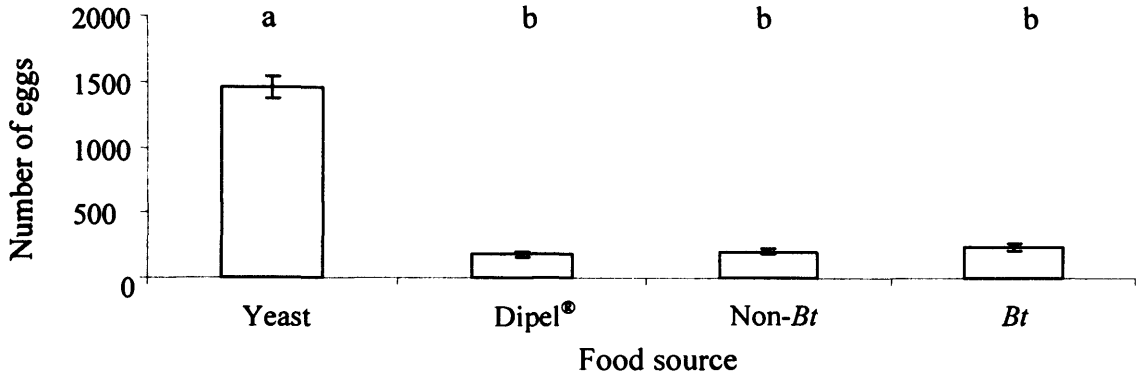

Figure 4.3 Total number of *F. candida* eggs (mean ± standard error) collected from plaster of Paris pots over four weeks. Dipel®, non-*Bt* and *Bt* refer to the leaf disc given to the *F. candida* and different lowercase letters indicate a significant difference between the food treatments.

Egg production from the original pots increased over the four weeks in all four treatments (Figure 4.4). Linear regression showed that there was a significant difference between the slopes of the four treatments ($F_{3,39}$=3.56, P=0.016) and the y-intercepts ($F_{3,39}$=10.83, P<0.001). Comparison of the three leaf disc treatments (after removal of the yeast data set) confirmed that there was no difference between their regression lines (slope $F_{2,29}$=1.80, P=0.170; y-intercept $F_{2,29}$=0.88, P=0.418). This indicates that the leaf treatments all had similar rates of increase in egg production whilst the yeast treatment was different.

Hatching rates in some of the pots were greater than 100%. This was probably caused by the original eggs hatching and resulting individuals reproducing during the four weeks the juveniles were raised. The total number of Collembola in each pot was then used to calculate the proportion of individuals in each size class. There were, however, no apparent patterns as the proportion of juveniles in each size category varied between cohort and treatment (Figure 4.5). Analysis was further complicated by the discrete nature of the pre-determined size groups and the non-normality of the data. No further useful information, therefore, could be extracted from this data set.

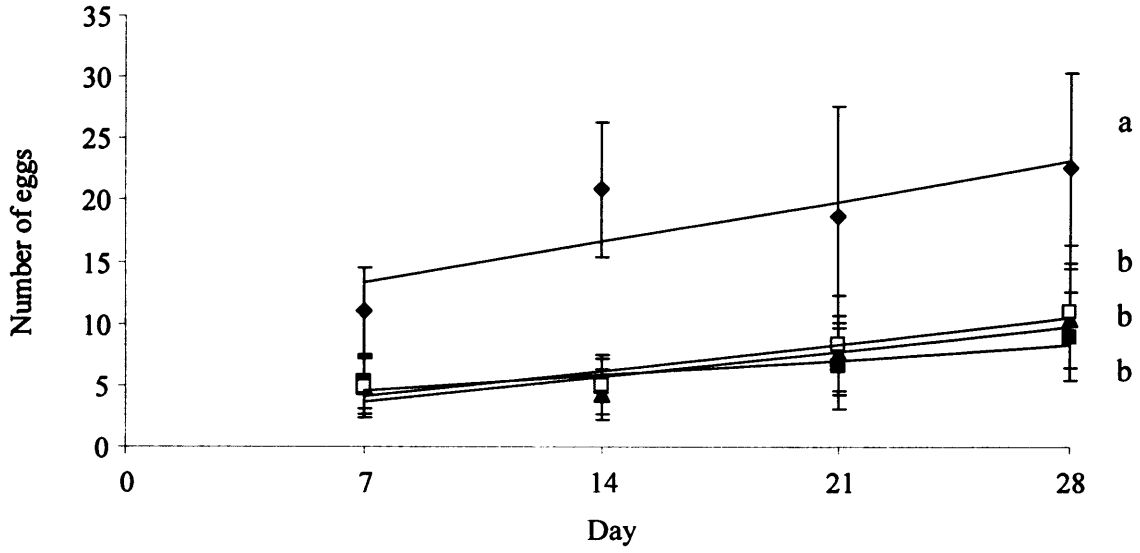

Figure 4.4 Number of *F. candida* eggs collected over time (square root transformed) with fitted regression lines and R^2 values. ♦ yeast (log eggs = 2.02 + 0.181(days), R^2=0.403), ■ Dipel® treated leaf discs (log eggs = 1.09 + 0.165(days), R^2=0.276), ▲ Non-*Bt* leaf discs (log eggs = 0.759 + 0.297(days), R^2=0.412), □ *Bt* leaf discs (log eggs = 0.851 + 0.271(days), R^2=0.302). Different lowercase letters indicate significant differences between the y-intercepts of the regression lines.

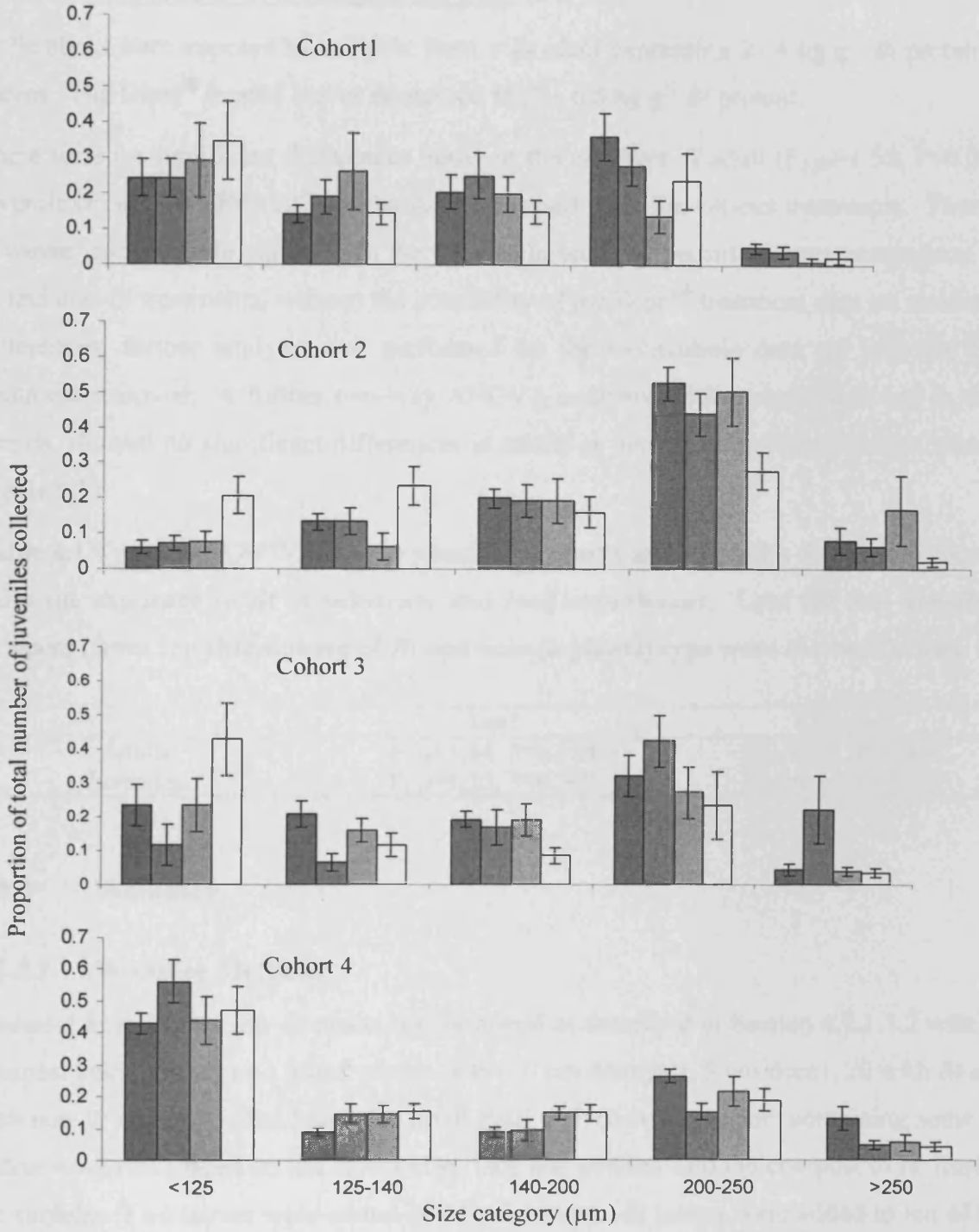

Figure 4.5 For each Cohort (1-4 indicates the week they were collected from the original ten adults) the proportion of juvenile *F. candida* (mean ± standard error) collected in each size category four weeks after egg collection. The *F. candida* were raised on four different diets.

□Yeast, ■ Dipel®, ■ non-*Bt* and ■ *Bt* leaf discs.

4.2.2.2.2 Exposure to *Bt* in compost and food

Collembola were exposed to compost from a *Bt* plant expressing 21.4 ng g^{-1} *Bt* protein in its leaves. The Dipel® treated leaves contained 80.3 ± 6.5 ng g^{-1} *Bt* protein.

There were no significant differences between the numbers of adult ($F_{3,39}$=1.58, P=0.218) or juvenile ($F_{3,39}$=1.29, P=0.307) Collembola collected from the various treatments. There was, however, considerable variation in the data collected. To permit a direct comparison of the *Bt* and non-*Bt* treatments, without the possibility of the Dipel® treatment data set masking any differences, further analysis was performed on the Collembola data set with the Dipel® treatment removed. A further two-way ANOVA analysis, with compost and leaf as the two factors, showed no significant differences in adults or juveniles numbers between treatments (Table 4.1).

Table 4.1 Two-way ANOVA on the number of adults and juveniles *F. candida* recovered from the exposure to *Bt* in substrate and food experiment. Leaf (*Bt* and non-*Bt*) and compost (from the rhizosphere of *Bt* and non-*Bt* plants) type were the two factors.

	Leaf	Compost
Adults	$F_{1,39}$=1.84, P=0.184	$F_{1,39}$=0.51, P=0.481
Juveniles	$F_{1,39}$=1.25, P=0.307	$F_{1,39}$=0.63, P=0.435

4.2.3 Woodlice

4.2.3.1 Woodlice Methods

Compost from *Bt* and non-*Bt* plants was prepared as described in Section 4.2.1.1.2 with 100 g compost being placed into round plastic pots (10 cm diameter, 5 cm deep), 20 with *Bt* and 30 with non-*Bt* compost. The base of a small Petri dish (5 cm diameter) containing some damp cotton wool was placed on the compost surface and pressed into the compost to be flush with the surface. Two leaves were added as a food source. *Bt* leaves were added to ten of the *Bt* compost replicates and non-*Bt* leaves added to the remaining ten. Ten of the non-*Bt* compost replicates had *Bt* leaves added, ten had non-*Bt* leaves added and ten had non-*Bt* leaves which had been soaked in Dipel® (Section 4.2.2.1.2). Leaf discs from each treatment were kept for later ELISA analysis (Section 2.2.5).

Porcellio scaber were obtained from Blades Biologicals (Kent, U.K.). The woodlice were sorted into two size groups: those weighing less than 15 mg (mean ± s.e., 3.1 ± 0.1 mg) and those weighing 15 mg or more (29.0 ± 0.7 mg). Five small and five large woodlice were weighed individually into each of the 50 containers and the lid replaced. All containers were placed in a controlled environment room at 16°C (16:8 h L:D cycle). The ratio of males to females was not controlled. Every two weeks the number of live woodlice was counted and live individuals weighed; the food source was replenished and the cotton wool re-wetted. Dead individuals were removed from the pots. To keep track of the two groups (small and large) identified at the beginning of the experiment woodlice that had died were also weighed to distinguish which specific individuals had died from each group each week. At the end of eight weeks the woodlice were checked to determine whether they were pregnant by looking for full brood pouches.

4.2.3.1.1 Analysis

The number of surviving woodlice was analysed using a one-way ANOVA. The number of pregnant adults per pot was used to calculate a mean pregnancy rate value per treatment which could also be analysed using one-way ANOVA (Section 4.2.1.1.4). Non-normality of the complete woodlouse weight data set prevented meaningful statistical analysis, so the data were divided into two groups and analysed seperately. The small and large groups identified at the start were still identifiable at the end of the study, although both groups, not surprisingly, had higher means (10 ± 0.6 and 39.0 ± 2.6 mg, respectively). Woodlouse weights after eight weeks were analysed using two one-way ANOVA. The mean rate of weight gain per pot was calculated using:

$$\frac{\text{(mean weight at t=8) – (mean weight at t=0)}}{8}$$

where t=0 is the start and t=8 the end of the experiment, respectively. These growth rates were also analysed using two one-way ANOVAs for both large and small weight groups.

4.2.3.2 Woodlice results

The woodlice were exposed to compost from *Bt* broccoli plants expressing 133 ± 60.5 ng g^{-1} *Bt* protein in their leaves. The Dipel® treated leaves contained 80.3 ± 6.5 ng g^{-1}.

There was no significant effect of the five different treatments on the number of pregnant woodlice present ($H_{4,49}$=6.66, P=0.155) and the total number of woodlice surviving after eight weeks was marginally non-significant ($F_{4,49}$=2.41, P=0.063). When the data were divided into small and large woodlice there was no significant effect on the number of large woodlice ($H_{4,49}$=2.37, P=0.499); the effect on the number of small woodlice was marginally non-significant ($H_{4,49}$=7.34, P=0.062, Figure 4.6a). There was no significant treatment effect on either the final weight ($F_{4,49}$=0.35, P=0.840) or weight gain ($H_{4,49}$=5.20, P=0.268) of the large woodlice. There was, however, a treatment effect on weight ($F_{4,49}$=8.61, P<0.001, square root transformed, Figure 4.6b) and rate of weight gain ($F_{4,49}$=8.17, P<0.001) for the small woodlice (Figure 4.6c). For these woodlice, individuals in the *Bt* leaf and non-*Bt* compost treatment were heavier and had faster weight gain than the two non-*Bt* leaf and Dipel® treatments. The woodlice in the *Bt* leaf and *Bt* compost treatment were, however, similar to the two non-*Bt* leaf treatments.

After removing the Dipel® treatment data set (Section 4.2.2.2.2) two-way ANOVA tests, with compost and leaf type as the two factors, indicated that, for the small woodlice, leaf, but not compost, caused differences in survival. Both leaf and compost affected weight and rate of weight gain. There was higher survival of small woodlice on *Bt* leaves than non-*Bt* leaves ($F_{1,39}$=8.48, P=0.006, Figure 4.7a); small woodlice feeding on *Bt* leaves were also significantly heavier ($F_{1,39}$=20.64, P<0.001, Figure 4.7b) and the rate of weight gain was faster ($F_{1,39}$=18.94, P<0.001, Figure 4.7c) than when feeding on non-*Bt* leaves. Woodlice living in *Bt* compost were less heavy ($F_{1,39}$=8.02, P=0.008, Figure 4.7b) and had a lower rate of weight gain ($F_{1,39}$=6.57, P=0.015, Figure 4.7c) than those in non-*Bt* compost. No significant interactions were detected between compost and leaf factors. Similar analysis on the large woodlice revealed no significant differences for survival, weight or rate of weight gain caused by either compost or leaf treatment.

Further analysis of the whole data set showed marginally non-significant differences in the number of pregnant woodlice in the *Bt* and non-*Bt* treatments (leaf $F_{1,39}$=3.14, P=0.089; compost $F_{1,39}$=3.19, P=0.07 using Sheirer-Ray-Hare adjusted P values due to non-normality of residuals, Figure 4.8a). The total number of surviving woodlice was also tested using a two-way ANOVA and this showed that leaf treatment ($F_{1,39}$=9.13, P=0.005), but not compost

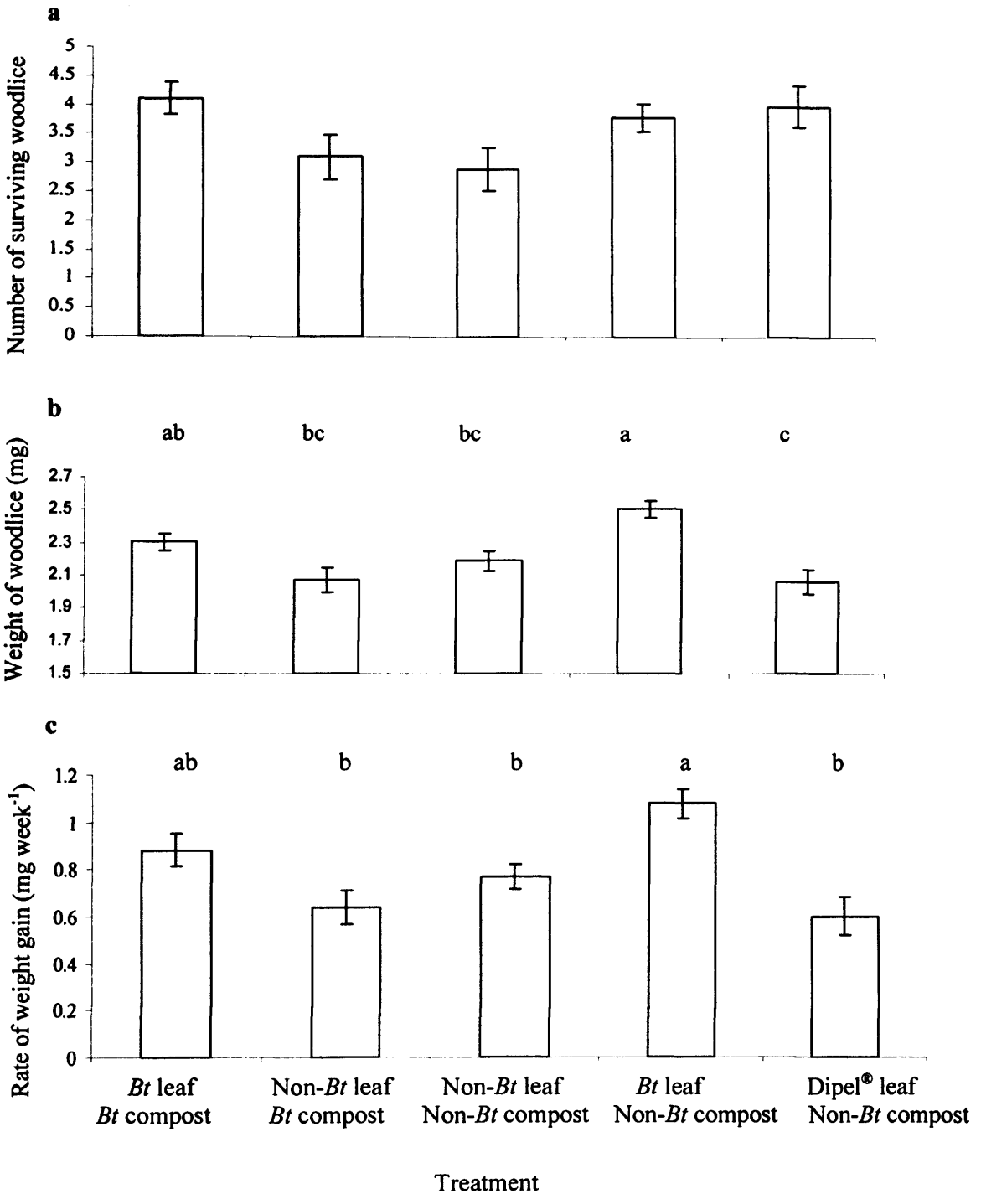

Figure 4.6 a) Number surviving, b) end weight (square root), and c) rate of growth of small woodlice (mean ± standard error). Different lowercase letters show significant differences between the treatments after one-way ANOVA. No difference was seen in the number surviving.

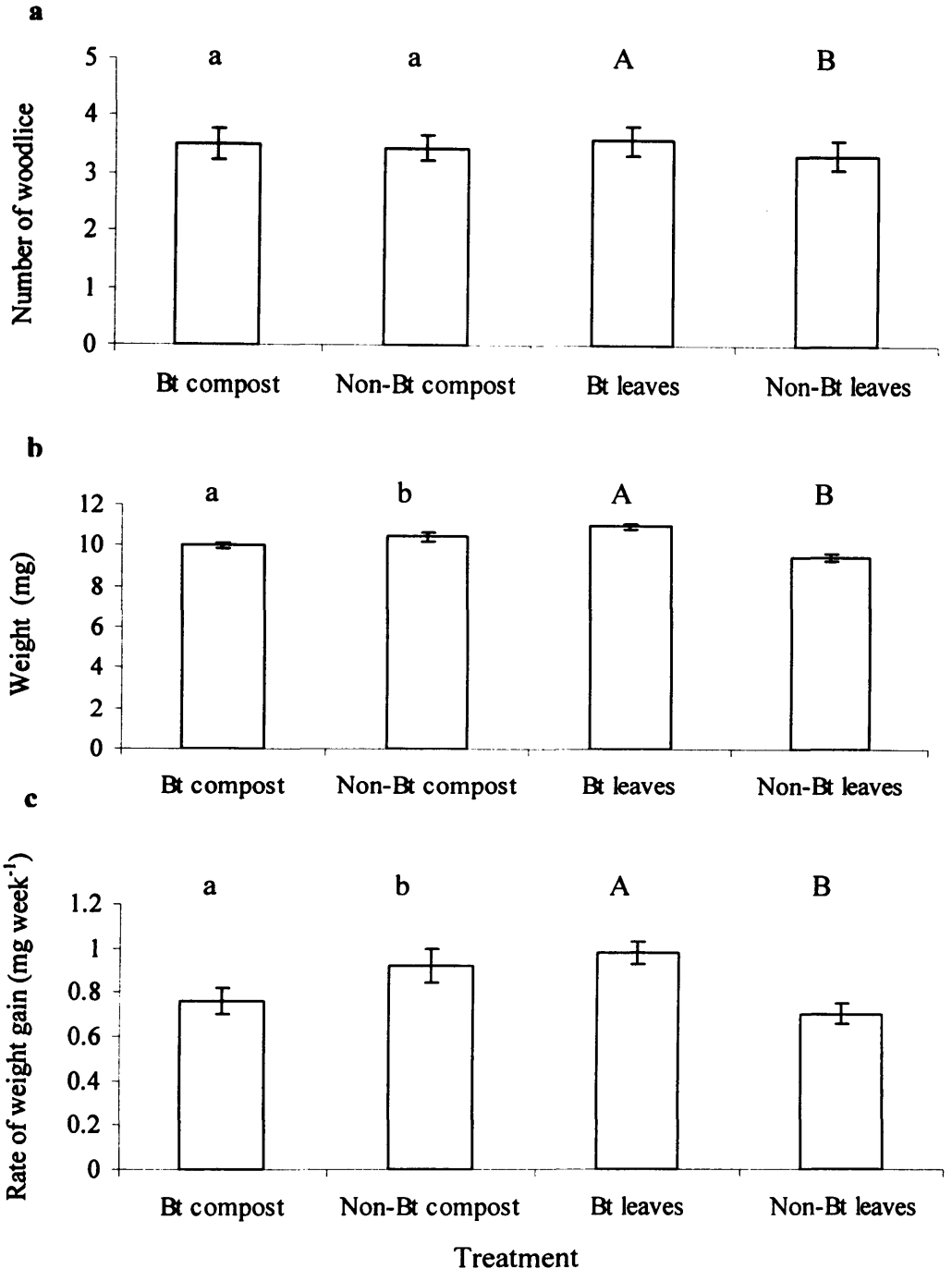

Figure 4.7 a) Number surviving b) weight and c) rate of growth of small woodlice (mean ± standard error). Different letters show significant differences between treatments following a two-way ANOVA using compost and leaf treatments as factors. Different lowercase letters indicate differences between the compost treatments and difference uppercase letters for the leaf treatments.

treatment ($F_{1,39}$=1.31, P=0.259), was a significant factor (Figure 4.8b). Considering the results of the two-way ANOVA tests on the two groups of woodlice the small woodlice group must be heavily influencing this result.

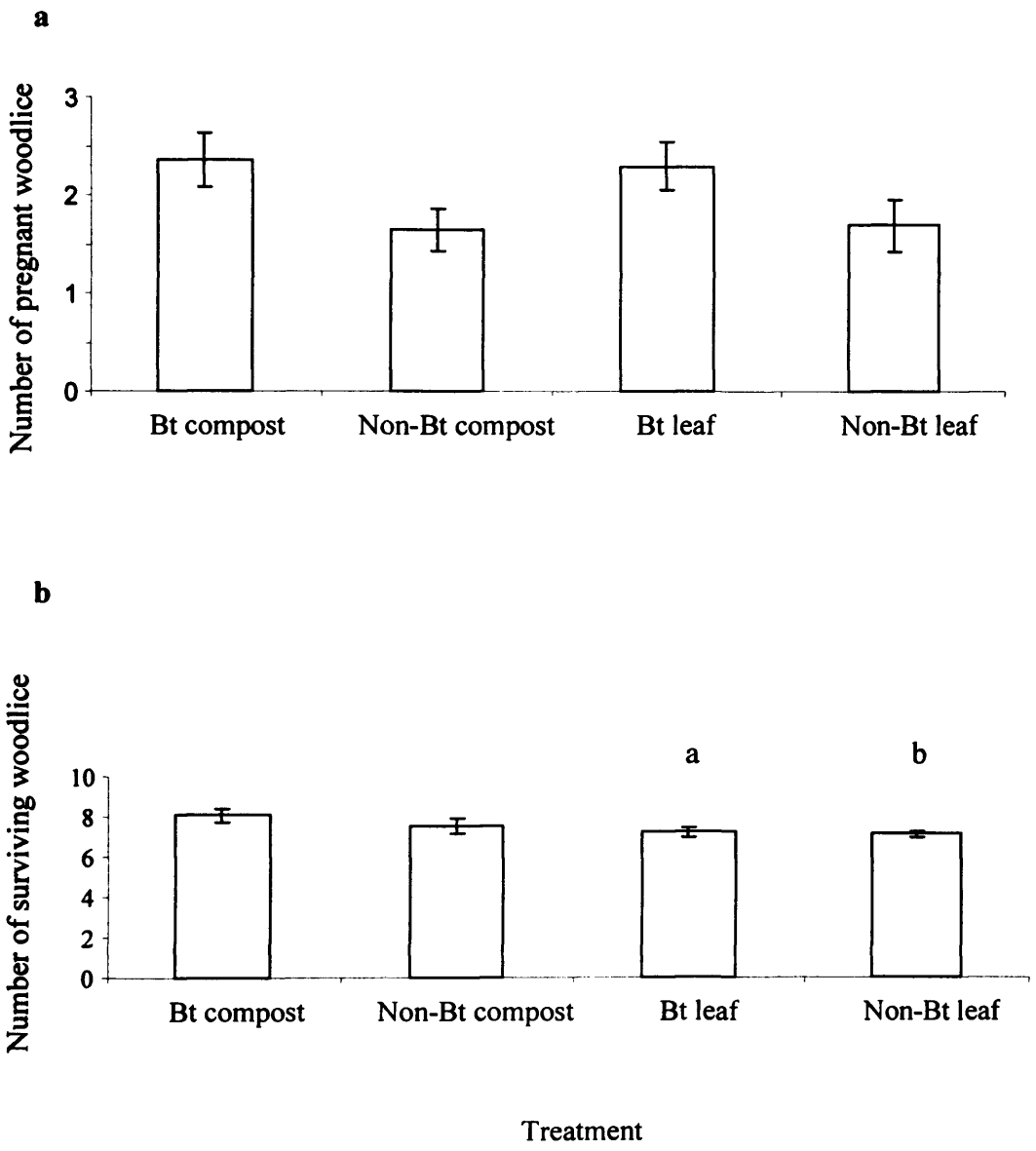

Figure 4.8 Number (mean ± standard error) of a) pregnant (no significant differences) and b) total surviving woodlice. Lowercase letters indicate significant differences between leaf treatments in one factor of the two-way ANOVA on the number surivivng, after the removal of the Dipel® data set. No significant differences were detected between the compost as a treatment factor.

4.2.4 Earthworm

4.2.4.1 Earthworm Methods

Dendrobaena rubidus were obtained from Wiggly Wigglers (Blakemere, Herefordshire, U.K.) and were kept in a large bucket (60 l) for three weeks in 10 l of coia (Wiggly Wigglers, bedding compost) with weekly addition of kitchen vegetable scraps and water. The earthworms were removed by manual searching and placed in a plastic container (12 x 30 x 20 cm) with no food or bedding material, but with a layer of wet filter paper for 12 hours to empty their guts.

80 g *Bt* compost was placed into 12 plastic containers (10 cm deep, 10 cm diameter) pierced at their base to provide drainage. Similarly, 80 g non-*Bt* compost was placed in another 18 identical plastic containers. A further 55 g of the coia bedding was then placed on top of the compost to fill the plastic containers. *Bt* leaves were added to six *Bt* compost and six non-*Bt* compost containers, non-*Bt* leaves to six *Bt* and six non-*Bt* compost containers, and non-*Bt* leaves which had been soaked in Dipel® (Section 4.2.2.1.2) were added to the remaining six non-*Bt* compost containers. Five individually weighed earthworms (0.67 ± 0.03 g) were placed in each container, which were then closed with a lid. The containers were placed on trays filled with water and allowed to soak for 15 minutes prior to placing on dry trays in a controlled environment room at 16°C (16:8 h, L:D cycle). The containers were covered with a black plastic bag throughout the experiment. Each week more leaves were added and the containers placed in trays filled with water for 15 minutes to maintain moisture. Some leaf discs were kept for ELISA analysis (Section 2.2.5).

After four weeks the earthworms were removed from the soil by manual searching and left on a layer of damp filter paper in another plastic container overnight. Earthworms were weighed individually the following day. Any cocoons found during manual searching were placed in a dish with some damp filter paper and returned to the controlled environment room and monitored weekly to determine hatching rates.

4.2.4.2 Earthworm Results

The earthworms were exposed to leaves and compost from *Bt* broccoli plants expressing 291.7 ± 74.8 ng g^{-1} *Bt* protein and Dipel® treated leaves coated with 80.3 ± 6.5 ng g^{-1} *Bt* protein. By the end of the four weeks it was noted that the coia bedding and compost had

been thoroughly mixed and that the leaves had been removed from the surface and integrated into the compost in all the pots. The number of earthworms surviving did not differ significantly between the treatments ($H_{4,29}$=0.88, P=0.928). There were also no significant differences between the treatments for the weight of the earthworms ($F_{4,29}$=1.04, P=0.405), the number of cocoons laid ($F_{4,29}$=1.72, P=0.177, square root transformed) and their hatching rates ($F_{4,29}$=1.40, P=0.263, Figure 4.9a). After four weeks the mean weight of the earthworms had increased to 0.706 ± 0.03 g.

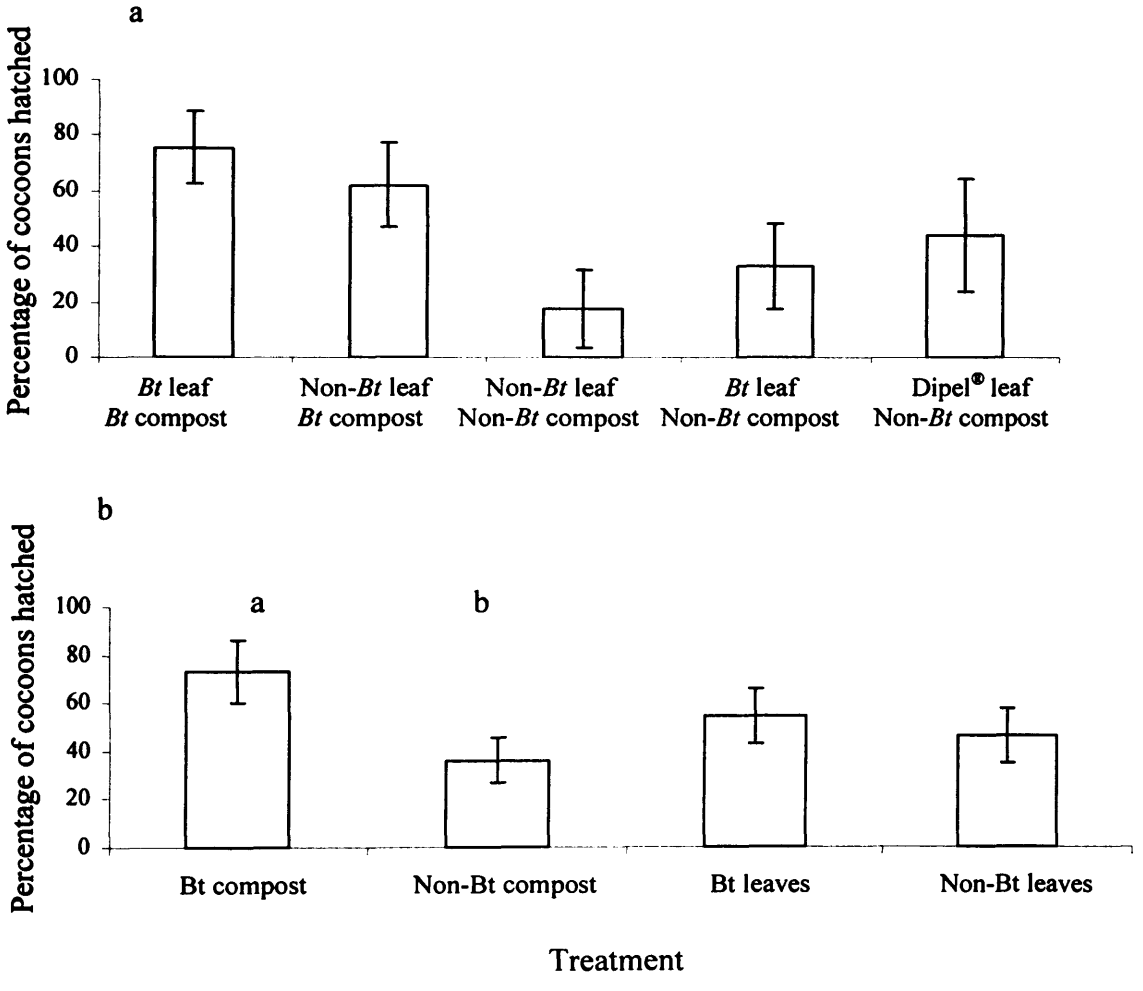

Figure 4.9 Percentage (mean ± standard error) of earthworm cocoons hatched after 2 weeks incubation. a) one-way ANOVA where no significant differences were detected, and b) two-way ANOVA results where lowercase letters indicate a significant difference between the treatments in the compost factor, there was no difference between the treatments in the leaf factor.

As with the Collembola experiment (Section 4.2.2.2.2) the Dipel® data set was removed and a two-way ANOVA analysis using compost and leaf type as variables carried out. Earthworm cocoons in *Bt* compost did have significantly higher hatching rates than those in non-*Bt* compost ($F_{1,23}$=0.27, P=0.024, Figure 4.9b) but leaf type did not have an effect ($F_{1,23}$=0.27, P=0.606). There was no interaction between the treatments. Two-way ANOVA analyses did not reveal any effects of leaf or compost treatment on survival, weight or cocoon production.

4.2.5 Slug

4.2.5.1 Slug Methods

Deroceras reticulatum were collected from a lawn in Llandaff, Cardiff (ST 144 790) and kept overnight without food. *Bt* and non-*Bt* compost was prepared as described in Section 4.2.1.1.2, along with some John Innes No. 2 compost in which no plants had been growing. 30-35 g *Bt* compost was placed into 20 plastic containers (10 cm diameter, 5cm deep). *Bt* leaves were added to half the replicates and non-*Bt* leaves to the remaining ten. This process was repeated with non-*Bt* compost. Ten replicates were also prepared containing 30-35 g non-*Bt* compost and leaves that has been soaked in Dipel® (Section 4.2.2.1.2) as well as ten replicates with John Innes No. 2 compost and non-*Bt* leaves. Leaf discs from each treatment were kept for ELISA analysis (Section 2.2.5).

Individual slugs were weighed and five placed in each container (mean weight ± standard error: 0.234 ± 0.007 mg). Each week more leaves were added and the soil moistened with a mister spray; the number of individuals and eggs laid were counted. Eggs were collected and placed on moist filter paper in new containers to monitor hatching rates. After eight weeks the remaining surviving slugs in the containers were individually weighed. The containers were kept in a controlled environment room at 16°C (16:8 h light:dark cycle).

4.2.5.2 Slug Results

The slugs were exposed to compost from *Bt* broccoli plants expressing 9.15 ± 7.3 ng g^{-1} *Bt* protein in their leaves. The Dipel® treated leaves had 80.3 ± 6.5 ng g^{-1} *Bt* protein.

The number of adult slugs surviving was not significantly different across treatments ($H_{5,59}$=4.53, P=0.467). The mean slug weight had increased to 0.277 ± 0.007 g by the end of

the experiment and there were significant differences between the treatments ($F_{5,59}$=4.02, P=0.004); the slugs in the Dipel® treatment were the heaviest whilst those in the non-*Bt* compost/non-*Bt* leaf treatment were the lightest (LSD=0.023, P<0.05, Figure 4.10a). There was a significant difference between the mean number of eggs in each treatments ($F_{5,59}$=3.02, P=0.018). The fewest eggs were laid in the Dipel® treatment which was significantly different to all the other treatments except the non-*Bt* leaf/non-*Bt* compost treatment (LSD=3.21, P<0.05, Figure 4.10b). Only four of the Dipel® treated pots and eight of the non-*Bt* leaf/non-*Bt* compost had eggs in; all the other treatments had eggs in all their pots.

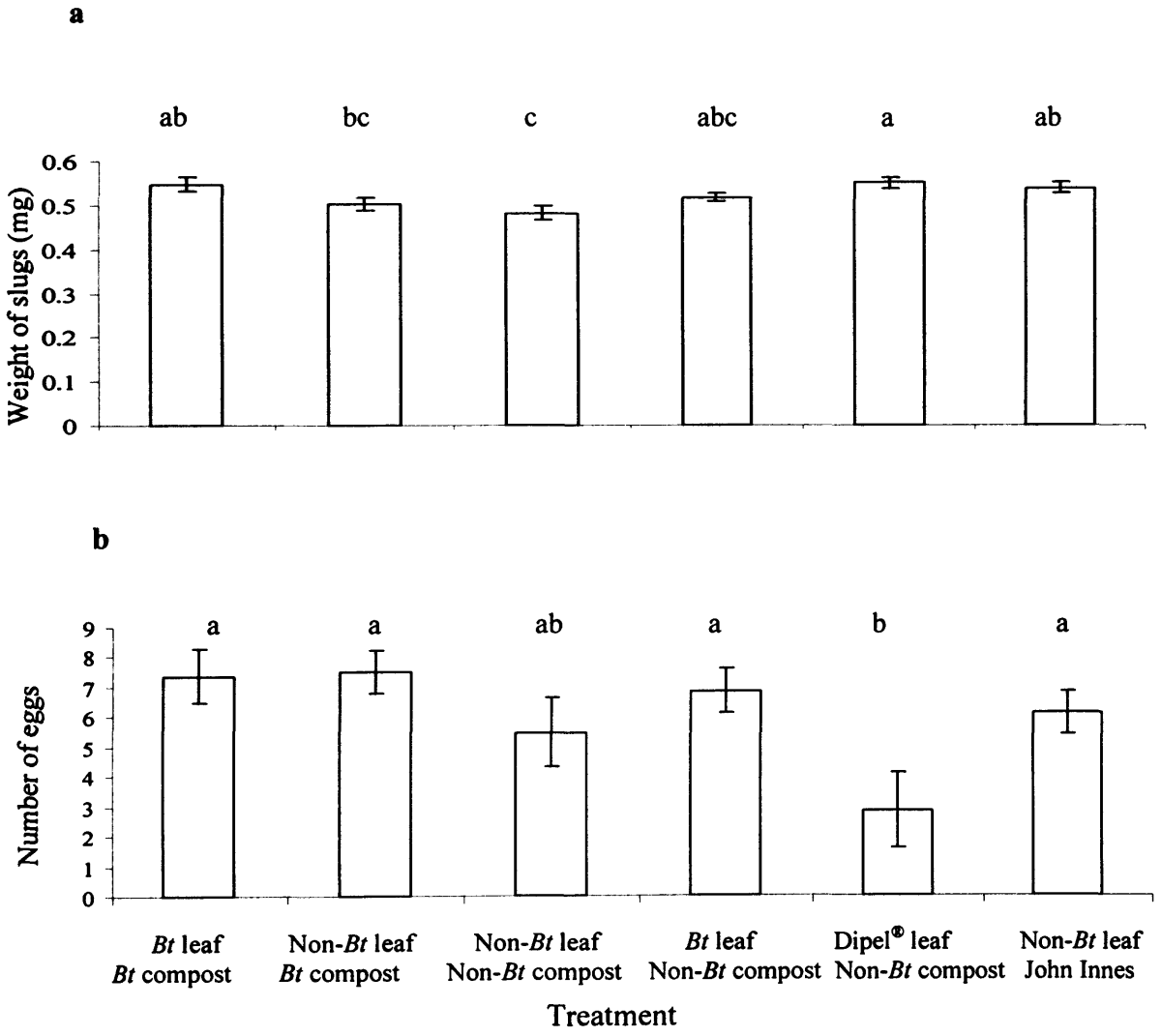

Figure 4.10 a) Weight (square root transformed) of slugs (mean ± standard error) and b) number of eggs (square root transformed) per treatment (mean ± standard error). Different lowercase letters indicate significantly different treatments.

The Dipel® treatment and the non-*Bt* leaf/John Innes compost treatment were removed from the data set for further two-way ANOVA analysis as described for the Collembola (Section 4.2.2.2.2). There was no significant difference in the number of eggs laid, as a result of either leaf treatment ($F_{1,39}$=0.13, P=0.720) or compost treatment ($F_{1,39}$=1.76, P=0.193), or adult survival (leaf $F_{1,39}$=0.31, P=0.570; compost $F_{1,39}$=0.62, P=0.420). Those slugs feeding on *Bt* leaves were significantly heavier ($F_{1,39}$=7.61, P=0.009, Figure 4.11) than those feeding on non-*Bt* leaves, but compost type did not have a significant effect on weight ($F_{1,39}$=3.01, P=0.091). There were no significant leaf/compost interactions in these two-way ANOVAs.

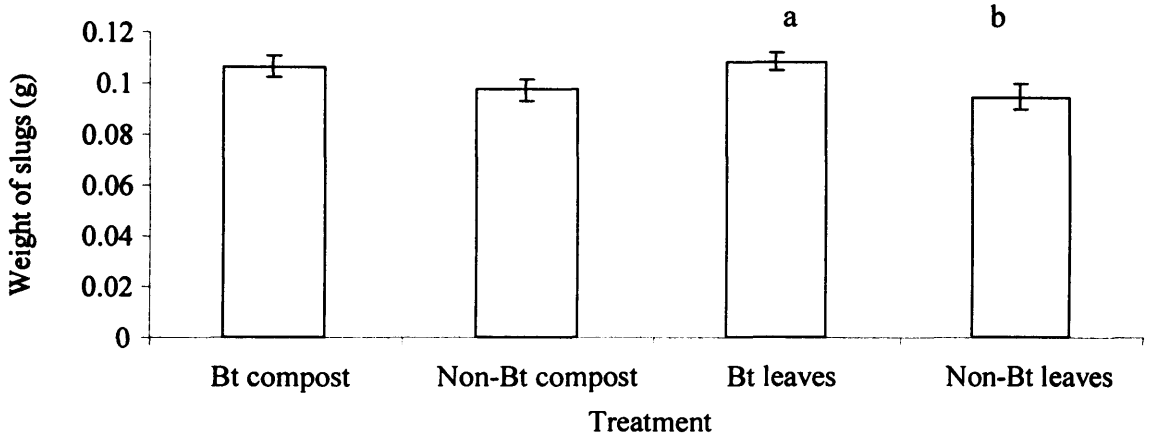

Figure 4.11 Weight (mean ± standard error) of slugs per pot (logarithmic transformed). Different lowercase letters indicate a significant difference between treatments in the leaf factor using a two-way ANOVA.

4.3 Discussion

The effects of the Cry1Ac protein produced from *Bt* broccoli on a diverse range of non-target species, across a range of several taxa, are varied (Table 4.2). *Bt* proteins in broccoli leaves used as a food source had a significantly positive effect on the survival, end weight and weight gain of small (but not large) woodlice, and the weights of slugs. *Bt* proteins in compost (Section 2.3.2) in which *Bt* broccoli had grown also increased hatching rates of earthworms from cocoons, but reduced the weight and rate of weight gain of small woodlice.

Table 4.2 Fitness parameters for the five non-target species with values (mean ± standard error) for *Bt* and non-*Bt* treatments. Factors used in the two-way ANOVA indicate leaf discs from *Bt* or non-*Bt* broccoli plants and compost in which *Bt* or non-*Bt* broccoli plants have grown. Non-significant results indicated by ns, significant results at P<0.05 by * and at P<0.005 by **. More details can be found in the relevant chapter (see Section column).

Non-target species	Fitness parameter measured	Unit	Factor used in two-way ANOVA	*Bt*	Non-*Bt*	P	Section
Nematodes	Survival	Number extracted	Compost ‡	13.3 ± 1.2	23.6 ± 4.2	ns §	4.2.1.2
Collembola	Survival	Number alive	Leaf ‡	8.3 ± 0.4	8.4 ± 0.3	ns §	4.2.2.2.1
	Reproduction	Total number of eggs produced over 4 weeks	Leaf ‡	237 ± 31	202 ± 22	ns §	
	Rate of egg production	Number of eggs per day	Leaf ‡	0.289	0.307	ns §	
Collembola	Survival	Number of adults	Compost	7.7 ± 1.24	6.6 ± 0.93	ns	4.2.2.2.2
			Leaf	7.2 ± 0.85	7.1 ± 1.32	ns	
	Reproduction	Number of juveniles	Compost	66.2 ± 22.8	45.9 ± 10.4	ns	
			Leaf	50.2 ± 9.81	61.9 ± 23.8	ns	
Woodlice	Survival	Number alive	Compost	8.05 ± 0.34	7.50 ± 0.40	ns	4.2.3.2
			Leaf	8.50 ± 0.25	7.05 ± 0.1	*	
	Reproduction	Number of pregnant woodlice	Compost	2.35 ± 0.28	1.65 ± 0.22	ns #	
			Leaf	2.30 ± 0.24	1.70 ± 0.27	ns #	
Small woodlice†	Survival	Number alive	Compost	3.5 ± 0.26	3.45 ± 0.23	ns	4.2.3.2
			Leaf	3.6 ± 0.25	3.35 ± 0.24	**	
	Weight	g	Compost	0.99 ± 0.01	1.00 ± 0.01	**	
			Leaf	1.03 ± 0.01	0.96 ± 0.01	**	
	Weight gain	g per week	Compost	0.76 ± 0.06	0.92 ± 0.07	**	
			Leaf	0.98 ± 0.05	0.70 ± 0.05	**	

† All large woodlice analyses were non-significant **‡ One-way ANOVA result** **# 0.05<P<0.09**

§ Significant differences were detected between other treatments as mentioned in text.

Table 4.2 continued

Non-target species	Fitness parameter measured	Unit	Factor used in two-way ANOVA	*Bt*	Non-*Bt*	P	Section
Earthworms	Survival	Number alive	Compost	4.83 ± 0.11	4.66 ± 0.14	ns	4.2.4.2
			Leaf	4.75 ± 0.13	4.75 ± 0.13	ns	
	Weight	g	Compost	0.68 ± 0.06	0.76 ± 0.06	ns	
			Leaf	0.71 ± 0.05	0.73 ± 0.06	ns	
	Reproduction	Number of cocoons	Compost	5.58 ± 0.89	5.41 ± 0.57	ns	
			Leaf	4.67 ± 0.45	6.33 ± 0.89	ns	
	Hatching rate	Percentage of cocoons hatched	Compost	73.0 ± 13.1	36.0 ± 9.3	*	
			Leaf	54.3 ± 11.3	46.3 ± 11.3	ns	
Slugs	Survival	Number alive	Compost	4.45 ± 0.17	4.25 ± 0.19	ns	4.2.5.2
			Leaf	4.25 ± 0.20	4.45 ± 0.15	ns	
	Weight	g	Compost	0.27 ± 0.01	0.25 ± 0.01	ns #	
			Leaf	0.29 ± 0.01	0.24 ± 0.01	*	
	Number of eggs	Number	Compost	61.1 ± 7.8	47.0 ± 7.8	ns	
			Leaf	56.65 ± 8.1	51.40 ± 7.7	ns	

 # 0.05<P<0.09

No other significant effects were detected in any of the other fitness parameters measured for these species or for the other species (nematodes and Collembola). With such a large data set there is always a possibility of finding a significant result by chance; in such cases, the Bonferroni correction may be applied. In this study, across so many different tests and taxa, this was not appropriate.

The lack of significant effects of the *Bt* protein on nematodes and Collembola is unsurprising considering how different their alimentary canals are to Lepidoptera (the target species). Other studies have failed to find effects of *Bt* proteins on nematodes and Collembola (e.g. Saxena *et al.*, 2001b, Duan *et al.*, 2004, respectively). One study has shown that there were fewer nematodes in *Bt* maize fields than non-*Bt* maize fields (Griffiths *et al.*, 2005) but it was suggested that this difference could have been related to soil moisture (soil surrounding *Bt* maize was drier than that around non-*Bt* maize) rather than a direct cause of the *Bt* protein. Another study, Clark & Coats (2006) reported lower reproduction of the Collembola *F. candida* on *Bt* maize than non-*Bt* maize but this difference was seen in only one of four *Bt* varieties tested; the authors believed this observation was not a direct effect of the *Bt* protein itself as all the reproduction rates (*Bt* and non-*Bt* maize) were significantly lower than the control food source (a grain derivative).

Despite there being no difference between the *Bt* and non-*Bt* compost treatments there were fewer nematodes present in all three soil treatments in which plants had been growing than the control treatment in which no plants had grown (the *Bt* and Dipel® treatments significantly fewer). This reduction in numbers could be due to the secretion of plant secondary metabolites (e.g. glucosinolates) from the plants into the soil and that these suppressed some nematode populations (Zasada & Ferris, 2004). Similarly, for the Collembola, different egg laying patterns were detected between the yeast and the leaf treatments but not between the *Bt* and non-*Bt* leaf treatments themselves. Romeis *et al.* (2003) showed that egg numbers were significantly higher for Collembola fed on yeast than on GM wheat and suggested that food type (plant vs. fungi) rather than quality (*Bt* vs. non-*Bt*) was more important to Collembola fitness.

Earthworm, woodlice and slug survival was unaffected by the presence of *Bt* protein in both leaves and compost. Small woodlice did have a higher survival rate when feeding on *Bt* leaves than when feeding on non-*Bt* leaves. They were also heavier and had faster weight

gain over the eight weeks when feeding on *Bt* leaves than on non-*Bt* leaves. Slugs were also heavier when feeding on *Bt* leaves than when feeding on non-*Bt* leaves. This result links with the increase in weight seen with the non-target Lepidoptera *Mamestra brassicae* when feeding on *Bt* broccoli (Section 3.3.2). Escher *et al.* (2000) showed that woodlice feeding on *Bt* maize had lower mortality whilst juveniles had greater weights as adults than those feeding on non-*Bt* maize. They attributed this difference to the lower lignin and higher soluble carbohydrate content of the *Bt* maize making it more nutritional than the non-*Bt*. A greater growth rate in *E. fetida* earthworms seen on *Bt* maize than non-*Bt* maize was also attributed, not to the *Bt* protein, but to differences in protein and sugar levels between the *Bt* variety and its non-*Bt* equivalent (Clark & Coats, 2006).

It seems unlikely that the difference in woodlice and slug weights seen in this study can be attributed directly to the *Bt* protein as the Dipel® results (Figure 4.6 and 4.10) do not follow a similar trend although this latter treatment had a higher concentration of *Bt* proteins. It seems more likely that the *Bt* gene inserted into the plant genome is affecting other metabolic pathways (reviewed in Filipecki & Malepszy, 2006) which affects the nutritional content of the plant. It would be necessary to analyse the *Bt* broccoli plants chemically to determine which metabolites, if any, are altered, possibly concentrating first on lignin and carbohydrates.

Lower lignin content in maize plants attracts a higher level of microbial colonisation and faster decomposition (Escher *et al.*, 2000), and woodlice would graze on these colonies. The effect of *Bt* plants on the soil microbial community is discussed in Chapter 6. The *Bt* broccoli seeds used in this study were bred by crossing *Bt* and non-*Bt* plants (Section 2.1.1). These seeds are therefore second generation with varied genotypes (Figure 2.1). This may also affect phenotypic properties such as metabolite levels e.g. secondary defence chemicals, nutritional values and root exudates which could all affect the non-target species investigated in this chapter. If the *Bt* gene links with a broccoli gene they will segregate together during gametogenesis resulting in a correlation between *Bt* protein production and the protein the broccoli gene encodes. By selecting the *Bt* plants with higher *Bt* protein expression we may also have inadvertently selected plants with a different nutritional value, for example, lower lignin content.

A full chemical analysis and comparsion of *Bt* and non-*Bt* broccoli plants is necessary to distinguish between a direct effect of the *Bt* protein and an indirect effect of the GM process. If compositional differences are uncovered, other than the presence of *Bt*, then an investigation would be necessary into whether it is the indirect effect of the change in chemical levels that is the cause of the positive impact of the *Bt* broccoli on woodlice and slug fitness parameters. A comparison of the non-target effects of the *Bt* broccoli with several different cultivars of broccoli would aid these investigations. For example, Wandeler *et al.* (2002) investigated the effect of two *Bt* and six non-*Bt* maize varieties on woodlice consumption rates. Consumption rates varied between the eight varieties but without correlation with *Bt* concentration or plant energy content (Wandeler *et al.*, 2002). For *Bt* plant risk assessment purposes (Section 1.4.8) the level of the impact of *Bt* broccoli on non-target species would need to be compared with the impact of several broccoli cultivars with different chemical compositions. If the impact of the *Bt* plant falls within a similar range seen with different broccoli cultivars, and it frequently does (Filipecki & Malepszy, 2006) then the risk of releasing the *Bt* broccoli would probably be deemed low.

The weight of earthworms was unaffected by the *Bt* protein in either plant tissue or compost. Saxena & Stotzky (2001b) also detected no differences in the weights of *L. terrestris* that had been feeding in soil planted with *Bt* maize and non-*Bt* maize for 40 days. In a longer exposure, Zwahlen *et al.* (2004) detected no differences in the weights of *L. terrestris* up to 160 days, but at 200 days those feeding on *Bt* corn weighed less than those earthworms feeding on non-*Bt* corn. This shows the importance of longer-term exposure studies. Clark & Coats (2006) showed that the effect of *Bt* maize on earthworm growth was limited to two of four GM lines tested so the result for the *Bt* broccoli used in this study is accurate but we cannot predict the same result would be reported for other *Bt* broccoli cultivars.

Reproduction parameters, especially progeny viability, are considered the more sensitive fitness parameters to monitor as it is affected by the accumulated impact of several of the parent's fitness parameters including longevity and mass (Lövei & Arpaia, 2005). Earthworm cocoons had a higher hatching rate in *Bt* compost than in non-*Bt* compost. This is in contrast to another study that reports a reduction in *A. caliginosa* cocoon hatching rates when the adults were in contact with soil in which ground up *Bt* maize had been added (Vercesi *et al.*, 2006). The *Bt* maize plants produced 9.6 $\mu g\ g^{-1}$ Cry1Ab proteins, much

higher than the Cry1Ac producing *Bt* broccoli plants used in this study (291 ± 74.8 ng g^{-1}). This could be a factor in the contrasting results of the two studies. It is, however, difficult to compare the effects of these two different earthworm species in contact with two different *Bt* proteins from two different GM plants.

The addition of cellulose to soil-free media increased *E. fetida* cocoon production and fine changes to phosphorus, carbon, nitrogen and sulphur levels resulted in measurable changes to cocoon production, with a positive correlation between the number of cocoons and phosphorus levels (Bouwman, 1997). These factors could be attributing to the differences in cocoon production measured in this study although further analysis of the *Bt* plants, their root exudates and the surrounding compost would be necessary to confirm if they altered the compost differently to their non-*Bt* equivalents. In contrast, a negative impact of *Bt* compost was observed in this study on small woodlice weight and weight gain. This effect may be related to moisture content. Griffiths *et al.* (2005) noted that soil surrounding *Bt* maize roots was drier than that around non-*Bt* maize plants. The compost used in this experiment was dried for the same amount of time (overnight) but the water content was not analysed to ensure they were equal. Juvenile (small) woodlice are probably more sensitive to these differences in moisture levels than the adult (large) woodlice and earthworms.

The data collected for the small woodlice produced the most number of significant differences (five out of six) and highlights the need to investigate the effects on both adults and juveniles. Woodlice reproduction is of particular interest for further investigation as with non-significance being marginal (Table 4.2) a longer study specifically on reproduction might reveal significant effects of *Bt* plants. On reflection it would have been appropriate to monitor all young produced during these experiments as for the Collembola, rather than concentrating on reproduction rates.

In most cases the results were significant for the leaf treatment but not the compost treatment. *Bt* proteins are degraded by micro-organisms and Sims & Holden (1996) showed that *Bt* proteins would disappear fairly rapidly from *Bt* soils, although other studies suggest *Bt* proteins may remain in some soils for longer (Section 1.4.5). By removing the plants the assumed continuous introduction of *Bt* protein to the compost (Section 2.4) was stopped, and as the soil was also dried overnight by the time the organisms were introduced there may have been no protein remaining in the compost. This could explain why the compost

treatment had only two significant effects (on one fitness parameter of two different species); it would be more realistic to place the organisms in microcosms with the plants still in place. A time-course study to investigate the degradation of *Bt* protein in soil (e.g. Tapp & Stotzky, 1998; Section 1.4.5) would be illuminating but with only a few compost samples testing positive (Section 2.3.2) priority should be given to developing a more sensitive ELISA.

The change in fitness of the species discussed in this Chapter seem most likely to be indirect effects of the GM process rather than a direct effect of the *Bt* protein itself. This is supported by the observation that the effect of Dipel® (containing the same *Bt* protein) did not follow the same trend as that of the *Bt* plant and *Bt* compost treatments. The indirect effects seen to woodlice, slugs and earthworms could be caused by changes to plant chemical composition, root exudation and soil conditions. Further investigations need to take place to explore these potential changes and any indirect effect would need to be compared with those changes caused by other agricultural factors; Griffiths *et al.* (2005), for example, showed that the difference in nematode numbers detected in *Bt* and non-*Bt* maize was less than the differences seen between different crop species.

The studies reported here were all small scale laboratory-based experiments with just one species being investigated at a time. In the field, over a longer period, where many species are interacting, the effects are likely to be different, especially when there is a choice of food source. For example, no differences were found between the numbers and diversity of 65 agriculturally important taxa of ground-dwelling arthropods (including araneids, heteropterans, carabids, cicindelines and staphylinids) in *Bt* and non-*Bt* cotton fields (Torres & Ruberson, 2006). The results collected in this study should also not be used to extrapolate from one species to the whole genus, family or order. If comparable results were detected in a field-scale test then commercial planting of these *Bt* broccoli plants would have to be considered carefully whether as a cash or trap crop (Section 3.5). The potential effects on some of these species could alter the natural balance of the field, for example, faster growing slugs would cause more damage to a crop and faster growing woodlice may alter decomposition rates. A change in numbers to any one of these species could also have a knock-on effect on higher trophic levels. This is discussed more thoroughly in Chapter 5.

5 Tri-trophic interactions: the effects of *Bt*-transformed plants on a predator of a non-target herbivorous pest (slugs)

5.1 Introduction

The direct and indirect effects of *Bt* plants on non-target invertebrates were considered in Chapter 4. Various studies (including the current study) have investigated the consequences of above-ground invertebrates coming into direct contact with *Bt* proteins through feeding on genetically modified plant foliage, sap and pollen (see Sections 1.4.7.1 and 1.4.7.2 and references therein), and of below-ground invertebrates encountering the protein through plant roots and protein exudates secreted into the soil (Section 1.4.7.4 and references therein). Non-target species closely related to the target organism are occasionally affected by the *Bt* proteins while species not so closely related seem to suffer no ill-effects.

Invertebrate species positioned at a higher trophic level in the food web may also come into contact with the *Bt* protein by, for example, directly ingesting pollen (Lundgren & Wiedenmann, 2002; Ludy & Lang, 2006). There are also less direct routes; for example, predators and parasitoids may be indirectly affected through ingestion of prey/hosts that have fed on genetically modified plants. *Bt* protein-effects on natural enemies could have important implications on the control of pest species; both parasitoids and predators act as efficient biocontrol agents (e.g. Bellows & Fisher, 1999; Rechcigl & Rechcigl, 2000).

Where such tri-trophic studies have been carried out, the majority have considered the effects of *Bt* proteins on predators (e.g *Chrysoperla carnea* Stephens (Neuroptera: Chrysopidae) Hilbeck *et al.*, 1998) or parasitoids (e.g. *Microplitis mediator* Haliday (Hymenoptera: Braconidae) Liu *et al.*, 2005a) of target organisms; little is known of effects on natural enemies of non-target species (Section 1.4.7.3 and references therein). A recent review of the effects of transgenic plants on natural enemies (Lövei & Arpaia, 2005) highlights the constraints of the relatively few (26) studies that have focused on easily cultured species from a limited number of taxanomic groups. Hymenoptera, especially parasitoids, are over represented whilst only five studies have researched the effects of *Bt* plants on Coleoptera, four of which look at *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae, Lövei &

Arpaia, 2005). Some groups, for example ants, are ignored. The majority of studies also focus on mortality and body mass, whilst Lövei & Arpaia (2005) suggest that reproduction and fitness of immature stages could be more sensitive to *Bt* proteins.

Predators and parasitoids of target species are likely to come into contact with the *Bt* protein through ingesting prey or parasitising hosts which have been feeding directly on the genetically modified plant. The mode of action of the *Bt* protein makes it unlikely that there will be marked direct effects on predators and parasitoids due to specificity of the protein (Section 1.2.2); natural enemy species are frequently members of a different order to that of the target pest and as, in most cases, the protein has been chosen to target only the pest organism no effects would be expected on either predator or parasitoid. Even in the presence of appropriate receptors in the natural enemy the protein is unlikely to have a direct effect because the alimentary canal enzymes found in the prey/host and its predator/parasitoid may digest the protein making it inactive, or the predator/parasitoid may require a higher concentration of the protein than the prey/host to be effective.

Tritrophic studies exploring the effects of the *Bt* protein on predators/parasitoids of target species (Section 1.4.7.3), despite differences in structure, crop type, *Bt* protein used and scale (from microcosm to field experiments), do show a distinct trend in findings. When detected, effects at the third trophic level are generally attributable to the *Bt* protein acting via an indirect route, usually due to changes in prey/host fitness (e.g. Hilbeck *et al.*, 1999; Walker *et al.*, 2007) or population level (e.g. Deng *et al.*, 2003).

Despite the expectations of limited effects of *Bt* plants on non-target species, as the results reported in Chapter 4 suggest, this may not be the case. If non-target herbivores of *Bt* plants are affected then it is possibile that these effects will filter into the third trophic level (predators and parasitoids of non-target herbivores). There remains a gap in our knowledge with studies that do consider these issues (Section 1.4.7.3 and references therein) focussing on predators and parasitoids of economically important non-target herbivores such as aphids and thrips (Section 1.4.7.3). Consequently, there is reason to suspect that the effects reported on natural enemies of target species should also be observed in natural enemies of non-target species. The effects may, however, be lower as the fitness or abundance of non-target prey/hosts should in general not be affected by the action of the *Bt* protein, although they sometimes are, for example, woodlice body weight (*Porcellio scaber*, Latrielle (Crustacea:

Isopoda)) (Table 4.2 and Escher *et al.*, 2000). Comparing the few (11) existing studies (Romeis *et al.*, 2006) is difficult as some are more realistic than others, for example, feeding purified *Bt* toxin directly to a predator in an artificial diet (e.g. Romeis *et al.*, 2004) is less realistic than feeding a predator prey that has been feeding on *Bt* plants (e.g. Zwahlen *et al.*, 2000).

Changes to the abundance and fitness of natural enemy species may lead to secondary effects on biodiversity in and around the *Bt* crop. This possibility highlights the need to investigate the effects of *Bt* plants on third (e.g. predators and parasitoids of herbivores), and higher (e.g. hyperparasitoids, Prutz *et al.*, 2004) trophic levels prior to making predictions of any long-term and wider ranging effects. In this chapter a relatively simple experiment was designed to measure the effects of *Bt* plant consumption on a predator of a non-target organism. The non-target pest slug *Deroceras reticulatum* Müller (Pulmonata: Limacidae) was found to be heavier when feeding on *Bt* broccoli (*Brassica oleracea* L. var. *italica* Plenk) leaves compared to those feeding on non-*Bt* broccoli leaves (Section 4.2.5.2). *Nebria brevicollis* Fabricius (Coleoptera: Carabidae), a predator of *D. reticulatum,* was chosen as an appropriate predator and the effects on its survival, weight and reproduction when fed on slugs that had ingested Cry1Ac broccoli plants were investigated. It was hypothesised that prey diet (*Bt* vs. non-*Bt*) would have no effect on beetle number, weight, foregut weight and number eggs due to the specific nature of the Cry1Ac protein.

5.2 Methods

5.2.1 *Deroceras reticulatum* collection and maintenance

Deroceras reticulatum slugs, less than 1 cm in length, were collected from a garden lawn in Llandaff, Cardiff (ST 144 790). The collected slugs were then split between three containers (20 cm^2, 5 cm deep) and fed on *Bt* broccoli leaves, non-*Bt* broccoli leaves or non-*Bt* leaves soaked in Dipel® (made up at 20 times the manufacturer's recommendation (15 g l^{-1})) for ten minutes before air drying for 30 minutes. After seven days of feeding at 16°C in a controlled environment room (16:8 h light:dark cycle) the slugs were frozen at -20°C until required. Two leaf discs (1 cm diameter, Section 2.2.2) from each treatment were cut and kept for ELISA analysis (Section 2.2.5).

5.2.2 *Nebria brevicollis* collection and maintenance

Beetles were collected by pitfall trapping. Plastic containers (15 cm deep, 7 cm diameter, with drainage holes pierced in the base) were dug into the ground so that the top was level with the soil surface. They were placed in a field recently reseeded to pasture at 2 m intervals, approximately 1 m from the hedge and 10-20 cm into the field, located at Goldfields Dairy Farm, Wenvoe, Cardiff (ST 113 709).

The traps were emptied every two days for three weeks in early Autumn and any *Nebria* species individuals collected were returned to the laboratory. Those identified as *N. brevicollis* were kept for future use; other species were returned to the field. *Nebria brevicollis* were placed at densities of 50 per plastic containers (20 cm^2, 5 cm deep) with 1 cm damp peat in the bottom and fed *Calliphora* sp. larvae (killed by freezing) every three or four days. Prior to the experiment *N. brevicollis* were starved for seven days to ensure gut evacuation.

5.2.3 Experimental design

Forty microcosms (10 cm diameter, 5 cm height) containing 1cm layer of John Innes No. 2 compost were set up with five treatments and eight replicates of each. Sixteen microcosms contained compost in which *Bt* plants had been grown (Section 2.2.1). This compost was dried and sieved (Section 4.2.1.1.2). Each microcosm was provided with approximately 50 mm^3 slug to eat. This standardization of slug volume was to control for the significant difference in the weight of slugs that had been feeding on different leaf treatments (see Section 4.2.5.2). Half of the 16 microcosms contained chopped-up tissue of *D. reticulatum* which had been eating *Bt* leaves (*Bt* slugs), the compost in the remaining eight contained *D. reticulatum* which had been feeding on non-*Bt* leaves (non-*Bt* slugs). The remaining 24 microcosms contained soil in which non-*Bt* plants had grown; eight had *Bt* slugs added to them, eight non-*Bt* slugs and eight *D. reticulatum* which had been feeding on Dipel® leaves (Dipel® slugs). Ten *N. brevicollis* beetles were weighed individually and added to each microcosm. The microcosms were then placed in a controlled environment room (16°C, 16:8 h light:dark cycle). Twice a week all 40 microcosms were sprayed lightly with de-ionised water to maintain humidity; any dead beetles were removed and living beetles were fed another section of chopped slug (5 mm^3 slug per individual beetle). After 28 days the

experiment was stopped, 24 h after the last monitoring day. The number of living beetles in each microcosm were counted, individually weighed and placed in micro-centrifuge tubes before being placed in a -20°C freezer. The microcosms were returned to the controlled environment room and seven, ten and 14 days after the beetles were removed the microcosm compost was manually searched for hatched beetle larvae.

24 h after freezing the adult beetles were dissected; beetle sex and, if female, the number of eggs in the ovariole was recorded. For each individual the foregut was also weighed and placed in clean micro-centrifuge tubes. Some of the remaining chopped slug sections and the leaf discs (Section 5.2.1) were also placed in micro-centrifuge tubes. Slug, beetle foreguts and leaf discs were analysed using an ELISA kit (Section 2.2.5). Only the foreguts of beetles that had been in contact with non-*Bt* soil, and that fed on *Bt* and non-*Bt* slugs were analysed with one alteration to the ELISA protocol. There was an alteration to the ELISA protocol as all the beetle foreguts weighed less than the ELISA kit's recommended weight of 20 mg: 100 µl extraction/dilution fluid was added to foreguts weighing up to 4 mg and an additional 25 µl per mg for heavier foreguts. This alteration was made to maximise the chance of detecting *Bt* protein in the samples. The samples were then ground up and placed in a micro-centrifuge for one minute at 13,000 rpm before loading into the plate wells and continuing with the recommended protocol (Appendix 1).

5.2.4 Data analysis

The number, weight, foregut weight and, in females, the number of eggs were analysed using the statistical program Minitab v14.1 (Minitab Inc., PA, USA). Insufficient numbers of *N. brevicollis* larvae were recovered for statistical analysis; only data on the adult beetles were considered. When the data (sometimes transformed) met the assumptions (normally distributed residuals and homogeneity of variances) one-way analysis of variances (ANOVA) were performed, in all other cases a non-parametric Kruskal-Wallis test was applied. Following this initial analysis, the Dipel® treatment was removed from the data set and a second set of one-way ANOVAs performed followed by two-way ANOVAs where the slug and compost type in each treatment were treated as variables. It was considered appropriate to remove this data set as the crux of the study was to investigate the effects of GM plants not the *Bt* proteins present in the Dipel®. Again the data were either transformed or a non-parametric test (Kruskal-Wallis or Sheirer-Ray-Hare tests) was performed.

5.3 Results

The *D. reticulatum* slugs were fed on *Bt* leaves from one *Bt* plant containing 3.26 ng g^{-1} *Bt* protein and Dipel® coated non-*Bt* leaves with 80.3 ± 6.5 ng g^{-1}. *Bt* protein was not detectable in the slugs nor in the *N. brevicollis* foreguts (threshold for positive sample: mean + three standard deviations of the non-*Bt* samples, Section 2.2.5).

No significant differences (P>0.05) were recorded between the five treatments when considering the number, final weight, foregut weight or the number of eggs of surviving beetles (one-way ANOVA, Table 5.1). Apart from the number of surviving beetles, for each parameter the mean value for each pot was calculated and each pot used as a replicate to avoid pseudoreplication. The only significant difference detected was that between the number of surviving beetles when fed on *Bt* and non *Bt* slugs (two-way ANOVA with Dipel® data set removed, Table 5.2). This suggested that while compost did not have an effect ($F_{1,7}$=0.66, P=0.423) slug treatment did ($F_{1,7}$=4.46, P=0.044); there was no interaction between the treatments. Fewer beetles survived on slugs which had eaten *Bt* leaves than on slugs which had eaten non-*Bt* leaves (Figure 5.1).

Table 5.1 Mean numbers of surving beetles, final weight, foregut weight and number of eggs per female beetle in each treatment (mean ± standard errors) with one way ANOVA/Kruskal Wallis results. *Bt*, non-*Bt* and Dipel® slugs had fed on leaves from *Bt* and non-*Bt* plants and non-*Bt* plants coated with Dipel® leaves. *Bt* and non-*Bt* compost come from plant pots in which *Bt* and non-*Bt* plants had grown.

	***Bt* compost, *Bt* slugs**	***Bt* compost, Non-*Bt* slugs**	**Non-*Bt* compost Non-*Bt* slugs**	**Non-*Bt* compost, *Bt* slugs**	**Non-*Bt* compost, Dipel® slugs**	**$F_{4,7}$, P**
Number of surviving adults	4.75 ± 0.90	5.75 ± 0.49	7 ± 0.87	4.75 ± 0.75	5.62 ± 0.50	1.64, 0.187
Beetle weight (mg)	52.41 ± 1.16	51.17 ± 1.03	50.14 ± 1.48	51.50 ± 2.02	53.97 ± 1.17	1.03, 0.404
Foregut weight (mg)	7.02 ± 0.23	5.82 ± 0.42	5.91 ± 0.53	6.57 ± 0.74	7.07 ± 0.38	1.47, 0.233
Number of eggs per female	1.73 ± 0.59	0.88 ± 0.43	1.17 ± 0.54	1.38 ± 0.59	1.09 ± 0.32	H=1.23, 0.874

Table 5.2 Mean numbers of surviving beetles, final weight, foregut weight and number of eggs produced per female beetle (mean ± standard errors) with two-way ANOVA/Sheirer-Ray-Hare results for each treatment and interaction between treatments. *Bt* and non-*Bt* slugs had fed on leaves from *Bt* and non-*Bt* plants. *Bt* and non-*Bt* compost came from plant pots in which *Bt* and non-*Bt* plants have grown. Significant results in bold.

	Bt compost	Non-*Bt* compost	*Bt* slug	Non-*Bt* slug	Interaction
Number of surviving adults	5.25 +0.51	5.87 + 0.63	**4.75 + 0.57**	**6.38 + 0.51**	
	$F_{1,7}$=0.66, P=0.423		**$F_{1,7}$=0.446, P=0.044**		$F_{1,7}$=0.660, P=0.423
Beetle weight (mg)	51.00 + 0.90	52.07 + 1.13	52.09 + 0.79	50.96 + 1.20	
	$F_{1,7}$=0.44, P=0.515		$F_{1,7}$=0.780, F=0.383		$F_{1,7}$=0.0, P=0.969
Foregut weight (mg)	5.89 +0.33	6.75 + 0.40	6.30 + 0.35	6.34 + 0.42	
	$F_{1,7}$=0.00, P=0.947		$F_{1,7}$=3.79, P=0.062		$F_{1,7}$=0.11, P=0.740
Number of eggs per female	1.30 + 0.37	1.27 + 0.39	1.55 + 0.41	1.02 + 0.36	
	$F_{1,7}$=0.00, P=0.937		$F_{1,7}$=0.780, P=0.411		$F_{1,7}$=0.30, P=0.586

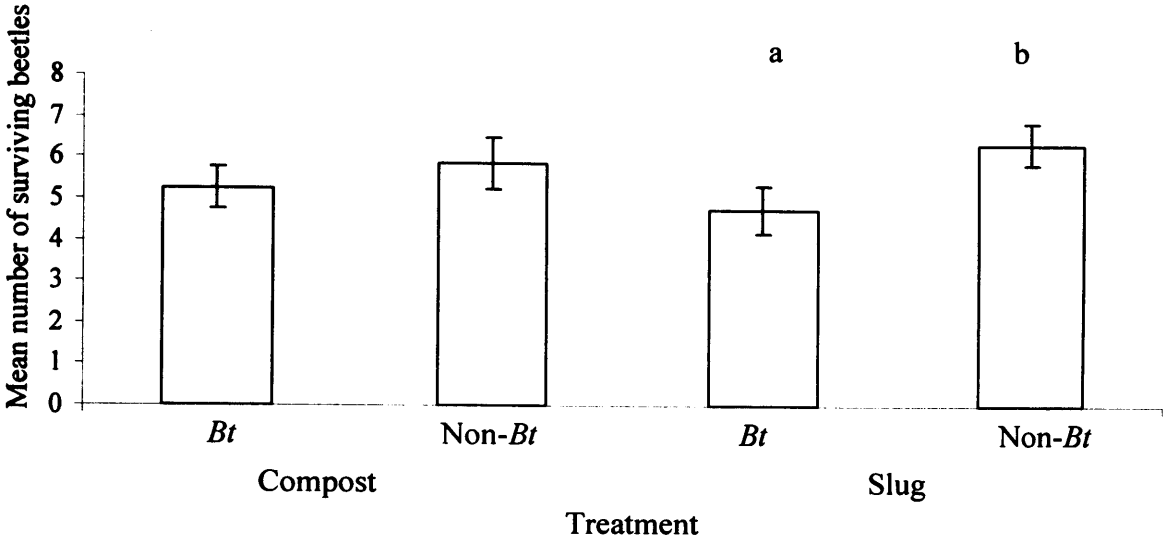

Figure 5.1 Number of surviving beetles after four weeks in each treatment (mean ± standard errors). Different lowercase letters indicate a significant difference between the treatments for the slug variable (fed on *Bt* and non-*Bt* leaves) using a two-way ANOVA with the Dipel® treatment removed.

5.4 Discussion

A significant difference was found in the survival of *N. brevicollis* individuals with more dying when fed on *Bt*-fed slugs compared to the number not surviving when feeding on non-*Bt* slugs. This is in contrast with other studies investigating the effects of *Bt* plants on predators; no difference between Coleoptera (carabid and staphylinid) numbers in *Bt* and non-*Bt* fields (Duan *et al.*, 2004; Torres & Ruberson, 2006) and an increase in predatory mite, spider and beetle (*Propylea japonica* Thunberg (Coleopotera: Coccinillidae) numbers in *Bt* fields (Deng *et al.*, 2003). Deng *et al.* concluded that the increase in predators was caused by an increase in prey species in the *Bt* crop field; in this experiment the apparent positive effect on the prey (increase in slug weight) has had a negative effect on the predator (decrease in survival), despite the experiment being designed to control for the differences in weight.

It is necessary to determine whether this difference in beetle survival is caused directly by the presence of the *Bt* protein in the slugs or is an indirect effect of the *Bt* plants. The ELISA did not detect *Bt* protein in the slugs and beetles, but the protein could still be passing intact from the plant, through the slugs, into the beetles. Bernal *et al.* (2002)

showed that *Bt* proteins derived from GM rice could be detected in the honeydew of the non-target insect, *Nilaparvata lugens* (Stål) (Delphacidae: Delphacinae), but there was no effect on two fitness parameters of its predator, *Cyrtorhinus lividpennis* (Reuter) (Heteroptera: Miridae) (survival to adult and developmental time for both males and females). Bernal *et al.* (2002) also reported no discernible effects of a *Bt* crop on *N. lugens* but they also did not analyse the predators for the presence of *Bt* proteins in the predators. The present study, with its mortality effects, found no *Bt* protein in the surviving beetle foregut but those beetles that died during the experiment (and were cannabalised) may have contained a detectable level of *Bt* protein in their foreguts.

The negative ELISA results for the surviving adult beetle foreguts could be accounted for by the 24 hour period between last feeding and death; the protein may have been digested by the beetle alimentary canal enzymes during this period. Analysis of *D. reticulatum* proteins within *Pterostichus melanarius* (Illiger) (Coleoptera: Carabidae) showed that ELISA detection of decayed slug remains was not possible at 12 hrs after ingestion (Calder *et al.*, 2005); it is possible that the *Bt* protein would also be digested within this period. Harwood & Obrycki (2006), however, did detect *Bt* proteins in slugs for up to 95 hours after a 3 h feeding period. These Cry1Ab-producing *Bt* maize (*Zea mays*, L.) plants did however, contain a much higher concentration of *Bt* protein (0.72 $\mu g\ g^{-1}$) than the Cry1Ac producing *Bt* broccoli plants (3.26 $ng\ g^{-1}$) used in this study.

ELISA has been used to detect Cry1Ab *Bt* protein in the alimentary canals of a range of predators, including spiders and beetles, found in a *Bt* maize field (Harwood *et al.*, 2005). Harwood *et al.* detected the protein in four Coleoptera species (all Coccinellidae) with mean concentrations ranging from 0.42 to 0.88 $\mu g\ g^{-1}$, whilst this study did not detect *Bt* protein in *N. brevicollis* guts. This difference in detection could be attributed to the different nature of the ELISA kits. ELISA kits have different specificities, probably due to the use of different antigens found on the surface of the different *Bt* proteins and the different antibodies used to detect them. It is possible that, for the present study, the ELISA kit's Cry1Ac target antigen is altered by the digestion process as it passes through the slug and the beetle, whilst the Cry1Ab target antigen used by the Envirologix kit (Portland, Maine, USA) in Harwood *et al.*'s (2005) experiment was slower to lose its stereo-chemical configuration. The latter authors also used the absorbance of the lowest concentration (0.5 $ng\ g^{-1}$) calibration standard as the threshold to calculate positives whilst in this present study the use of mean plus three standard deviations of the non-*Bt* samples is considerably more conservative. Harwood *et al.* (2005) did not, however, quantify the

level of *Bt* protein in the maize fields where their arthropod samples originated so it cannot be demonstrated that the higher concentration of *Bt* protein detected in the guts is related to higher levels in their *Bt* plants.

Cry1 proteins, such as those found in these *Bt* broccoli plants, are not known to be active against Coleoptera; the Cry3 proteins are usually used to target beetles (e.g. Donovan *et al.*, 1992). If the Cry protein in the *Bt* plants were the cause of the lower survival of *N. brevicollis* then a similar trend would be expected with the slugs fed on Dipel® treated leaves. If the Cry1 proteins were active then significantly lower weights would have been expected in the beetles feeding on both *Bt* and Dipel® slugs. This is particularly true of foregut weight as the beetles would have stopped feeding once the protein had been digested (Section 1.2.2). Combined with the negative ELISA results it seems unlikely that the lower survival of beetles is a direct effect of the *Bt* protein and other indirect causes should be considered.

Some, as yet unknown, "quality" of *Bt* slugs appears to be affecting survival of the *N. brevicollis*; this could be related to why slugs feeding on *Bt* leaves were heavier than those feeding on non-*Bt* leaves (Section 4.2.5.2). The data show that there were no differences in either the weight of the surviving beetles or the weight of their foreguts. This suggests that although there was a difference between the *Bt* and non-*Bt* slugs, the beetles are not ingesting or assimilating more slug tissue to compensate for this difference. Compensatory mechanisms have been detected in other species; for example Hilbeck *et al.* (1999) showed that *Chrysoperla carnea* larvae took a longer developmental time when fed *Spodoptera littoralis* Boisduval (Lepidoptera) larvae which had ingested *Bt* proteins at sub-lethal concentrations. With hindsight it would have been prudent to attempt to collect, weigh and analyse the foreguts of the dead beetles for *Bt* protein content. Unfortunately, many of the dead beetles had already been cannibalised by the surviving beetles before they were removed from the microcosms.

The slug *Tandonia budapestenis* is toxic to an agriculturally important slug predator *P. melanarius* but when cut open *T. budapestensis* were not toxic suggesting the presence of a toxin in the skin or mucus (Symondson, 1997). Many gastropods for example, nudibranchs (Avila & Paul, 1997) and snails (Hesbacher *et al.*, 1995) accumulate metabolites in their mantles and mucus in order to deter predators. These metabolites may be accumulated from their food source, for example, snails accumulate two anti-feedants (atranorin and parietin) from lichen (Hesbacher *et al.*, 1995) whilst nudibranchs

get a chemical from feeding on sea fans which is toxic to fish (Cronin *et al.*, 1995). The beetle predators in this study were not deterred from feeding as no *D. reticulatum* remains were found in the microcosms. This, however, may have been due to lack of food choice. If, as considered in Chapters 3 and 4, genetic modification has altered the composition of the broccoli plants in ways other than the production of *Bt* proteins, this effect may well filter through to the beetles via the slugs. The exact mechanism of toxicity to *N. brevicollis* remains uncertain and requires chemical analysis of the *Bt* and non-*Bt* plants, paying particular attention to plant defence metabolites found in Brassicas (e.g. glucosinolates) and analysis of slug mucus.

Measuring the fitness of an organism is fraught with difficulties. In this present study a range of parameters including mortality and weight were used. A more sensitive parameter of fitness is to measure reproduction; this is an indicator of the accumulated measure of several parameters of the mother's fitness (Lövei & Arpaia, 2005). Comparison between the number of eggs laid, their hatching success and larval developmental times between the treatments would have been informative. Unfortunately not enough *N. brevicollis* larvae were recovered for statistical analysis; only ten larvae were recovered from a total of 40 pots. Adult *N. brevicollis* may have cannibalised their own eggs and larvae, and, in hindsight, it may have been better to have removed the adults and place them in new microcosms at regular intervals during the experiment. Another possibility would have been to use a matrix of hydroleca pebbles balanced over a mesh for the adult beetles to lay their eggs. Any eggs laid then fall through the mesh and may be counted. This method has been successfully employed to monitor reproduction of *P. melanarius* (Thomas, 2002) but does remove beetle contact with compost. While the present study suggests that there was no effect of *Bt* compost on the adult fitness parameters measured, there remains uncertainty as to the effect on egg hatching and beetle larvae and this should be investigated further. Data were, however, collected on the number of eggs found in each dissected female and this showed that there was no significant difference between the treatments. Extrapolation of these results suggest that there would have been no difference in the number of eggs laid but they do not indicate whether there would have been a difference in hatching rates or the survival of the larvae.

Increased mortality could reduce the numbers of *N. brevicollis* in a field regardless of effects on reproduction. A 40 % mortality rate can have significant long-term population effects (Lynch *et al.*, 2001) and, in this study, almost 50 % mortality was seen in the treatment where beetles were fed slugs that had fed on *Bt* plants (Table 5.2). By reducing

the numbers, or even removing the presence, of this predator it can be surmised that there may be alterations to the populations of other invertebrates, especially other prey and competing predatory species. This may have subsidiary effects on the biodiversity of a *Bt* field.

Data from prey choice experiments show that the number of predators were negatively affected by *Bt* plants if only one prey (*Plutella xylostella* L. (Lepidoptera), the target species) was present but numbers were not affected if an alternative prey (aphids) was also available (Schuler *et al.*, 2005). Parasitoids will also choose to hunt on non-*Bt* plants, facilitated by the herbivore-induced volatiles produced by the damaged plants, as they are more likely to find healthy hosts there than on less damaged *Bt* plants, (Schuler *et al.*, 2004). These two studies by Schuler suggest that *N. brevicollis*, as a generalist predator, might avoid slugs that are toxic and would probably find alternative prey. Extrapolation of these data to the field is, however, complicated, especially as the data collected are from a short-term laboratory experiment. The change in number of predators detected in this study must also be put into context with other practices in agriculture. For example, Coleoptera numbers are not affected by *Bt* potatoes but the application of permethrin (a synthetic insecticide) to potato fields lowered the spider populations to 31 % of the numbers in a non-treated field (Duan *et al.*, 2004) and similarly *Bt* maize affected population numbers of three predators (Musser & Shelton, 2003) and spiders (Ludy & Lang, 2006) less than pyrethroid insecticides.

6 The effects of *Bt* broccoli on soil micro-organisms.

6.1 Introduction

In Chapter 4 and 5 the effects of *Bt* toxins produced by genetically modified (GM) broccoli (*Brasssica olearcea* L. var. *Plenk*) (*Bt* broccoli) on several invertebrate species was investigated. This chapter concentrates on the effect, if any, of the root exudates from *Bt* broccoli plants on soil micro-organisms. Micro-organisms are major contributors to nutrient cycling and decomposition processes, and investigation into the impact of GM plants on their existence and diversity is essential (Bruinsma *et al.*, 2003; Kowalchuk *et al.*, 2003; Lilley *et al.*, 2006). Studies not involving GM plants have shown that soil type (Wieland *et al.*, 2001), plant species (Germida *et al.*, 1998), cultivar (Chiarini *et al.*, 1998) and crop management (Cookson *et al.*, 1998) can all cause changes to the soil micro-organism community. Change in agricultural practices, such as changing from pesticide spraying to growing pest resistant *Bt* plants, may therefore, also result in changes to the micro-organism community.

The effect of GM plants on soil micro-organisms can be either direct or indirect (Liu *et al.*, 2005c). A direct effect of *Bt* plants will depend on the toxicity of the protein and its availability to micro-organisms. Although, the nature of the *Bt* protein's specificity and its method of action of binding to invertebrate gut receptors (Section 1.2.2) makes toxicity to micro-organisms unlikely, the protein may become available to micro-organisms through root exudates (Saxena *et al.*, 1999). To what extent this is likely remains uncertain due to differences in protein production by different plants, the binding of the protein to clay particles and humic acids in the soil, and differences in the protein's dispersion rates (Section 1.4.5). Indirect effects caused by modification to a plant's metabolic pathways can introduce novel substrates to the soil systems (Bruinsma *et al.*, 2003) or result in changes to the plant proteins and root exudation (Liu *et al.*, 2005c). For example, Escher *et al.* (2000) showed a change in the lignin composition of maize from its parental line once modified. Such unintentional changes, arising either from gene insertion or the tissue culturing process, may influence micro-organism communities (Liu *et al.*, 2005c).

There have been relatively few studies that have looked at the effect of *Bt* plants on soil micro-organisms; those that exist show no significant effect on total number of species (e.g. Saxena & Stotzky, 2001b), no significant effect on biomass as estimated from

phospholipid fatty acid analysis (Griffiths *et al.*, 2005; 2007) or a detectable effect that is less than that caused by changes in the crop management programmes (Wei-xiang *et al.*, 2004a, b). Such studies have, however, considered only the total number of culturable soil micro-organisms; culturing, isolating and identifying different species would have been highly time consuming and complex. In addition, a large number of micro-organisms are not culturable in the laboratory and may be overlooked by these methods.

Changes in micro-organism community composition can be detected by using methods that investigate substrate utilisation such as fatty acid methyl ester (Germida *et al.*, 1998) and community level physiological (Griffiths *et al.*, 2005) profiling. These methods, however, still require an initial culturing step and identify only changes in microbial activity. They may only be used to hint at a change in the micro-organisms present (Liu *et al.*, 2005c). An analysis of microlipids may also be used as an indicator of micro-organism community composition change (e.g. phospholipid fatty acid analysis, Griffiths *et al.*, 2005; 2007). This method, although indicating change at a more structural, and thus more sensitive, level than community level physiology profiling has, however, been criticised on two fronts: changes in fatty acids can be caused by environmental factors, and micro-organisms share the same fatty acids (Liu *et al.*, 2005c).

Certain studies investigating the effects of GM plants on micro-organisms have detected changes in specific bio-chemical activities (Table 1.4); for example, an increase in phosphatase activity (Wei-xiang *et al.*, 2004a, b), a decrease in urease activity (Sun *et al.*, 2003), an increase in herbicide persistance (Accinelli *et al.*, 2004) and a decrease in decomposition activity (Escher *et al.*, 2000). No adverse effects of growing *Bt* maize on microbial activity, when measuring nitrogen mineralisation, and nitrification and respiration rates, has however, also been reported (Devare *et al.*, 2007). Changes in bio-chemical activity could all be related to changes in the groups of micro-organisms present in the soil (Wei-xiang *et al.*, 2004a, b), however enzyme activity can also be influenced by other organisms and soil type (Liu *et al.*, 2005c). On this basis it is plausible to hypothesise that, even when no change is detected in the total number of micro-organisms present, there may be a shift in the actual species composition of the micro-organism community present in the soil surrounding *Bt* plants rhizosphere and that this compositional shift may result in the changes in biochemical activity.

As well as not identifying the exact change in microbial community composition, the analytical approaches used in the studies above rely on traditional "ideas" of species to

determine species richness and therefore biodiversity. The comparison of nucleic acids using molecular methods to identify operational taxonomic units (OTUs) is now the preferred method of looking at bacterial biodiversity (Torsvik *et al.*, 1990). Molecular techniques can detect all microbial DNA present in the soil, are culture independent and, if required, DNA can be sequenced and used for identification purposes. Different OTUs are identified by differences in their DNA sequence. Donegan *et al.* (1995), for example, used restriction endonucleases to create DNA fingerprints of the micro-organisms in soil from *Bt* and non-*Bt* cotton. Differences were detected, but a further treatment using purified *Bt* toxins showed no effect; the authors hypothesised that the genetic manipulation of the cotton created a further change in the plant characteristics which caused the differences observed. This concurs with some of the results in Chapter 4 and 5 where alterations to invertebrate fitness when feeding on *Bt* broccoli plants could not be attributed to the *Bt* protein alone.

In another study Baumgarte & Tebbe (2005) showed that inter-crop differences in the micro-organism communities of *Bt* and non-*Bt* corn was less than differences found between the crop fields. They used a technique called single strand conformation polymorphism (SSCP) where DNA molecules are denatured into single strands and subsequently form secondary structures according to their sequence. These secondary structures have different electrophoretic mobility and move through a gel at different speeds. The number of bands detected can be used as an indicator of the number of OTUs in the samples, and different banding patterns indicate a difference between the bacterial communities.

Other DNA fingerprinting methods do not require denaturing of DNA before the electrophoresis but identify the moment when the two strands disassociate during electrophoresis. The percentage of guanine-cytosine bonds (%GC) in DNA affects the moment at which the two strands disassociate. A higher %GC indicates stronger association between the two strands so a higher temperature or stronger denaturant is needed to separate them and stop them moving through the gel. In temperature gradient gel electrophoresis (TGGE) the gel is subjected to a slowly increasing change in temperature whilst density gradient gel electrophoresis (DGGE) uses a gradient of denaturant in the gel (usually a mix of urea and formamide).

Although TGGE and DGGE have been used to detect variation in the microbial communities in different crops (Wieland *et al.*, 2001; Smalla *et al.*, 2001), and are both

popular methods for looking at microbial complexes in various substrates, it appears that to date, only one study has used TGGE to look for differences in the micro-organisms present in the rhizosphere of *Bt* and non-*Bt* crops. Castaldini *et al.* (2005) detected differences between *Bt* and non-*Bt* corn in their eubacteria community, heterotrophic bacteria and mycorrhizal colonisation. In this chapter, three molecular fingerprinting techniques, SSCP, TGGE and DGGE, were used to look for differences in the microbial populations surrounding *Bt* and non-*Bt* broccoli plant roots.

6.2 Material and methods

6.2.1 Experimental design and sampling

Soil was collected from Field 75, at Long Ashton Research Station (Bristol, UK, Grid Reference ST 532 704). The soil was of a gleyic luvisol type and comprised 21% clay, 44% sand and 35% silt (Fortune *et al.*, 2005). The larger organic matter and organisms (greater than 2 cm long) were removed by hand before the soil was thoroughly mixed in large shallow trays (10 x 70 x 70 cm). The soil was then separated into 14 cm diameter plant pots (20 cm tall). Soil cores (1 cm diameter, 0.5 cm deep) were taken from the centre of each pot (t=0 sample) and a seed was planted at the bottom of these wells prior to being refilled. In total, 12 *Bt* broccoli (*Brassica oleracea* L. var. *italica* Plenk) seeds and 12 non-*Bt* broccoli seeds (Green Comet) were planted (Section 2.1.1). The pots were kept in a greenhouse with temperatures always greater than 16°C (Section 2.2.1), watered twice a week for the first four weeks and then as necessary.

Four horizontal soil cores (0.1 x 1 cm) were taken from each plant pot at one, two, three and six months (i.e. 16 samples per plant) and stored at –80°C. The cores were taken from half-way down the pot (Figure 6.1) and a sterilised coloured bead was inserted into each core space to identify where a sample had been taken. At six months, two leaf discs (1 cm diameter) from the unfurled leaf nearest the top of the stem were also sampled from each plant (Section 2.2.2).

6.2.2 Quantification of *Bt* protein

The four samples taken at each time point were combined and ground-up using a sterile pestle in a 1.5 ml micro-centrifuge tube. Twenty milligrams of soil was removed and placed in another 1.5 ml micro-centrifuge tube and mixed with 0.5 ml extraction buffer and processed using an ELISA kit (Section 2.2.5). The leaf samples taken at six months were also processed using the ELISA. Samples were denoted as positive for *Bt* protein if

they exhibited an optical density greater than the threshold of mean plus three standard deviations of the optical density of non-*Bt* samples. The concentration of *Bt* protein present in these positive samples was calculated from the optical density using the equations provided with the ELISA kit (Appendix 1).

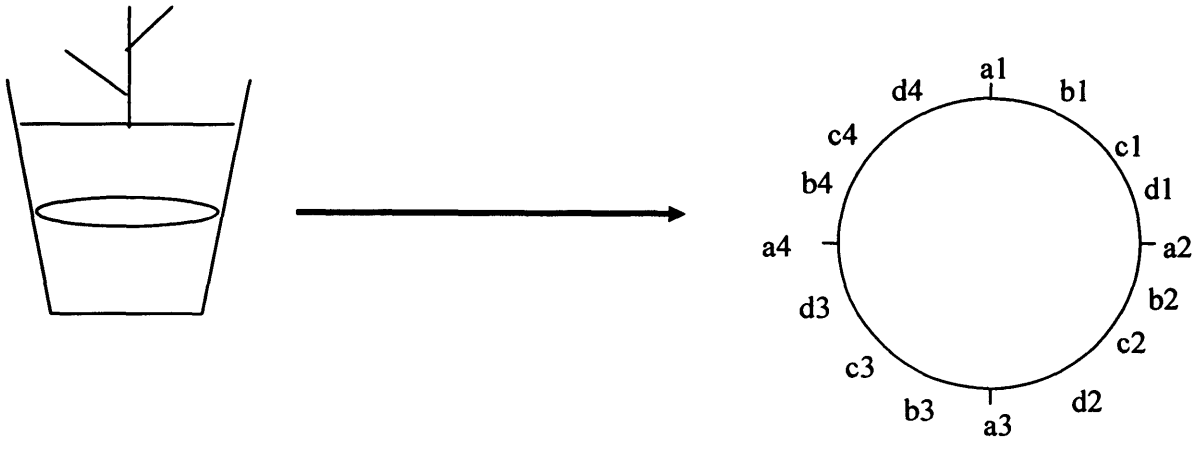

Figure 6.1 Position of soil samples within the plant pot and at each time point a) one month, b) two months, c) three months, d) six months.

6.2.3 DNA extraction and purification

500 milligrams soil was removed from the combined soil cores (Section 6.2.1) and a "FastDNA® Spin kit for soil" from Q-biogene (Carlsbad, CA, USA) used to extract DNA. The kit instructions (Appendix 2) were followed except at the final step where DNA was eluted with 150 µl rather than 50 µl in order to re-suspend all the silica. Eight microlitres of extracted DNA was mixed with 2 µl 6x loading dye and run on 1.5% agarose gel for 30 minutes at 100 V (Section 2.2.4).

6.2.4 Polymerase chain reaction

Three primer pairs were tested: pAf and pHr, p27f and p1429r, and p3f and p2r (Table 6.1). All three pairs amplify sections of 16S rDNA and were chosen as general eubacterial primers. They have also been used to amplify bacterial DNA successfully in complex substrates including soil (Bruce *et al.*, 1992), sea sediments (Newberry *et al.*, 2004) and biofilms in waste water treatment reactors (Muyzer *et al.*, 1993). Polymerase chain reaction (PCR) amplification was carried out on an Applied Biosystems Geneamp PCR system 9700 (Warrington, UK) in 0.5 ml microstrip tubes (Abgene, Epsom, UK) using components from a Taq DNA polymerase kit (Qiagen, Sussex, UK). PCR

amplification was attempted on 10^{-1}, 10^{-2} and 10^{-3} dilutions of the initial DNA extracts. A negative control was always run with each PCR (i.e. where the DNA template was replaced by RNAse free water) and a positive control of *Mycobacterium tuberculosis* (Koch) DNA.

Table 6.1 Sequences of the three oligonucleotide primer pairs used and the size of fragment they amplify.

Primer	Oligonucleotide Sequence 5' – 3'	Fragment size (bp)	Reference
pAf	AGA GTT TGA TCC TGG CTC AG	1500	Bruce *et al.*, 1992
pHr	AAG GAG GTG ATC CAG CCG CA		
p27f	AGA GTT TGA TCM TGG CTC AG[1]	1502	Newberry *et al.*, 2004
p1429r	GGT TAC CTT GTT ACG ACT T		
p3f	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	233	Muyzer *et al.*, 1993
p2r	ATT ACG CGG CTG GCT GG		

[1] M= A or C

6.2.4.1 Primers pAf and pHr

Two microlitres template DNA was mixed with 3 mM MgCl, 50 pmol each primer, 200 µM dNTPs, 0.5 U Taq polymerase, 0.5 µl bovine serum albumin (10 mg ml^{-1}), 1 x PCR buffer and made up to a 25 µl reaction with RNase free water. The thermal profile consisted of an initial denaturing at 94°C (3 minutes), then 30 cycles of denaturing at 94°C (3 minutes), annealing at 55°C (1 minute) and extension at 72°C (2 minutes), before a final extension step at 72°C (10 minutes).

6.2.4.2 Primers p27f and p1429r

Two microlitres template DNA was mixed with 2.25 mM MgCl, 50 pmol each primer, 100 µM dNTPs, 0.5 U Taq polymerase, 0.5 µl bovine serum albumin (10mg ml^{-1}), 1 x PCR buffer and made up to a 25 µl reaction with RNase free water. The thermal profile was initial denaturing at 95°C (2 minutes), then 35 cycles of denaturing at 92°C (45 seconds), annealing at 50°C (2 minutes) and extension at 72 °C (1 minute), before a final extension step at 72°C (10 minutes).

6.2.4.3 Primers p3f and p2r

These primers amplify a smaller fragment of DNA than the other two pairs (Table 6.1) but the fragment length is ideal for SSCP despite it reducing the number of base pairs that may be used to detect differences. A GC clamp on the p3f primer also increases the

likelihood of detecting single base pair changes (Myers *et al.*, 1985; Sheffield *et al.*, 1989).

Three microlitres template DNA was mixed with 3 mM MgCl, 50 pmol each primer, 200 µM dNTPs, 0.5 U Taq polymerase, 1x PCR buffer and made up to a 25 µl reaction with RNase free water. The thermal profile consisted of an initial denaturing at 94°C (5 minutes), then 35 cycles of denaturing at 94°C (45 seconds), annealing at 55°C (1 minute) and extension at 72°C (3 minutes), before a final extension step at 72°C (10 minutes). This gave a double band profile on the agarose gel which was optimised by altering the magnesium chloride concentration to 3.5 mM and the addition of 0.5 µl BSA (10 mg ml^{-1}).

6.2.4.4 Multiplex

The QuantiTect Multiplex PCR kit (Qiagen) is normally used to amplify several different sequences using different pairs of primers simultaneously. The supplied buffer is optimised so that where several PCRs may be required, with different reaction mixes and thermal profiles, just one will do. The optimisation of the buffer also means that it can be used where some factors were found to be inhibiting the reaction, eliminating many optimisation steps. 25 microlitre reactions were prepared containing 1 µl of each primer (2 µM, primer pair p3f and p2r), 12.5 µl multiplex buffer, 4 µl template DNA (diluted 100x) and 6.5 µl water. The thermal profile comprised of an initial denaturing step at 95°C (15 minutes), then 35 cycles of denaturing at 94°C (0.5 minutes), annealing at 48°C (1.5 minutes) and extension at 72°C (1.5 minutes), before a final extension step at 72°C (10 minutes).

6.2.5 Separation methods

6.2.5.1 SSCP

The submerged gel electrophoresis apparatus (SEA 2000®, Elchrom Scientific, Switzerland) filled with 1 x TAE buffer (4.84 g tris-chloride, 1.14 ml acetic acid, 2 ml 0.5M EDTA) and a Ministat compatible control unit (Huber, Germany) was used to cool the buffer to 9°C. Three microlitres of PCR product was denatured by mixing it with 7 µl formamide-NaOH solution (1 ml formamide, 10 µl 1 M NaOH) before heating for 4-5 minutes at 95°C in an Applied Biosysyems Geneamp PCR system 9700. It was then placed immediately in ice cold water for four minutes. Eight microlitres denatured

samples, with bromophenol blue, were loaded onto a GMA™ (gene mutation analysis) mini gel (Elchrom Scientific). The gel was run at 9°C for 12 h at 72V.

The gel was placed in a plastic container (10 x 30 x 30 cm) covered in foil on a shaker and 10 mM TAE buffer (33 ml 1 x TAE buffer with 66 ml deionised water) was added with SYBR® gold (Invitrogen, Paisley, UK) (1:100000) for 30 minutes to stain the DNA. The gel was then de-stained for a further 30 minutes in distilled water. The gel was visualised on a UV light transluminator (312 nm).

6.2.5.2 TGGE

The running gel comprised of 4.5 ml acrylamide, 750 µl 50 x TAE buffer (242 g tris-chloride, 57.1 ml acetic acid, 100 ml 0.5 M EDTA), 12.6 g urea, 6 ml formamide, 8 ml water and 750 µl 80% glycerol. These constituents were mixed in a plastic 50 ml beaker before the addition of 30 µl Temed and 300 µl APS (0.1 g ammonium persulphate in 100 µl water) to polymerise the solution. The gel was then immediately applied between the plates using a syringe and allowed to set. After 2 h a stacking gel was prepared (Table 6.2) and polymerised with 5 µl Temed and 50 µl APS. This stacking gel was then added on top of the original gel and a comb inserted into it to create wells.

Table 6.2 Components of the various gels prepared for TGGE and DGGE.

Component	25%	40%	60%	90%	Stacking
40% acrylamide/BIS	5 ml	5 ml	5 ml	5 ml	750 µl
50x TAE buffer	0.4 ml	0.4 ml	0.4 ml	0.4 ml	125 µl
Formamide	2 ml	3.2 ml	4.8 ml	7.2 ml	
Urea	2.1 g	3.36 g	5.04 g	7.56 g	
Distilled water	Up to 20 ml	Up to 20 ml	Up to 20 ml	Up to 20 ml	4.1 ml

Whilst the stacking gel set, the electrophoresis buffer tank and samples were prepared. 6.85 L water and 125 ml 50 x TAE buffer was placed into the tank and heated to 45°C. Fifteen microlitres PCR products were mixed with 5 µl 6x loading dye. After 1.5 h the comb was removed from the gel and the wells washed out with deionised water. The gel was attached to the core apparatus and lowered into the tank. Twenty microlitre samples were loaded into each well. Electrophoresis began at 100 V until the samples reached the junction of the two gels. Voltage was then reduced to 55 V. The temperature was set to increase by 1°C per hour up to 60°C.

The gel was removed from the tank and the top plate carefully peeled away from the gel within a plastic container (30 x 30 x 10 cm) with 400 ml liquid from the tank and 6 µl

SYBR® gold. The whole container was then covered in silver foil and placed on a shaker for 20 minutes. The gel was carefully separated from the backing plate and photographed under a UV transluminator (312 nm).

6.2.5.3 DGGE

Two gels were prepared with 25% and 60% denaturant (Table 6.2). These mixes were polymerised with 30 µl Temed and 300 µl APS (0.1 g ammonium persulphate in 100 µl water). The gels were then placed in syringes attached to the Model 475 gradient delivery system which was used to apply the gels at the correct ratio in-between the two plates. After 1.5 h, a stacking gel (Table 6.2) was polymerised with 5 µl Temed and 50 µl APS, and loaded on top of the denaturant gel and the comb inserted.

The electrophoresis buffer tank was filled with 6.85 l water and 125 ml 50x TAE buffer, and heated to 53°C. Five microlitres loading dye (6x) was added to 15 µl PCR product. The comb was removed from the stacking gel after 1.5 h and the wells rinsed with deionised water. The gel was then attached to the core apparatus and lowered into the buffer tank. Twenty microlitres sample was then loaded into the wells and the gel run at 68 V. After 12 h the gel was removed from the tank and carefully peeled away from the plastic backing. It was stained using the same method as for the TGGE gels (Section 6.2.5.2).

The bands were grouped at the bottom of the gel so the denaturant gradient was later altered to 40-90% (Table 6.2). All the TGGE/DGGE apparatus was part of the D-code universal mutation detection system (Biorad, Hemel-Hempstead, UK) and was attached to a Biorad powerpac basic to supply the electric current; the associated manuals were used for methodology.

6.3 Results

6.3.1 Quantification of *Bt* proteins

All the non-*Bt* plants tested negative for *Bt* protein. The *Bt* plants displayed considerable variation in *Bt* concentration and using the threshold of 0.05 ng g^{-1} calculated from the non-*Bt* plant samples (Section 2.2.5) ten of the twelve plants were determined as producing Bt protein; five containing a high concentration of *Bt* (298.8 ± 172.1 ng g^{-1}) and five containing a low concentration of *Bt* (2.42 ± 1.27 ng g^{-1}). The two plants testing negative and all their associated samples were disregarded. Only six of the 50 soil samples (10 plants, 5 time points) from the *Bt* plants tested positive (7.35 ± 2.67 ng g^{-1},

threshold calculated from non-*Bt* samples was 3.14 ng g^{-1}); insufficient data made further statistical analysis impossible.

6.3.2 Molecular results

The "FastDNA® Spin kit for soil" kit (Q-biogene) extracted the DNA successfully (Figure 6.2). Primers pAf and pHr did not amplify the DNA extracted from the soil despite optimisation of the process for the positive control. The other two primer pairs, however, did amplify the DNA when it was prepared using at least a 10^{-2} dilution (Figure 6.3). The clearest bands produced (i.e. the same brightness as the positive control) were from the p3f and p2r primer pair in combination with the multiplex kit using a 10^{-2} dilution of the DNA template (Figure 6.4). Only two bands were ever visualised on the SSCP gels (Figure 6.5), the majority of the DNA appeared to remain in the wells. The TGGE gels when illuminated had poor resolution resulting in smears of staining (Figure 6.6) so could not be quantified. Of the three methods DGGE was the most successful and when the gel was illuminated there was a clear banding pattern for each of the samples (Figure 6.7). This result could not, unfortunately, be reproduced on further attempts. From this one gel it could be seen that, although there were differences in the intensity of the bands, there was no obvious differences in the banding patterns between the *Bt* and non-*Bt* samples.

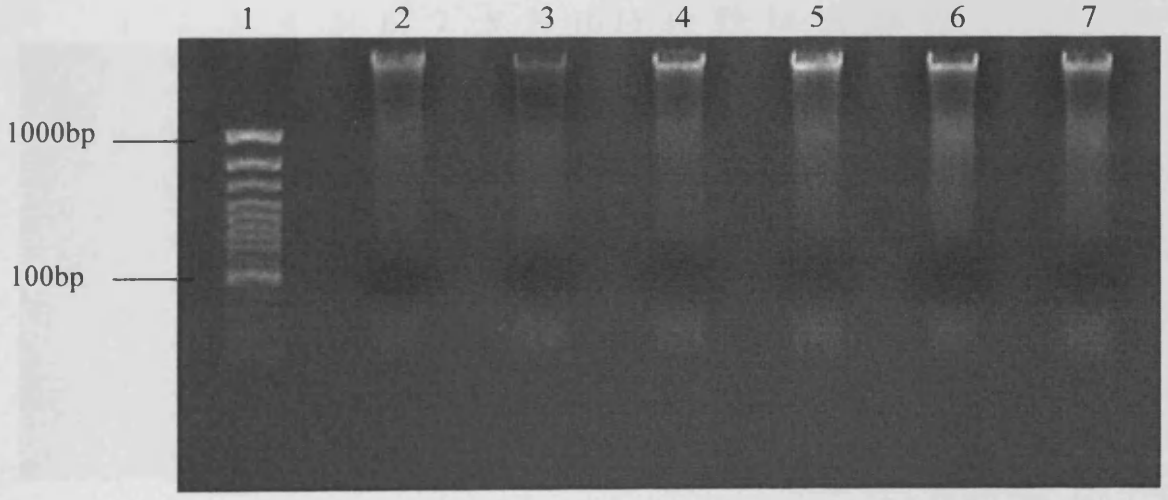

Figure 6.2 Agarose gel with ethidium bromide staining to show DNA extractions had worked. Lane 1 DNA ladder, Lanes 2-4 extracts from *Bt* soil, Lanes 5-7 extracts from non-*Bt* soil.

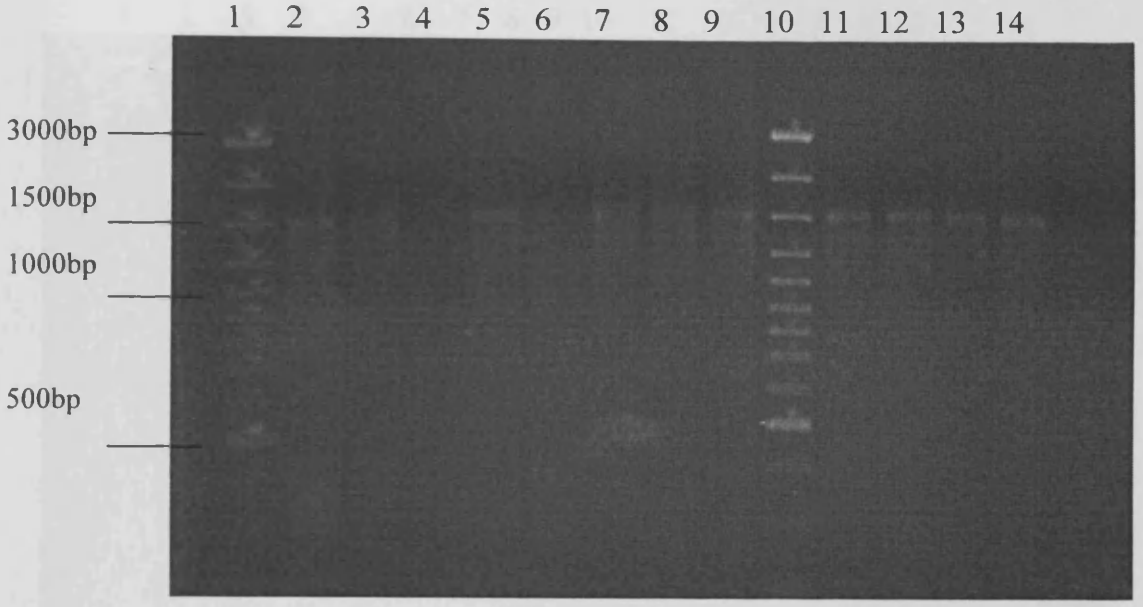

Figure 6.3 Agarose gel with ethidium bromide staining. PCR using primers p27f and p1249r, fragment length 1502bp. All sample are extracts from *Bt* soil at t=0. Lane 1 and 10 DNA ladder, Lanes 2-5 10^{-1} dilution, Lanes 6-9 10^{-2} dilutions, Lanes 11-14 10^{-3} dilutions.

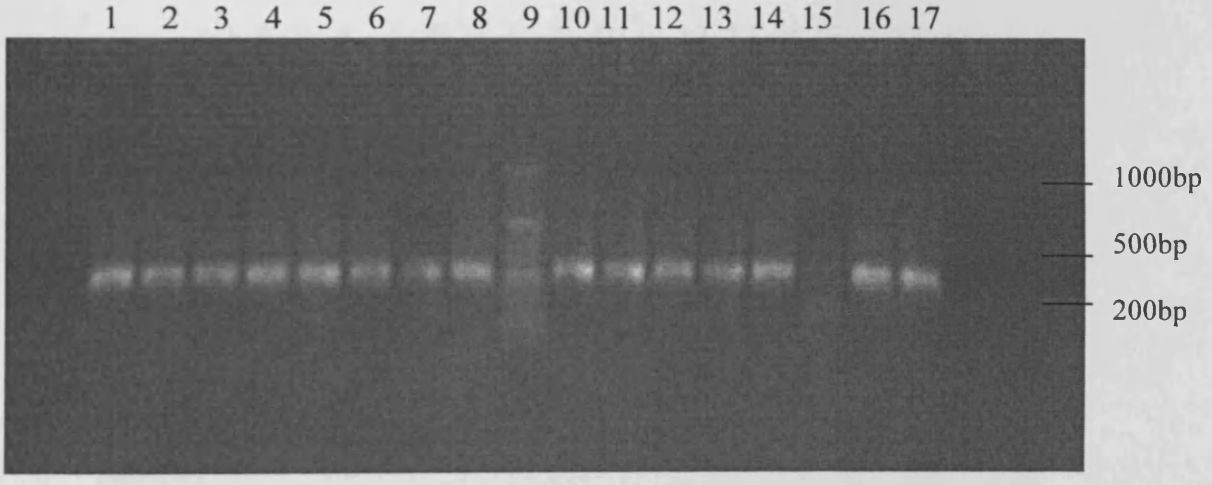

Figure 6.4 Agarose gel of multiplex PCR using primers p3f and p2r with ethidium bromide staining. Lane 1-4 non-*Bt* soil extracts, Lanes 5-8, 10-14 *Bt* soil extracts, Lane 15 negative control, Lanes 16 and 17 positive control and Lane 9 DNA ladder. All extracts from t=0 and diluted 10^{-2}.

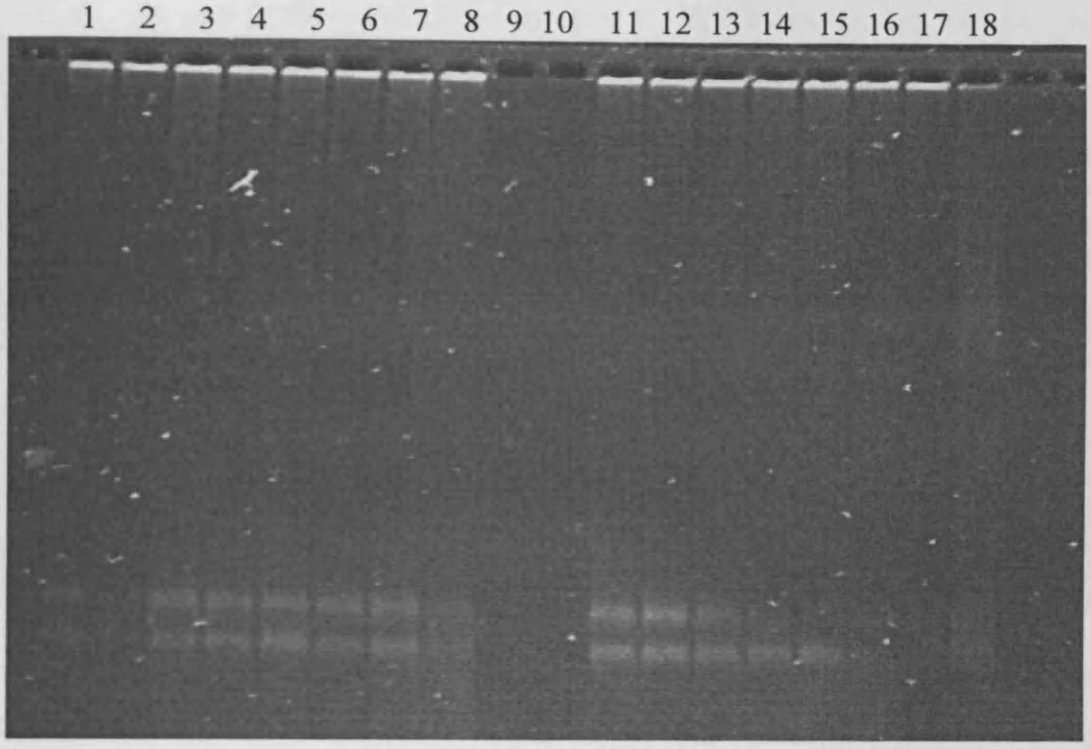

Figure 6.5 SSCP gel with SYBR® gold staining. Multiplex PCR using primers p3f and p2r on 10^{-2} dilutions of extractions. Lane 1-8 non-*Bt* soil, Lanes 11-18 *Bt* soil (Lanes 9 and 10 blank).

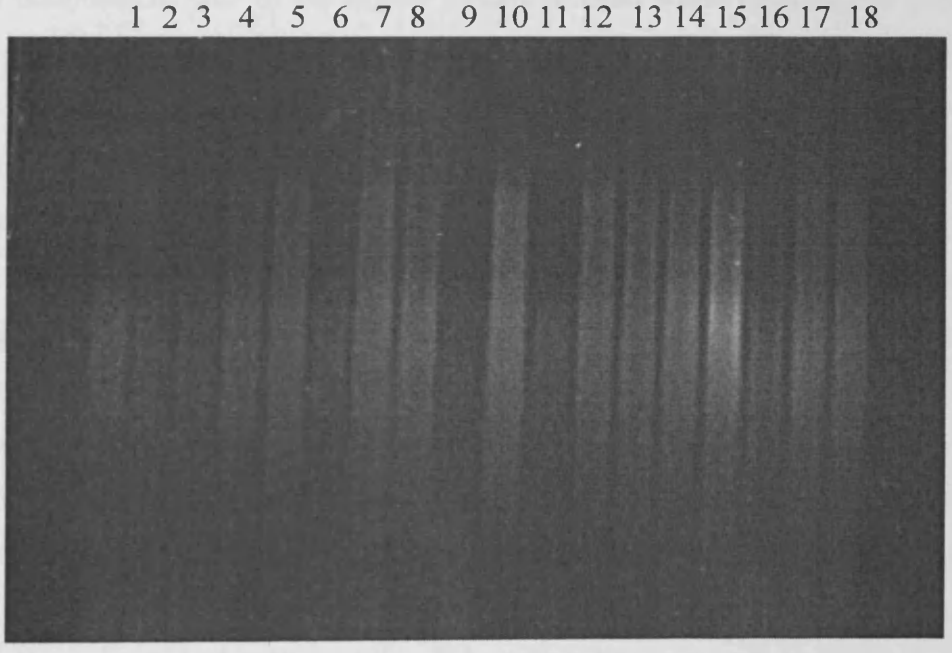

Figure 6.6 TGGE gel with SYBR® gold staining. Multiplex PCR using primers p3f and p2r using 10^{-2} dilutions of DNA extracts. Lanes 1-9 non-*Bt* soil extracts, Lanes 11-18 *Bt* soil extracts.

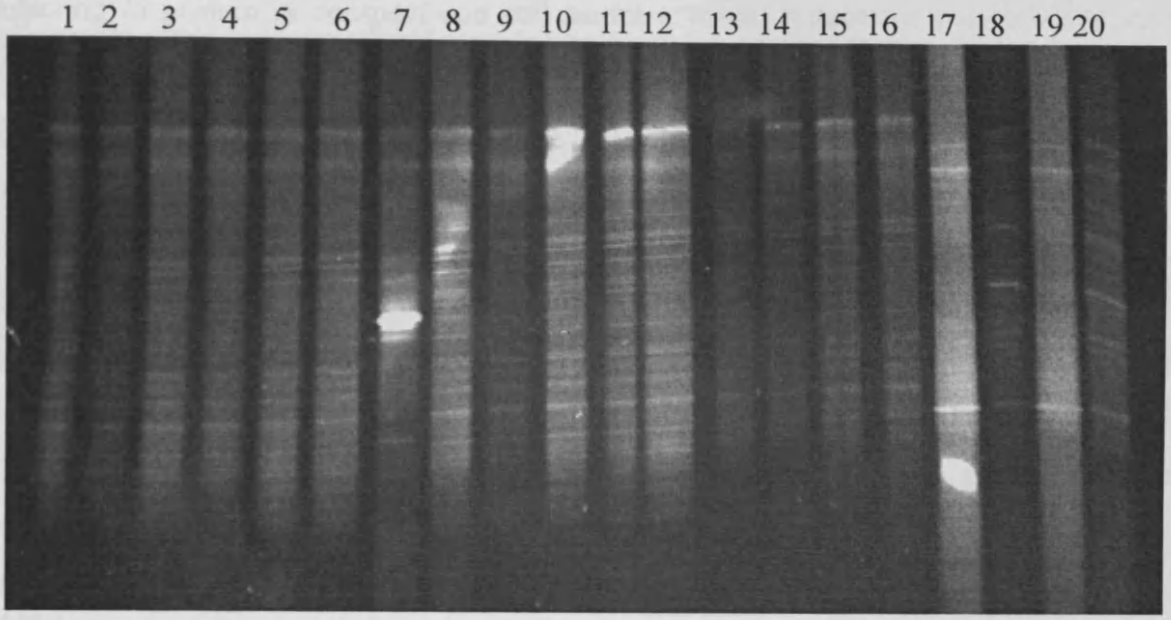

Figure 6.7 DGGE gel with SYBR® gold staining. Multiplex PCR using primers p3f and p2r using 10^{-2} dilutions of DNA extracts at t=0. Lanes 1-10 non-*Bt* soil extracts, Lanes 11-20 *Bt* soil extracts.

6.4 Discussion

Bt proteins were detectable in six of the soil samples in which *Bt* broccoli had grown. This was undoubtedly due to the higher threshold used when detecting *Bt* protein in soil from the field site compared to John Innes compost (Section 2.3.2). The higher optical densities of non-*Bt* field soil samples may either be a consequence of some cross-reactivity of the ELISA antibodies with the Long Ashton soil not detected in the John Innes compost, or *B. thuringiensis* and its associated proteins may be already present at the Long Ashton field site. The levels detected in the soil samples from the field (7.35 ± 2.67 ng g^{-1}) were higher than those detected in the compost (0.05 ± 0.02 ng g^{-1}, Section 2.3.2), this could be due to the different properties of the two substrates. The field soil had a relatively high clay content (21%; Fortune *et al.*, 2005) and *Bt* proteins will bind to clay particles (Tapp & Stotzky, 1995; 1998). If the ELISA is inefficient at detecting protein in substrates other than plant material this may not only explain why so few soil samples tested positive but also explains why the compost samples had a lower concentration recorded; the *Bt* proteins did not bind to compost particles and may have either leached out of the plant pots when watered or been available for degradation. While testing this ELISA kit's efficiency using soil samples spiked with purified *Bt* proteins would on reflection have been beneficial, designing an ELISA capable of

detecting *Bt* protein in compost and soil samples would reduce the possibility of cross-reactivity (e.g. Tapp & Stotzky, 1995).

The background concentration of *Bt* in the soil rhizosphere (7.35 ± 2.67 ng g^{-1}) is within a similar range to the concentration of *Bt* protein detected in soil from around *Bt* maize plants in some other studies (4.4 ng g^{-1} (Hopkins & Gregorich, 2003) and up to 10 ng g^{-1} (Baumgarte & Tebbe, 2005)), but is significantly less than the 95 μg g^{-1} detected by Saxena & Stotzky (1999) in root exudates from *Bt* maize (*Zea mays*, L.) grown in a soil-free medium. This difference could be attributed to the *Bt* protein binding to soil particles (Tapp & Stotzky, 1995) and not being extracted effectively by the extraction buffers provided with the ELISA kit. *Bt* broccoli may also exude less *Bt* protein than *Bt* maize resulting in lower concentrations in the soil; Saxena *et al.* (2004) used immunological methods to detect *Bt* proteins in soil in which *Bt* maize, potato (*Solanum tuberosum* L.) and rice (*Oryza sativa* L.) had grown but not in soil in which *Bt* oilseed rape (*Brassica napus*), cotton (*Gossypium* spp.) and tobacco (*Nicotiana tabacum* L.) had grown.

In all cases the template DNA had to be diluted before amplification was successful; this step is thought also to dilute substances in the extract, such as humic acids that are known to inhibit Taq polymerase (Nesme *et al.*, 1995). The highest level of luminescence, and thus DNA, were identified on gels from the primer pairs p3f and p2r (Muyzer *et al.*, 1993) in combination with the multiplex primer kit.

Several techniques were tried to separate the amplified micro-organism DNA and to detect differences between *Bt* and non-*Bt* samples. SSCP produced two bands whilst TGGE produced more bands but they were unquantifiable. The DGGE method was successful (Figure 6.7) but proved unrepeatable. The one "good" gel showed no differences in the banding patterns between the *Bt* and non-*Bt* samples. This was as expected as they are all from the first time point (i.e. before the plant is growing and exuding *Bt* proteins) and suggests that the process works and could be used with confidence on the other time points. DGGE and TGGE are awkward procedures requiring many steps and ensuring quality control across many gels is difficult. Again, with more time it may be possible to establish one of these techniques to work to a high enough standard to produce quantifiable bands that could then be analysed. A computer program such as Gelcompar, Applied Math (Baumgrate & Tebbe, 2005) should be used to count and analyse the bands more accurately than by eye.

Had it been possible to determine differences between *Bt* and non-*Bt* soil bacteria assemblages it would have been appropriate to use more specific primers (general eubacteria primers were used) to explore differences in specific groups of bacteria. Sheppard *et al.* (2005), for example, used TGGE to detect differences in the methanogenic bacteria present in sewage treated and untreated soils. Key groups to investigate are mycorrhizal and wood-decaying fungi, nitrogen-fixing bacteria especially symbionts, and nitrifying bacteria (Bruinsma *et al.*, 2003; Kowalchuk *et al.*, 2003). Further steps would be necessary to identify which micro-organisms were different between the *Bt* and non-*Bt* samples. Sequencing of DNA in excised gel bands could be one suitable method of identifying OTUs as the melting behaviour of the DNA relates only to the proportion of GC present and not the actual sequence. It cannot be assumed that if any differences were detected that they would be long term; Dunfield & Germida (2003) used restriction endonucleases DNA fingerprinting to detect a difference in the micro-organism assemblages in herbicide-tolerant and non-transgenic oilseed rape fields but this did not persist after the growing season.

The variety of techniques used in various studies (e.g. Saxena & Stotzky, 2001b; Germida *et al.*, 1998; Sun *et al.*, 2003; Castaldini *et al.*, 2005) to determine the effect of GM plants on micro-organism communities makes comparison of the results difficult but highlights the disadvantages associated with each method (Bruinsma *et al.*, 2003). These studies also show that sometimes the change in micro-organism communities is short-term, often lasting only as long as the plant is growing (e.g. herbicide-toleant oilseed rape, Dunfield & Germida, 2003) and not season to season (e.g. lectin producing potatoes, Griffiths *et al.*, 2000) and that this can vary with the plant and soil type. When investigating the effects of GM plants (and other changes in agricultural practice) a single practical method appears insufficient, and an approach where several methods are integrated is better (Bruinsma *et al.*, 2003; Liu *et al.*, 2005c). Various reviews have also argued that investigations into changes to keynote indicator groups must be prioritised (Bruinsma *et al*, 2003; Kowalchuk *et al.*, 2003) although understanding how the broader community reacts to the growth of *Bt* plants is also important (Bruinsma *et al.*, 2003).

The understanding of any potential risks of GM plants to micro-organism communities, uncovered using any of these methods, is limited by our current knowledge of soil systems and the reactions of micro-organism communities to other agricultural changes (Bruinsma *et al.*, 2003). Using phospholipid fatty acid analysis, for example, the difference between micro-organisms communities in grass and maize was significant but

the difference between *Bt* and non-*Bt* maize was not (Griffiths *et al.*, 2005) and the difference in community level physiology caused by the presence of *Bt* maize in comparison to non-*Bt* maize was no greater than that caused by a pyrethroid pesticide (Griffiths *et al.*, 2006); even the age of plant (Baumgarte & Tebbe, 2005) and growth stage (Griffiths *et al.*, 2007) can affect the bacterial community more than the presence of *Bt* from GM plants. Any significant change in the micro-organism community must, therefore be interpreted carefully with respect to current knowledge of systems and the effect of current agricultural practices (Bruinsma *et al.*, 2003; Liu *et al.*, 2005c).

7 Discussion

7.1 Interplant variability of *Bt* concentration in *Bt* broccoli

Broccoli (*Brassica oleracea* L. var. *italica,* Plenk) plants able to withstand attacks by *Plutella xylostella* L. larva (Earle *et al.*, 1996), the cause of substantial damage to brassica crops worldwide (Earle *et al.*, 2004), were developed by introducing a *Bt* Cry1Ac protein gene. The plants used in this study were bred by crossing the transgenic line with Green Comet (a non-*Bt* broccoli) individuals. This resulted in a segregating population where only 50% of the plants were resistant to *P. xylostella* (Earle *et al.*, 1996). The *Bt* protein should also control other Lepidoptera species. Plants with the *Bt* gene were selected from this mixed population using an ELISA but when their control of three other Lepidoptera species was tested the results were variable (Chapter 3); *Pieris brassicae* L. died on *Bt* plants but *Mamestra brassica* L. and *Agrotis segetum* (Denis & Schiff) larvae did not. Whether this variation was a consequence of the nature of the experiments, particularly in their short-termness, and that a longer exposure would have resulted in the death of the larvae, remains unknown. Regardless, death would not have occurred before a substantial amount of damage would have been caused to the plants. Different Lepidoptera species are known to be vulnerable to different concentrations of *Bt* protein (Höfte & Whiteley, 1989); *Bt* cotton (*Gossypium* spp.), specifically Bollgard®, for example is resistant to the main US cotton pest *Heliothis virescens* Fabricius but the transgene does not provide complete resistance to two Australian cotton pests (*Helicoverpa armigera* Hübner and *H. punctigera* Wallengren) more tolerant to *Bt* protein (Fitt, 2003).

The specific line of *Bt* broccoli used in this study was produced as part of a process exploring transformation techniques for brassicas and inheritance of the *Bt* gene (Earle *et al.*, 1996). Pest resistance, rather than commercial application, was therefore the main topic of interest. It is unlikely that this particular *Bt* broccoli segregating population could be considered for use as a commercial crop as only a proportion (70%, Section 2.3.1) of plants were shown to contain the *Bt* gene using polymerase chain reaction (PCR). Protection against herbivory was limited to just one of three major British pests (Chapter 3). This segregating population may, however, be suggested as a trap crop, attracting pests and keeping them away from the main cash crop, for example, as suggested for *Bt* kale (*Brassica oleracea* L. var. *acephala*) (Cao *et al.*, 2005) and *Bt* maize to attract pests away from sugarcane (*Saccharum* spp.) fields (Keeping *et al.*, 2007) (Section 3.4).

The PCR and ELISA results correlated (Section 2.3.1) but the ELISA kits also enabled quantitative determination of the concentration of *Bt* protein in the broccoli plants with a *Bt* gene and was therefore used throughout the rest of the study. The ELISA highlighted the marked variation in concentration (0.01-8200 ng g^{-1}) between plants expressing *Bt* proteins at a level above the threshold but not within a plant (Section 2.3.2). Differences up to ten-fold have also been reported for concentrations of Cry1Ab protein within *Bt* maize (*Zea mays*, L.) and *Bt* maize back-crosses (Fearing *et al.*, 1997) and hybrids of oilseed rape and *B. rapa* had similar levels of *Bt* protein as the original transformed plants which varied up to three fold (Zhu *et al.*, 2004). The variation in the present study could be related to *Bt* gene copy number present in the plants (the segregating population indicated by the PCR (Section 2.3.1) would contain both homo- and hetero-zygotes for the *Bt* gene, Table 2.6). This should be avoided in future experiments by selecting for homozygous lines. Different homozygous lines could then be compared to each other to investigate the effects of genetic background on *Bt* protein levels. Gene copy did not affect β-glucuronidase expression significantly in GM white clover (*Trifolium alba* L.) but expression was affected by genetic background (i.e. parent plants (Scott *et al.*, 1998, Section 2.4)). The genetic background of the broccoli plants used in this study may, therefore, be a contributing factor to the variation in *Bt* protein levels.

As hybrids these plants more accurately portray escape of the *Bt* gene into wild populations (Section 1.4.4) than commercial use. In the UK this would be plausible as there are many wild brassicas with which broccoli could produce hybrids. Hybridisation though can be reduced by creating isolation distances and a 50 m gap reduces hybridisation between herbicide-tolerant oilseed rape and *B. rapa* to 0.04% (Weekes *et al.*, 2005). Hybrids might be more invasive than non-*Bt* crops as the *Bt* gene could confer a selective advantage (e.g. more seeds than wild relatives (Vacher *et al.*, 2004)). This study, however, indicates that the *Bt* gene is not expressed at a consistently high enough level to stop herbivory by two Lepidoptera species so it seems that in this case there is unlikely to be a selective advantage for the hybrid plants. A brassica hybrid was shown to be not competitive (Halfhill *et al.*, 2005): invasiveness is unlikely. The introduction of the *Bt* gene, though, could alter other plant metabolic pathways (reviewed in Filipecki & Malepszy, 2006) and this could result in some other selective advantage. The alteration in another pathway may be a contributing factor to the heavier, although non-significant, weight of *A. segetum* in this study when fed on leaves containing *Bt* protein.

7.2 *Bt* proteins in broccoli root exudates

ELISA kits were also used to detect *Bt* protein in compost and soil in which some of the *Bt* broccoli plants had grown (Section 2.3.2 and Section 6.3.1). Although levels were generally low this suggests that, unlike the closely related oilseed rape (*Brassica napus*, L.) (Saxena *et al.*, 2004), *Bt* broccoli exudes *Bt* proteins into the soil allowing soil dwelling biota to come into contact with the *Bt* protein. This evidence also conflicts with the conclusion that Cry1A proteins are not exuded (Saxena *et al.*, 2004; Section 1.4.5) but differences between this study and the present one could be due to efficiency of the *Bt* protein detection systems used rather than differences in exudation. Decomposers are especially vulnerable to this *Bt* protein as they also feed on senescent leaves and plant residues incorporated into the soil after harvesting (Tapp & Stotzky, 1995); proteins were detectable in senescent leaves from these *Bt* broccoli plants (Section 2.3.2).

The concentrations of *Bt* protein in soil from the field in which *Bt* and non-*Bt* plants had grown was higher (7.35 ± 2.67 ng g^{-1}, Section 6.3.1) than that found in John Innes no. 2 compost (0.05 ± 0.02 ng g^{-1}, Section 2.3.2). This is probably related to the binding properties of the *Bt* protein altered by the presence of humic acids (Crecchio & Stotzky, 1998) and the type of clay particles present (Tapp & Stotzky, 1995, Section 1.4.5). As *Bt* proteins bind to clay particles the protein may have leached out of compost but remained in the field-collected soil prior to extraction using the ELISA. As only 54 % of the compost samples from plants with the *Bt* gene tested positive for *Bt* protein it would be beneficial to test the extraction efficiency of the ELISA kit by spiking soil and compost samples with known concentrations of purified *Bt* protein (e.g. Tapp & Stotzky, 1995). It may also be worthwhile growing *Bt* broccoli in a hydroponic system (e.g. Saxena *et al.*, 1999) to confirm the levels of *Bt* protein in root exudates, especially as the closely related *B. napus* did not exude Cry1Ab proteins (Saxena *et al.*, 2004). In the current study the use of a threshold (mean plus three standard deviation of non-*Bt* samples, Section 2.2.5) prevented the presence of false positives.

A different ELISA antibody could be developed to be used specifically for monitoring *Bt* proteins in soil. This might reduce the relatively high thresholds used in this study to avoid false positives from cross-reactivity with other soil components. To achieve this, antibodies would have to be screened against an array of soil types to check for cross-reactivity but such new antibodies could be used to detect not only *Bt* proteins in soil but also to establish the level of exposure for non-target species (e.g. Harwood *et al.*, 2005;

Section 5.4). Although, the development of a monoclonal antibody can be time-consuming once developed it would almost certainly be less costly than buying the commercial kits used in this study. An ELISA, however, does not confirm whether the *Bt* protein is active; the antibody could be binding to an intact antigen or a fragment of the whole protein. Larvicidal immunoassays with a target species to show if the protein is active (e.g. Saxena *et al.*, 2004) may be a preferable method of detection than the ELISA.

7.3 The effect of *Bt* broccoli on non-target species

The effects of the protein were monitored for several fitness parameters (e.g. survivial, weights and reproduction) on a wide spectrum of species within six orders (Coleoptera, Collembola, Haplotaxida, Isopoda, Pulmonata and Rhabditida) of four different phyla (Annelida, Arthropoda, Mollusca and Nematoda). As far as I am aware the effects of *Bt* proteins from GM plants on molluscs have never been investigated before; making predictions was therefore difficult. Species within other taxa have been investigated with non-significant effects detected (Table 1.3), except an increase in weight of the isopod *Porcellio scaber* L. (Escher *et al.*, 2000), a decrease in earthworm (*Aporrectodea caliginosa* Savigny) cocoon hatching rates (Vercesi *et al.*, 2006) and a change in nematode numbers (Griffiths *et al.*, 2005; 2006). The differences between *Bt* and non-*Bt* treatments in the present study were also found to be mostly non-significant (Table 5.1). The present study differs from the others in that, for the first time, rather than trying to compare results from many studies with different approaches and different *Bt* plants, a relatively standard, comparable approach has been used on a disparate range of taxa. Physiologically, and due to protein specificity, the non-significant effects recorded are perhaps not entirely unexpected; the species tested were from a variety of orders different from the target species (Lepidoptera) and would have receptors in their guts different from those required for binding to the active toxin produced by the *Bt* broccoli plants (Section 1.2.2).

Some statistically significant results were, however, detected when testing for the effects of *Bt* broccoli plants on decomposers and soil dwelling organisms. Slug (*Deroceras reticulatum*, Müller) and woodlouse (*P. scaber*) weights were significantly higher when feeding on *Bt* leaf treatments than non-*Bt* leaf treatments. Significant effects were found on reproduction and in young organisms; for example, small woodlice survival, end weight and rate of weight gain and earthworm (*Dendrobaena rubidus,* Savigny) hatching rates. Except for the end weight of the small woodlice on *Bt* compost, these parameters

were all significantly greater on *Bt* treatments (both leaf and compost). That more significant results were detected in this study than other published studies (Section 1.4.7) underlines the need to investigate more taxa and more parameters. Whilst the majority of previous studies into the effects on non-target soil-dwelling species have concentrated on "lethal" parameter effects (e.g. differences in survival) and may have overlooked effects on other parameters (Lövei & Arpaia, 2005), in this study "sub or non-lethal" parameter effects were also considered.

Very few studies of the effects on *Bt* plants on non-target organisms note the concentration of *Bt* protein in the GM plants used; some examples are *Bt* maize with 0.72 $\mu g\ g^{-1}$ (Harwood & Obrycki, 2006) and 9.6 $\mu g\ g^{-1}$ (Vercesi *et al.*, 2006). The variation in concentration of *Bt* protein in the plants detected in this study made replication difficult but *Bt* concentration was determined and recorded throughout. The concentration in the plants used was always above the nominated threshold (except for an extra treatment in the *P. brassicae* study, Section 3.2.6.2) of mean plus three standard deviations of the non-*Bt* samples (Section 2.2.5). Significant effects were found on slug fitness parameters at relatively low (9.15 ± 7.3 ng g^{-1}) concentrations of *Bt* protein (Section 4.2.5.2). A proposed higher concentration (Section 7.1) in commercial broccoli plants could lead to more marked effects.

The majority of effects of *Bt* plants on non-target organisms were linked to *Bt* broccoli leaves rather than compost from around *Bt* plant roots. This would be unsurprising had the effects been negative as the *Bt* proteins require ingestion to have an effect on the gut. The results were, in fact, positive suggesting that it is not a direct effect of the *Bt* protein that was being observed but an indirect consequence. Differences in plant chemical composition have been detected between *Bt* and non-*Bt* maize in compunds such as lignin (Escher *et al.*, 2000; Flores *et al.*, 2005; Saxena *et al.*, 2001a), sugar (Escher *et al.*, 2000; Clark & Coats, 2006), nitrogen (Escher *et al.*, 2000) and protein (Clark & Coats, 2006) levels. These could all alter the decomposition rate and nutritional value of the plants. The latter is more likely to be measurable in young, actively growing (e.g. small woodlice as seen in this study) rather than mature members of a species. This may also lead to an alteration in adult fitness with changes in reproductive parameters.

The chemical composition of *Bt* broccoli should be further investigated to analyse the causes of the increase in weight seen in specific species. The differences in plant chemical composition, as noted in Sections 3.4 and 4.3, could be related to either the

genetic variability of the transgenic line (a F2 generation) or somaclonal variation from the cell culturing techniques used to produce the original transgenic plants (reviewed in Filipekci & Malepszy, 2006). The end product may also have been altered by the different packaging mechanisms that plants employ (Goldburg & Tjaden, 1990); non-toxic pea (*Pisum sativum*, L.) amylases expressed in beans (*Phaseolus vulgaris*, L.), for example, were found to be toxic to mice (Prescott *et al.*, 2005, Section 1.4.6).

Any increase in woodlouse and slug fitness could have an impact on food chains and biodiversity in the field especially their predators. There was an apparent negative effect of *Bt* plants on the predatory beetle; there were fewer surviving *Nebria brevicollis* (Fabricius) that had fed on slugs that had been feeding on *Bt* broccoli leaves than on slugs feeding on non-*Bt* broccoli leaves (Section 5.3). Compost in which *Bt* plants were grown did not appear to have an effect on the beetles. The more sensitive parameter: reproduction (egg production by the beetles) was not affected at this third trophic level (Section 5.3). Other studies have discovered no effects of *Bt* proteins on beetles e.g. predatory ladybirds (*Coleomegilla maculata*, DeGeer) in the laboratory (Duan *et al.*, 2002) and the numbers of carabids and staphylinids in a field (Duan *et al.*, 2004). The *Bt* protein was not detectable in this study in *N. brevicollis* beetle guts (Section 5.3) but the protein could have been altered by the slug or beetle digestive processes; it is, however, unlikely that it is the *Bt* protein causing a direct effect. *D. reticulatum* may accumulate and secrete metabolites from broccoli, including the *Bt* protein, in its mucus to deter predators. This is known to happen with snails accumulating anti-feedants from lichen (Hesbacher *et al.*, 1995; Section 5.4). It could be speculated that the increase in slug fitness (i.e. heavier weights) may also have led to other changes, perhaps leading to increased mucus production making feeding on, and digestion of, individual slugs more difficult for *N. brevicollis*. This effect is unlikely to be detected in the field as the predatory beetles would feed on a variety of prey, and may avoid prey which are difficult to capture and digest.

The increase in slug fitness, and decrease in predator fitness, could have an important impact on *Bt* crop yields. If a true indicator of what could happen in the field, when higher *Bt* concentrations are produced by plants, or outside fields where slugs feed on *Bt* hybrids, these results suggest that the reduced crop damage resulting from regulating Lepidoptera larval feeding could be cancelled out by an increase in damage caused by a herbivorous slug pest. In this case the grower would have to implement more slug control measures which would involve a financial cost. This increase in slug numbers is unlikely

to be suppressed by *Nebria* beetles (and possibly other natural enemies) as, at least for the experimental beetle, their numbers are adversely affected by feeding on slugs that have fed on *Bt* broccoli plants (Section 5.3). *Nebria* spp. are generalist predators and reducing their numbers could impact control of other pest species and again result in higher pesticide usage. *Bt* plants are supposed to help reduce chemical inputs so this would appear to be counter-productive.

Although inconclusive, the microbial study (Chapter 6) provided a preliminary investigation of the interaction between *Bt* plants, specifically their root exudates and micro-organisms. Micro-organism DNA was extracted and amplified, and the parameters optimised for analysis using denaturant gradient gel electrophoresis. Future progress demands the development of a more reproducible method to provide sufficient and appropriate data for analysis of the operational taxonomic units. This would allow determination of any differences between *Bt* and non-*Bt* soils, and whether they change over time as more *Bt* protein is exuded into the soil. Differences are likely to be detected as many studies looking at biochemical changes in the soil indicate changes in the micro-organism activity (Table 1.4). Changes in micro-organism composition have been previously discovered (Baumgarte & Tebbe, 2005) using a similar molecular techniques to the one in this study but there remains a need to investigate the DNA sequences in the bands that are different, and use the sequences to identify the micro-organisms present (Section 6.4). Changes to keynote species such as nitrifying bacteria (Kowalchuk *et al.*, 2003) could lead to a loss of important soil ecosystem functions (e.g. decomposition and nutrient cycling). Without a greater knowledge of variations in soil communities due to other changes in agricultural management (Bruinsma *et al.*, 2003) and within a growing season (Griffiths *et al.*, 2007) it will, however, be difficult to contextualise these changes and assess the risk of planting *Bt* crops.

7.4 Setting the effects of *Bt* broccoli on non-target species in context.

With so many statistical tests reported in this study there is a probability of finding significance purely by chance. There are, however, a sufficient number of significant results to require them to be placed in a broader context. In particular, these effects are of one *Bt* protein, expressed in one specific broccoli cultivar, in microcosms where there are no species interaction or food choice. To investigate if these effects run true for all *Bt* broccoli a comparison of different *Bt* broccoli cultivars and different genetic constructs

inserted into the same cultivar would be appropriate; different *Bt* constructs may, for example, have the gene inserted under the control of different promoters and will be transformed into different positions in the genome.

In the field the test organisms used in this study may not come into contact with the same level of *Bt* protein as in the reported laboratory tests. The variability in protein levels between *Bt* broccoli plants could be a significant factor in the results. Once a commercial line of *Bt* broccoli is proposed its level of *Bt* protein expression and exudation should be compared with the concentrations reported in this study and theories drawn accordingly.

The effects of *Bt* broccoli plants must also be compared with other broccoli management practices. Even if the *Bt* plants were found to have a significant effect in the field this may be of a lower impact on non-target species than other pest control management practices. Other studies have shown that the effect of *Bt* plants is sometimes less than that caused by other crop management schemes, for example, the use of pesticides (Duan *et al.,* 2004; Meissle & Lang, 2005; Griffiths *et al.*, 2006). Different varieties of the same crop can also have quite different chemical compositions so may also have different effects on non-target species. For example, crop type (maize versus grass) had more of an effect on micro-organism biomass than *Bt* proteins (*Bt* versus non-*Bt* plants (Griffiths *et al.*, 2005)) and the effect of *Bt* maize on earthworm growth was limited to two out of four GM lines tested (Clark & Coats, 2006).

7.5 Future work

The first obvious step would be to design a *Bt* broccoli cultivar that produces the *Bt* proteins in all seeds and at a non-variable level high enough to kill the majority of target pests (see Section 2.4 for possible approaches). Once designed, similar laboratory assays as to those conducted here would need to be carried out along with field plot experiments, to investigate the effect of these new *Bt* broccoli plants on non-target species. Farm scale trials comparing *Bt* and non-*Bt* plants and other crop management practices (e.g. Dipel® and chemical based pesticides), following similar procedures to the herbicide-tolerant UK farm scale trials (The Royal Society, 2003), would eventually be desirable.

The studies presented here represent more accurately what might happen to biodiversity if the *Bt* gene escaped into nature by hybridisation. Some further steps are therefore needed to test if the gene is likely to escape from *Bt* fields into wild populations and become invasive (e.g. Crawley *et al.*, 1993; 2001) before considering the effects on a larger scale

on non-target species for longer periods of time. As reproduction was the most frequently affected parameter (Table 4.2), and appears to be more sensitive (Lövei & Arpaia, 2005), this should be studied in detail over several generations. Studies involving other species are also necessary to see if the results detected here are confined to these species, particular groups within an order, or are further-reaching.

The control non-*Bt* plant to be used for these studies should be considered carefully. In this study the commercially available broccoli "Green Comet" was used, however these are F1 hybrids and the *Bt* plants were a backcross so had variable genetic backgrounds (Table 2.2). It could be argued that the differences detected here are caused not by the presence of the *Bt* gene but these background differences. The plants grown from the hemizygous seed that did not test positive for the *Bt* protein could also have been used as a further treatment (as they were for *P. brassicae,* Section 3.2.6.2). They would have the same background variability as the *Bt* plants. By using the Green Comet, this study allowed the comparison of the *Bt* plants with a currently used agricultural crop which could be more valuable for risk assessment purposes.

It may be possible to investigate several broccoli cultivars in parallel (e.g. Clark & Coats, 2006; Wandeler *et al.*, 2002). This would allow determination of whether differences in other plant metabolites was the indirect cause of the effects on non-target species seen in this study rather than a direct effect of the *Bt* protein itself. If lignin is the crucial factor it must be considered that lignin content not only affects nutritional value for herbivores but also microbial colonisation and thus decomposition (e.g. Escher *et al.*, 2000). It would be necessary to investigate whether leaf decomposition rates would also affect dissipation and deactivation of the *Bt* protein (e.g. Tapp & Stotzky, 1998, Section 1.4.5).

The investigations carried out in this study have proved to be important, relatively detailed, preliminary studies into the possible effects of *Bt* broccoli on some non-target soil biota. By using ELISA it was possible to determine the level of exposure for the non-target organisms and measure various fitness parameters. Significant effects were uncovered but at this stage it is not possible to state with absolute certainty whether these effects would be seen in the field. The level of exposure in the field remains unknown and whether these statistically significant but small effects would translate into a noticeable higher impact to biodiversity than current agricultural practices is yet to be determined.

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Appendices

Appendix 1 ELISA protocol.

Bt-Cry1Ab/Cry1Ac

• Intended Use

For the detection and quantitation of Bt Cry1Ab and Cry1Ac (MON810, Bt11, Bt176) endotoxin residues in corn tissues and cotton leaf tissues. For use with other sample matrixes, contact the company for application bulletins and/or specific matrix validation guidelines.

• Principle

The Abraxis Bt Cry1Ab/Cry1Ac Microtiter Plate Kit is a "sandwich" enzyme linked immunosorbent assay (ELISA). In the assay system, standards, controls, or sample extracts are added to wells coated with monoclonal antibodies raised against Cry1Ab/Cry1Ac endotoxin. Any endotoxin residues found in the standard or sample extracts bind to the antibodies on the wells. The "sandwich" is completed by the addition of polyclonal antibodies raised against the same endotoxin. An enzyme labeled conjugate is then added and the enzyme activity bound to the wells is measured using a substrate to develop a colored product. Since the formation of a "sandwich complex" occurs only in the presence of a Cry1Ac/Cr1Ab molecule, the enzyme activity of the bound sandwich complex is directly proportional to the amount of endotoxin in the sample.

A dose response curve of absorbance of the colored product formed vs. concentration is generated using results obtained from the standards. Concentration of Cry1Ab/Cry1Ac present in the control and sample extracts are determined directly from this curve.

Lighter color = Lower concentration
Darker color = Higher concentration

• Reagents

The Abraxis Bt Cry1Ab/Cry1Ac Kit contains the following items:

1. Cry1Ab/Cry1Ac Antibody coated wells
Monoclonal antibody specific for Cry1Ab/Cry1Ac endotoxin adsorbed to plastic wells.
8 strips of 12 antibody coated wells
and strip holder (1).

2. Cry1Ab/Cry1Ac Antiserum solution
Polyclonal antibody (rabbit) specific for Cry1Ab/Cry1Ac endotoxin.
One vial containing 11 mL

3.Goat anti-rabbit Enzyme Conjugate (100x)
Horseradish peroxidase (HRP) labeled goat anti-rabbit. Supplied as a liquid concentrate 100x with preservative and stabilizers.
One vial containing 0.25 mL

4. Conjugate Diluting Buffer
Buffered solution with preservative and stabilizers used to dilute the conjugate
One bottle containing 12 mL

5. Bt Standards (Cry1Ab)
Five concentrations (0, 0.25, 0.5, 1.0, 2.0, 4.0 ng/mL) of Bt calibrators in a buffered solution with preservative and stabilizers. Each vial contains 2.0 mL.

6. Control (Cry1Ab)
A concentration (approximately 1.5 ng/mL) of Bt Cry1Ab in a buffered solution with preservative and stabilizers. One vial containing 2.0 mL.

7. Extraction Solution/Sample Diluent (5x)
Buffered solution 5x concentrate with preservative and stabilizers without any detectable Bt endotoxin.
One bottle containing 30 mL.

8. Color Solution
A solution of hydrogen peroxide and 3,3',5,5'-tetramethyl benzidine in an organic base.
One bottle containing 12 mL

9. Stopping Solution
A solution of diluted acid.
One bottle containing 6 mL

10. Washing Buffer (5x) Concentrate
Preserved buffered 5x concentrate.
One bottle containing 110 mL

• Reagent Storage and Stability

Store all reagents at 2-8°C. Do not freeze. Reagents may be used until the expiration date on the box.

Consult state, local and federal regulations for proper disposal of all reagents.

• Materials Required but Not Provided

In addition to the reagents provided, the following items are essential for the performance of the test:

Precision pipets capable of delivering 20, 100, 500, and 1000 uL , and tips

Disposable Tissue Extractors, Abraxis PN 510010

Test tubes for dilution of sample extracts

Marking pen (indelible)

Tape or Parafilm®

Timer

Vortex mixer

Distilled or deionized water for diluting Wash buffer and the 10x Cry1Ab/Cry1Ac Extraction/Dilution Buffer

Storage bottles with 300 mL capacity for the storage of 1x Extraction/Dilution buffer and 1000 mL capacity for storage of 1x Wash buffer

Microplate or strip reader capable of reading absorbance at 450 nm

Wash bottle (Nalgene cat # 03-409-10E or equivalent) if performing manual plate washing

Test tube rack

• Materials Recommended but Not Required

Multi-channel pipette (100 uL)

Reagent reservoirs for multi-channel dispensing

Automated plate washer

• Sample Information

This procedure is recommended fo use with corn tissues and cotton leaf tissues. For testing of Cry1Ab and Cry1Ac in corn and cotton seeds, and in bulk corn grain, refer to application bulletin. Other samples may require modifications to the procedure and should be thoroughly validated.

Samples containing gross particulate matter should be filtered using a low protein binding filter such as Pall Gelman Sciences cat # 4184 or equivalent. Alternatively the samples can be centrifuged at 5000 x g for 5 minutes.

Sample Extraction

1. Take 2 leaf punch samples (approximately 10 mg each) by snapping the tube cap of the Sample extraction Device down on the leaf. Insert the pestle into the tube and grind the tissue by rotating the pestle against the sides of the tube with a twisting motion. Continue the process for about 30 seconds, or until the leaf tissue is well ground. *To prevent sample contamination, a new extraction device and pestle must be used with each plant tissue sample.*

NOTE: If a quantitation level of Cry1Ab/Cry1Ac endotoxin is needed (quantitative assay), the weight of each leaf punch sample must be determined and recorded.

2. Add 0.5 mL of the 1x Sample Extraction/Dilution Buffer to the tube.
3. Repeat the grinding step (described in step 1) to mix tissue with the Extraction/Dilution Buffer. Allow the solids to settle in each tube for a few minutes before proceeding.

Sample Dilution

If the Cry1Ab/Cry1Ac concentration of a sample exceeds 4 ng/mL and a quantitatively results is desired, the sample is subject to repeat testing using a diluted sample. A ten-fold or greater dilution of the sample is recommended with an appropriate amount of Sample Extraction/Sample Diluent buffer. For example, in a separate test tube make a eleven-fold dilution by adding 100 uL of the sample to 1000 uL of Sample Extraction/ Sample Diluent buffer. Mix thoroughly before assaying. Perform the assay according to the Assay Procedure and obtain final results by multiplying the value obtain by the dilution factor e.g. 11.

• Reagent Preparation

1. **Wash Buffer**
In a 100 mL container, dilute the wash buffer concentrate 1:5 by the addition of distilled or deionized water (i.e., 100 mL of wash buffer concentrate plus 400 mL of H_2O). This solution is used to wash the antibody coated wells. Store refrigerated when not in use.

2. **1x Cry1Ab/Cry1Ac Extraction/Dilution Buffer**
Add the entire contents of the 5x bottle supplied in the kit to 120 mL of distilled or deionized water in a suitable size container. Store refrigerated when not in use.

3. **Anti-Rabbit HRP Conjugate**
Dilute the conjugate 1:100 with the conjugate diluent buffer just prior to use. (i.e., 0.10 mL conjugate + 9.90 mL of conjugate diluent buffer). Dilute only the amount needed per assay, diluted conjugate is not to be stored.

All reagents must be allowed to come to room temperature prior to use.

• Procedural Notes and Precautions

As with all immunoassays, a consistent technique is the key to optimal performance. To obtain the greatest precision, be sure to treat each well in an identical manner.

Add reagents directly to the bottom of the well while avoiding contact between the reagents and the pipet tip. This will help assure consistent quantities of reagent in the test mixture.

Avoid cross-contaminations and carryover of reagents by using clean pipets for each sample addition and by avoiding contact between reagent droplets on the tubes and pipet tips.

If more than 3 strips are going to be run, the use of a multichannel pipette is recommanded.

If fewer than all eight strips are used, reseal the unused strips with the desicant in the foil bag provided. Store refrigerated.

Use the well identification markings on the plate frame as a guide when adding samples and reagents. In a qualitative assay, the zero standard, four non-zero calibrators , a control and 84 sample extracts may be run in one plate. For a quantitative assay, the zero standard and four calibrators and a control along with 42 sample extracts may be run in duplicate wells on one plate.

Do not use any reagents beyond their stated shelf life. Each component used in any one assay should be of the same lot number and stored under identical conditions.

Appendix 1 continued

Avoid contact of Stopping Solution (diluted sulfuric acid) with skin and mucous membranes. If this reagent comes in contact with skin, wash with water.

• Limitations

This Abraxis Assay will detect Cry1Ab/ Cry1Ac and other related endotoxins to different degrees. Refer to specificity table for data on several of the Cry endotoxins.

• Quality Control

A control solution at approximately 1.5 ng/mL of Cry1Ab is provided with this Assay kit. It is recommended that it be included in every run and treated in the same manner as unknown samples. Acceptable limits should be established by each laboratory.

• Assay Procedure

Read Reagent Preparation, Procedural Notes and Precautions before proceeding.

1. Add 100 uL of standard zero, 100 uL of each Calibrator or Control, and 100 uL of each diluted sample extract to their respective wells. Follow the same order of addition for all reagents. Cover plate to prevent contamination and evaporation.
2. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill contents.
3. Incubate at ambient temperature for 30 minutes.
4. After incubation, carefully removed the covering and vigorously shake the contents of the wells into a sink or other suitable container. Flood the wells completely with Wash Buffer then shake to empty. Repeat this wash step two times. Tap the strips on to a stack of paper towels to remove residual wash buffer. Alternative, perform these three washes with a microtiter plate or strip washer.
5. Add 100 uL of Cry1Ab/Cry1Ac antibody solution.
6. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill contents.
7. Incubate at ambient temperature for 30 minutes.
8. Repeat step 4.
9. Add 100 uL of enzyme conjugate.
10. Thoroughly mix the contents of the wells by moving the strip holder in a rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill contents.
11. Incubate at ambient temperature for 30 minutes.
12. Repeat step 4.
13. Add 100 uL of color solution.
14. Incubate at ambient temperature for 20 minutes.
15. Thoroughly mix the contents of the wells by moving the strip holder ine rapid circular motion on the benchtop for a full 20-30 seconds. Be careful not to spill contents
16. Add 50 uL of stopping solution. This will turn the well contents to yellow.
17. Read results at 450 nm within 15 minutes after adding the Stopping Solution. Set the plate reader to blank on the zero standard wells.

• Results

Data Interpretation

Average the absorbance readings for the duplicate calibrators, control, and samples.

Semi-Quantitative Results

Compare the OD's of the diluted sample extracts to those of the calibrators to obtain an estimate of the amount of Cry1Ab/ Cry1Ac endotoxin in the sample extract.

Quantitative Results

For a quantitative Cry1Ab/Cry1Ac assay, a linear curve fit for the standard curve should be used if the plate reader used has data reduction capabilities. Otherwise, calculate the results as described in the manual calculations.

Manual Calculations

1. Average the absorbance value for each of the calibrators, control, and samples.
2. Construct a standard curve by plotting the mean OD of each calibrator on the vertical linear (Y) axis against its corresponding Cry1Ab concentration on the horizontal linear (X) axis on the graph paper provided.
3. Determine the endotoxin concentration for controls and samples by finding its OD value and its corresponding concentration level on the graph. Multiply the results by dilution factor incurred during extraction (500 uL + *x* mg leaf tissue)/1000 to report as micrograms (ug) of endotoxin per gram of tissue.

• Performance Data

Precision

Cry1Ab fortified samples were analyzed both within a single assay, and in differentassays. The following results were obtained using buffer, leaf and kernel extracts:

Buffer

Control	1	2	3
Mean (ppb)	1.04	2.12	3.94
% CV (within assay)	6.3	5.6	3.4
% CV (between assay)	3.9	4.0	1.9

Leaf Extract

Control	1	2	3
Mean (ppb)	1.30	2.08	3.96
% CV (within assay)	5.4	7.9	7.7
% CV (between assay)	3.2	5.1	4.7

Corn Kernel Extract

Control	1	2	3
Mean (ppb)	1.24	2.18	4.04
% CV (within assay)	6.7	3.5	8.1
% CV (between assay)	4.6	2.6	7.9

Limit of Detection

The Abraxis Bt Cr1Ab/Cry1Ac Assay limit of detection is 0.125 ng/ml (ppb) Cry1Ab in corn leaf extract. The Limit of Detection (LOD) was determined by calculating 3 standard deviations (OD units) from a negative corn leaf sample population and by interpolating from a Cry1Ab standard curve.

Recovery

Corn leaf extract samples were fortified with various levels of Cry1Ab endotoxin and then assayed using the Abraxis Cry1Ab/Cry1Ac Assay. The following results were obtained:

Amount of Cry1Ab Added (ppb)	Recovery Mean (ppb)	Recovery S.D. (ppb)	Recovery %
0.750	0.718	0.026	96
1.50	1.49	0.092	99
3.0	2.95	0.090	98
Average			98

Specificity

The Abraxis Cry1Ab/Cry1Ac detects the presence of various Bt endotoxin to differing degrees. The following table shows the concentration of various Bt endotoxin equivalent to the given concentration of Cry1Ab.

Bt Endotoxin (ng/ml)				
Cry1Ab	0.50	1.0	2.0	4.0
Cry1Ac	0.28	0.49	0.95	2.0
Cry1F	18	24	57	460
Cry9C	72	317	1429	>2700
Cry2A	>2500	>2500	>2500	>2500

• Ordering information

Abraxis Cry1Ab/Cry1Ac Kit, 96T	PN 510001
Sample Extraction/Dilution Buffer	PN 510002
Disposable Extraction Device	PN 510010

• Assistance

For ordering or technical assistance contact:

Abraxis LLC
Sales Department
54 Steamwhistle Drive
Warminster, Pennsylvania, 18974

(215) 357-3911 * Fax(215) 357-5232
WEB: www.abraxiskits.com
Email: info@abraxiskits.com

• General Limited Warranty

Abraxis LLC warrants the products manufactured by the Company, against defects and workmanship when used in accordance with the applicable instructions for a period not to extend beyond the product's printed expiration date. Abraxis makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose.

Appendix 2 FastDNA® SPIN kit for soil. BIO 101® systems

Add up to 500 mg of soil to lysing matrix E tube.

Add 978 µl sodium phosphate buffer and 122 µl MT buffer.

Secure tubes in FastPrep® instrument and process for 30 seconds at speed 5.5.

Centrifuge lysing matrix E tubes at 14,000 rpm for 30 seconds.

Transfer supernatant to a clean tube. Add 250 µl PPS reagent and mix by shaking the tube by hand 10 times.

Centrifuge at 14,000 rpm for 5 minutes to pellet precipitate. Transfer supernatant to a clean 15 ml tube. (Resuspend binding matrix suspension before use.) Add 1 ml binding matrix suspension to the supernatant.

Place on a rotator or invert by hand for 2 minutes to allow binding of DNA to matrix. Place tube in a rack for 3 minutes to allow settling of silica matrix.

Remove 500 µl of supernatant without disturbing the binding matrix. Discard the supernatant. Resuspend binding matrix in the remaining amount of supernatant. Transfer approximately 600 µl of the mixture to a SPIN™ filter and centrifuge at 14,000 rpm for 1 minute. Empty each catch tube and add the remaining supernatant to the SPIN™ filter and spin again.

Add 500 µl SEWS-M to the SPIN™ Filter and centrifuge at 14,000 rpm for 1 minute. Decant flow through and replace SPIN™ Filter in catch tube. Centrifuge at 14,000 rpm for 2 minutes to "dry" the matrix of residual SEWS-M wash solution.

Remove SPIN™ Filter and place in fresh kit supplied catch tube. Air dry the SPIN™ filter for 5 minutes at room temperature.

Add 50 µl DES (DNase/pyrogen free water) and gently stir matrix on filter membrance with a pipette tip or vortex/finger flip to resuspend the silica for efficient elution of the DNA. Centrifuge at 14,000 rpm for 1 minute to transfer eluted DNA to catch tube.