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EFFECTS OF PLANTS GENETICALLY MODIFIED FOR INSECT RESISTANCE ON NONTARGET ORGANISMS

Maureen O'Callaghan,[1] Travis R. Glare,[1]
Elisabeth P.J. Burgess,[2] and Louise A. Malone[2]
[1]*AgResearch, PO Box 60, Lincoln, Canterbury, New Zealand;*
email: maureen.ocallaghan@agresearch.co.nz; travis.glare@agresearch.co.nz
[2]*HortResearch, Private Bag 92169, Auckland, New Zealand;*
email: eburgess@hortresearch.co.nz; lmalone@hortresearch.co.nz

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■ **Abstract** Insect resistance, based on *Bacillus thuringiensis* (Bt) endotoxins, is the second most widely used trait (after herbicide resistance) in commercial genetically modified (GM) crops. Other modifications for insect resistance, such as proteinase inhibitors and lectins, are also being used in many experimental crops. The extensive testing on nontarget plant-feeding insects and beneficial species that has accompanied the long-term and wide-scale use of Bt plants has not detected significant adverse effects. GM plants expressing other insect-resistant proteins that have a broader spectrum of activity have been tested on only a limited number of nontarget species. Little is known about the persistence of transgene-derived proteins in soil, with the exception of Bt endotoxins, which can persist in soil for several months. Bt plants appear to have little impact on soil biota such as earthworms, collembolans, and general soil microflora. Further research is required on the effects of GM plants on soil processes such as decomposition. Assessment of nontarget impacts is an essential part of the risk assessment process for insect-resistant GM plants.

INTRODUCTION

Genetic modification of plants is a powerful technology, with the potential to transform agriculture. Although the steady growth in global plantings of genetically modified (GM) plants (52) attests to their usefulness for many farmers and their acceptance in many markets, the imposition of moratoria in several countries reflects skepticism and public concern about a range of issues around GM, including potential impacts on the environment. In response to this concern, governments have established regulatory bodies to oversee the deployment of GM organisms commercially and in research. Many governments and companies have also funded research aimed at assessing the nature and likelihood of any adverse effects of GM

plants on the environment including invasiveness, effects on nontarget species, potential for transgenes to "escape" into the environment by horizontal and vertical gene transfer, and development of resistance to transgene-derived proteins (20). Here we summarize the current state of research on the effects of insect-resistant (IR) GM plants on nontarget plant-feeding insects, beneficial species such as pollinators and natural enemies, and soil biota.

Crops and Traits for Insect Resistance

Insect resistance, conferred via expression of a variety of *Bacillus thuringiensis* (Bt) delta-endotoxins, is the second most commonly used trait, after herbicide resistance, in commercial GM crops (52). Four Bt delta-endotoxin genes (*cry1Ab*, *cry1Ac*, *cry2Ab*, and *cry9C*) are currently used commercially in maize and cotton to protect against lepidopteran pest attack (92). Many more Bt GM plants expressing delta-endotoxins are being developed, particularly annual crop species that are grown on a large scale around the world. A vegetative insecticidal protein (not a delta-endotoxin) from Bt (VIP3A) is also being field-tested (74). Protease inhibitors (PIs) and lectins, whose ranges of insecticidal activity are generally broader than those of Bt toxins, are also being used in many experimental crops. Biotin-binding proteins (18, 59), toxins from bacterial symbionts of entomopathogenic nematodes (60), chitinases (106), enzymes controlling aromatic aldehyde synthesis (37), spider venom peptides (75), *Aedes aegypti* trypsin modulating oostatic factor (100), enhancin from insects (19), plant defensins (2), and plant hormones (93) are some more recent insecticidal transgenes being investigated for agricultural use. A more detailed list of crops genetically modified for insect resistance is provided in a supplemental table (follow the Supplemental Material link from the Annual Reviews home page at http://www.annualreviews.org).

Assessing Environmental Impacts of Insect-Resistant Plants

Since the mid-1990s, when the first GM crops were commercialized, a reasonably comprehensive set of testing methods has been devised for assessing the likely impacts of IR GM plants on nontarget organisms. These tests begin in contained, indoor conditions and often use transgene products rather than GM plants and progress to more realistic conditions, involving microcosms, field trials, modeling, and finally farm-scale evaluations or commercial crop monitoring. A "tiered toxicity" approach, which has its origins in chemical pesticide testing, is generally taken. The responses of a nontarget organism to a range of concentrations of the transgene product are considered along with estimates of its likely exposure to the product in the field. Nontarget organisms are selected on a range of criteria (e.g., abundance in the field, ease of handling in the laboratory, taxonomic certainty, value to the agroecosystem, endangered status), and those for which toxicity is demonstrated are then subjected to more detailed investigation (24, 36, 87).

EFFECTS OF INSECT-RESISTANT GENETICALLY MODIFIED PLANTS ON NONTARGET PLANT-FEEDING INSECTS AND THEIR ENEMIES

Insects as Ecosystem Service Providers

Daily (28) first coined the term "ecosystem service providers" for organisms that make essential contributions to the functioning of ecosystems and the human economy. Species representing pollinators, natural enemies (including predators and parasitoids), and detritovores are among the insect ecosystem service providers that have been subjected to tests with GM plants or the proteins they express. Insects without obvious ecological roles but which are considered representative of valued biodiversity (e.g., endangered or culturally valued species) have also been candidates for study. Recent results with above-ground nontarget insects are presented immediately below and those with soil-dwelling organisms are presented in the section titled Effects of Insect-Resistant Genetically Modified Plants on Soil Biota.

Pollinators

Among the many insect pollinators of agricultural crops, honey bees are the best known. Even where bees are not essential for successful crop production (e.g., self-fertile plants), they often improve fruit or seed yields (26, 44) and have the additional advantage of producing honey. Furthermore, agricultural crops can provide food not only for honey bees but also for wild bees, such as bumble bees, and other pollinators.

Neither Bt cotton nor Bt maize requires bees for pollination, but cotton nectar is attractive to them and produces a useful honey. Maize pollen may be collected when other pollen sources are scarce. Pre-release honey bee biosafety tests have been conducted for each Bt crop registered in the United States (102), including Cry9C maize and Cry3A potatoes. Each test involved feeding bee larvae and sometimes adults with purified Cry proteins in sucrose solutions at concentrations that greatly exceeded those recorded from the pollen or nectar of the GM plants in question. In each case, no effects were observed (102). The rationale for requiring larval and not adult bee tests is questionable, because adult bees ingest considerable quantities of pollen in their first few days post emergence. Larvae, particularly later instars, also consume pollen along with jelly secreted by nurse adult bees, but only recently have there been attempts to quantify pollen ingestion by individual larvae (65).

Other studies with bees fed purified Bt proteins, or pollen from Bt plants, or bees allowed to forage on Bt plants in the field have confirmed the lack of effects noted by the U.S. Environmental Protection Agency (EPA) (67, 68, 76). Post-release monitoring programs are now underway to assess impacts of North American GM crops on pollinators under commercial field conditions (42, 43).

The effects of other IR proteins and GM plants on honey bees and bumble bees have been investigated in a series of laboratory-, glasshouse-, and field-based studies reviewed recently (68, 76). Of these, only serine PIs affect honey bees

(12, 17) and bumble bees (66), causing changes in bee digestive proteases and some reductions in survival when ingested at high concentrations. Whether bees in the field would be exposed to such levels of PIs will depend on expression levels in the pollen of the GM PI-plants and the amounts and types of pollen foraged. Conditions in the hive such as social interactions, presence of disease, and environmental factors may also influence impacts of PIs on bees in the field.

Natural Enemies

Predators and parasitoids are significant regulators of insect pest populations, and integrated pest management systems strive to harmonize pesticide use with the preservation or augmentation of these natural enemies. In recent years there have been many studies on potential tritrophic impacts of GM plants as well as field surveys of natural enemy abundance on GM crops, and these are discussed below.

Natural enemy survival depends upon a supply of host insects, so reductions in host numbers feeding on GM plants will affect population densities of natural enemies, as will any pest control measure. In addition, GM plants could have a direct "prey-mediated" effect on individual natural enemies via ingestion of GM pollen, other plant tissue, or active recombinant protein in the bodies of their prey. Indirect effects could also result from prey being smaller, sicker, or less palatable for having fed on the GM crop. This complexity has meant that establishing cause and effect in GM plant/natural enemy studies has not been a straightforward task. Caution may be required in the interpretation of some results. Comparison with the effects of existing pest control measures is also a valid approach but is not always possible. Studies published before 2001 have been summarized previously (45); the following section updates that report with respect to IR GM plants.

Bt COTTON Prior to registration of Cry1Ac cotton, the EPA ascertained that purified Cry1Ac was not toxic to green lacewing larvae (*Chrysopa carnea*), adult lady beetles (*Hippodamia convergens*), or adult parasitoids (*Nasonia vitripennis*) and concluded that these natural enemies were unlikely to be affected by ingesting Bt cotton pollen or nectar, or prey that may have fed on Bt cotton plants (102). Subsequently, potential negative tritrophic impacts on the predators *Orius tristicolor* and *Geocoris punctipes* (but not on *Nabis* spp. or *Zelus renardii*) (78) and the parasitoids *Cotesia marginiventris* and *Copidosoma floridanum* (3) have been demonstrated in laboratory studies. Reports from field studies of arthropod abundance on Bt cotton have also been mixed, with decreases (109), increases (98), and no change (71) in the fitness or survival of some species noted.

Bt MAIZE Before Bt maize was released, laboratory tests showed that purified Cry1Ab was not toxic to the green lacewing, *C. carnea*, the lady beetle, *H. convergens*, or the parasitoid *Brachymeria intermedia* (102). Subsequent tritrophic studies with several different Cry1Ab-maize-fed prey species and the green lacewing confirmed that the toxin itself does not affect this predator but that the suboptimal quality of Bt-maize-susceptible prey species may have an impact. When given a

two-way choice, the lacewings preferred non-Bt-maize-fed *Spodoptera littoralis* caterpillars to Bt-fed larvae (69). Green lacewings experienced delayed development and reduced survival when fed Bt-maize-fed *S. littoralis* caterpillars, which were sublethally affected and carried measurable quantities of Cry1Ab in their bodies (35, 79). However, consuming *Tetranychus urticae* mites, which were not Bt susceptible but carried measurable quantities of Cry1Ab after sucking the cell contents of GM maize, had no effect on green lacewings (35). Similarly, maize-cell-sucking thrips *Anaphothrips obscurus* had no effect on the development or survival of their predator *Orius majusculus* (114). Sap-sucking aphids (*Rhopalosiphum padi*) neither contained the Bt toxin nor had any effect on the predator (35). A recent study in which green lacewings were fed high doses of Cry1Ab toxin showed no negative impacts and confirmed that negative tritrophic effects observed with this predator and some prey fed Bt maize were entirely prey mediated (83). Prey-mediated effects have also been observed with *Parallelorhogas pyralophagus* that parasitize *Eoreuma loftini* (Crambidae) stemborers fed Bt maize (7). Pollen from another variety of Bt maize, which contained the *cry3Bb* gene but did not express the protein at measurable levels in pollen, did not affect the fitness of the lady beetle *Coleomegilla maculata* (34, 64).

Field surveys, which do not distinguish population-level, prey-mediated, or direct effects, have shown little impact of Bt maize on predator species numbers or densities. Of nine predator species (including green lacewings), only *Coleomegilla maculata* larvae were found at significantly lower densities on Bt sweetcorn (107). This lady beetle and 11 other natural enemy species or groups of species (including a *Chrysopa* sp.) were not affected by Bt maize in a recent Ohio study (53). The predators *C. maculata*, *Harmonia axyridis*, and *Orius insidiosus* were less affected by Bt sweetcorn than by pyrethroid sprays (70). Field studies to date appear to confirm the EPA's original predictions, and those of subsequent researchers, of minimal impacts of Bt maize on natural enemies.

Other Insect-Resistant Plants and Proteins

Negative effects of other Bt crops and toxin combinations have been similarly difficult to find. Cry1Ac oilseed rape ingested by the nontarget aphid *Myzus persicae* had no effect on the ability of the parasitoid *Diaeretiella rapae* to control this pest (88). Cry3A potato fed to the Colorado potato beetle had no effect on its carabid predator, *Lebia grandis* (81). Field studies with Cry3A potatoes showed no significant impacts on beneficial predator species, compared with the reductions noted with the regular permethrin applications required with a conventional crop (80). Cry1Ab rice plants did not affect the development or survival of the predator *Cytorhiunus lividipennis* feeding on brown planthoppers, *Nilaparvata lugens*, which were not susceptible to Cry1Ab but excreted it with their honeydew (6). Field tests with rice expressing Cry1Ab and Cry1Ac showed no effects on the population dynamics of five spider species (61).

PIs are generally benign to natural enemies when expressed in GM plants or fed to prey in artificial diets. However, cowpea trypsin inhibitor (CpTI)-injected tomato

moth caterpillars (*Lacanobia oleracea*) given to the predator *Podisus maculiventris* resulted in reduced nymph growth and adult female weights, but experiments with the same insects fed CpTI potatoes showed no negative effects on the predator (4). Parasitoids, *Eulophus pennicornis*, were adversely affected by both CpTI-injected and CpTI-potato-fed *L. oleracea* prey (5). Transient, minor changes in the masses of adult carabid beetles (*Nebria brevicollis*) fed prey reared on aprotinin-diet have been recorded (16). Stinkbug predators (*Perillus bioculatus*) feeding on Colorado potato beetles reared on oryzacystatin (OCI) potatoes compensate for any effects of this PI by upregulation of their digestive proteases (10, 11). Other tritrophic studies with PI-plants have shown no effects of CpTI-strawberry-fed prey on carabid beetles (46), or of OCI oilseed rape on *Harmonia axyridis* predators of *Plutella xylostella* caterpillars (40) or *D. rapae* parasitoids of *M. persicae* aphids (88).

The snowdrop lectin, *Galanthus nivalis* agglutinin (GNA), has been the subject of many natural enemy studies. Some negative effects have been observed, although the impacts in field situations have not yet been ascertained. Experiments using prey fed artificial GNA diets have not always produced results consistent with those using GNA-expressing plants. For example, dose-dependent effects on the development of the aphid parasitoid *Aphidius ervi* were noted when parasitoids were fed *M. persicae* aphids raised on a GNA-containing diet, but not aphids raised on GNA potatoes (23). Similarly, a diet containing GNA produced small negative effects on the parasitism of *Diatraea saccharalis* caterpillars by *Cotesia flavipes*, but no adverse effects were observed when the prey fed on GNA sugarcane (90, 91). Negative effects have been recorded for the predator *P. maculiventris* on tomato moth caterpillars injected with GNA (reduced growth) or fed GNA potato (reduced fecundity) (4). GNA-potato-fed prey had a negative effect on egg viability of the lady beetle *Adalia bipunctata*, but prey fed a GNA diet increased the fertility of this predator (33). Prey that were fed GNA diets or GNA potato were less favored or resulted in smaller, shorter-lived adult parasitoids *P. pyralophagus* (99), *Aphelinus abdominalis* (22), or *Eulophus pennicornis* (5). The parasitoids *Aphidius colemani*, *Trichogramma brassicae*, and *Cotesia glomerata* had reduced longevity when fed sucrose solutions containing GNA (82).

In summary, the research cited here suggests that effects of IR GM plants on natural enemies of herbivorous insects depend on the potential for the natural enemy to be exposed to the IR protein and its inherent susceptibility to that protein. Thus, Bt plants that are insecticidal only to select groups of insect species (e.g., Lepidoptera) are unlikely to have direct effects on natural enemy species outside this group, as has been shown for lacewings and Bt maize (35). IR proteins with ranges of activity wider than those of Bt may have a greater chance of affecting natural enemies, but research with PIs and lectins so far has suggested that these effects are not major. There is no evidence that IR proteins accumulate along food chains; in fact, the available data report dilution instead (16, 22, 23, 48). This is not surprising given the high lability of proteins in biological systems relative to secondary metabolites and suggests that the degree of exposure of natural enemy species to IR proteins will not be great. The risks may, in fact, be far less than

those posed by insecticides that operate via contact or that are concentrated along food chains or by resistant cultivars developed by traditional breeding.

Insects of Cultural or Aesthetic Value

In recent years the concept of biodiversity as an indicator of ecosystem health has become widely accepted (21), and in many countries biodiversity itself is seen as having intrinsic value (62). Some insects (butterflies, bees, and endangered species) are regarded positively almost globally, but there are also regional differences in perceptions of valued species. In countries with high levels of endemism, certain insects may be regarded as symbols of the country's unique and precious native fauna. At the very least, insects may be seen as food for valued birds. For some cultures, insects may be traditional food, feature in folk history, or contribute to a culture's sense of identity. All these factors may have a bearing on the selection of nontarget invertebrate species for testing the biosafety of GM plants in the future.

To date, the monarch butterfly (*Danaus plexippus*) remains the most-studied example of a culturally valued insect. The observations that pollen from Bt corn line N4640 (presumably Event 176, but not stated) dusted onto milkweed leaves caused mortality of monarch larvae (63) and that Cry1Ab-expressing pollen of Event 176 Bt-maize pollen could drift onto milkweed plants and do likewise (55) prompted considerable public concern. A comprehensive suite of further studies (49, 57, 72, 77, 95, 111) showed that commercial crops of Bt maize in the United States at that time (mostly MON810 or Bt11, neither of which expresses Cry1Ab in their pollen) posed no significant risk to monarch populations, but that varieties with high pollen expression levels (e.g., Event 176) or different Bt toxins would need further assessment (89). Event 176 Bt-maize pollen may also have negative effects on caterpillars of *Papilio polyxenes* (108, 111), *Pieris brassicae*, *Pieris rapae*, and *Plutella xylostella* (39), but not on milkweed tiger moth larvae *Euchaetes egle* (56).

Obviously, toxicity tests are considerably more difficult to conduct with endangered species. Observations on the distribution of its host plant and the timing of larval emergence were used to conclude that Bt-maize pollen would not threaten the endangered Karner blue butterfly (*Lycaeides melissa samuelis*) in the United States (102). Field surveys of endangered insect abundance in and near large-scale GM field plots (104) may be the best approach to assessing impacts on such insects. Where native insects may have particular cultural value, studies to understand their distribution and ecology in agricultural habitats, and methods for conducting laboratory bioassays with them, may help with GM plant risk assessment.

EFFECTS OF INSECT-RESISTANT GENETICALLY MODIFIED PLANTS ON SOIL BIOTA

There is the potential for soil-dwelling organisms to be exposed to transgene-derived proteins released into the soil as exudates from the roots of living GM crop plants (9) and through contact with plant material left in the ground after

harvest. Soil-borne communities are dominated by microorganisms, which account for greater than 80% of the total biomass (excluding plant roots) (58). The microbial communities exist within complex soil food webs together with numerous and varied soil-dwelling invertebrate species (e.g., earthworms, Collembola, and nematodes). Together, these communities carry out soil ecosystem processes such as nutrient cycling and decomposition, processes that have major ecological and agricultural significance. Evaluation of the impacts of GM plants on soil organisms is therefore an essential part of environmental risk assessment of GM plants. Although many studies have examined effects of GM plants "above ground," comparatively little research has been directed toward impacts on soil ecosystems and processes.

Assessing the Impacts of Genetically Modified Plants on Soil Biota

Testing the effects of GM plants on nontarget soil organisms and processes is beset with difficulties (47). The heterogeneity of the soil environment, the complexity of the communities present in soil, and the aggregation and patterns of movement of soil fauna and flora present a significant challenge to the design of ecologically relevant test methods. From necessity, studies have often examined effects on a single soil-dwelling organism under specific environmental conditions and often over a short period. Although it is possible to select organisms representing processes, functions, and taxonomic groups for laboratory testing of effects of GM plants, there are obvious limitations to this approach (54). For example, reliable indicator species for important ecological processes such as decomposition or nitrogen mineralization have not been identified. Instead of measuring effects of GM plants on other members of the ecological community, direct measurements of ecosystem processes, such as decomposition or soil respiration, can be made. This approach focuses attention on establishing the impact, if any, of the GM crop on ecosystem function.

Persistence of Transgene-Derived Proteins in Soil

The potential impacts of GM plants on soil organisms depend, at least in part, on the persistence of the transgene-derived protein and its biological activity in soil. With the exception of Bt toxins expressed in cotton and maize, persistence of transgene-derived proteins in soil has rarely been studied. Bt endotoxin from maize enters the soil from both root exudates (84, 85) and postharvest residues (86). Bt toxins can bind to clay particles and humic acids from soils and can retain their insecticidal activity in that state (27). Estimates on the persistence of Bt toxins in soil vary. In a recent laboratory study, Bt endotoxin in decomposing Bt maize and soil mixtures was rapidly degraded, with no detectable levels after only 14 days (51). The authors suggested that much of the Bt endotoxin in crop residues is highly labile and quickly decomposes in soil, but that a small fraction may be protected from decay in relatively refractory residues. These findings contrast

with a field study in which no degradation of the Cry1Ab toxin was detected in the first month after plant litter was buried in soil (112). Inconsistencies between studies may be partly explained by differing experimental procedures (e.g., use of dried, ground plant samples versus fresh material), but they also reflect the complexity of the plant-soil ecosystem. Persistence depends on the interactions between many variables such as biotic activity, soil type, crop management practices, and environmental conditions, and therefore may vary between sites and seasons.

The persistence of proteinase inhibitor (PI) in buried GM plant litter has also been monitored immunologically (32). The concentration of PI in GM plant litter had declined to 0.05% of the initial levels after 57 days in soil and could not be detected in subsequent samples. The fate of other transgene-derived proteins in soil remains unknown, in part because of the lack of techniques available to monitor their presence and persistence. Although direct measurement of the persistence of transgene-derived proteins is essential, studies are also needed on the biological activity of transgene-derived proteins persisting in soil, such as larvicidal bioassays (27, 112).

Earthworms

Earthworms play a vital role in decomposition of plant litter, and the physical and chemical changes to soil resulting from earthworm activity can significantly affect the soil's biological properties and processes. Despite their obvious importance in the soil ecosystem, little research has been carried out on the effect of IR GM plants on earthworms. Tests on the effects of purified Bt toxin on earthworms suggest that Bt plants have little impact on earthworms. For example, there were no growth effects or mortality in *Eisenia foetida* exposed to 120 times the amount of Cry3A toxin expected in one kilogram of soil in a plot of Bt potatoes (102).

Saxena & Stotzky (86) showed that earthworms can ingest Bt toxin bound to soil. In this study, Cry1Ab was detected in the guts and casts of earthworms kept for 45 days in jars containing Bt maize biomass. No deleterious effects of Bt maize on earthworms (*Lumbricus terrestris*) were detected in these pot trials. Zwahlen et al. (113) found that adult *L. terrestris* lost 18% of their initial weight after 200 days when fed Bt maize compared with a 4% weight gain when fed non-GM maize. The authors were uncertain whether this effect was caused by the Bt toxin and suggested that it may have resulted from a difference in the nutritional quality of the plant material ingested by the earthworms. Genetic manipulation or the tissue culturing of plants may cause changes in plant characteristics, with GM leaves and non-GM leaves reported to differ in several characteristics, such as C:N ratio, lignin content, and carbohydrate content (38, 41). While adverse effects on earthworms are unlikely, further studies are needed to rule out the possibility of long-term sublethal effects of Bt toxin on earthworms. Studies on the effects of other IR proteins, which are less specific in their mode of action, are also required.

Isopods

No adverse effects of Bt maize expressing Cry1Ab protein were found on woodlice *Porcellio scaber* in a soil-free laboratory system (38). Woodlice did not differentiate between Bt maize and non-GM maize in their food preference, and numbers of offspring produced did not differ between the two treatments. Initial weight increases of offspring were significantly higher in the non-GM treatment, but adult *P. scaber* showed greater weight increase when feeding on Bt maize leaves. Differential weight gains between the treatments may have resulted from differences in the nutritional quality of the GM and non-GM maize leaves.

Microarthropods

Collembolans are recognized as key indicator species of soil fertility and health, as they play a vital role in the breakdown and recycling of crop residues, and abundant populations of these microarthropods are generally present in well-managed agricultural soils. Collembolans are often found in the root zone of plants and can be exposed to transgene-derived proteins exuded into the rhizosphere by roots. As collembolans are active in the decomposition of organic matter, they may also be exposed to transgene-derived proteins that remain in crop residues.

No adverse effects have been reported in laboratory studies carried out with selected species of Collembola, which are easily maintained in laboratory cultures. Bt cotton and Bt potato leaf residues fed to *Folsomia candida* had no effect on time to oviposition, egg production, or final body length (110). Similarly, purified Bt toxins and leaf tissue of Bt cotton, Bt corn, and Bt potatoes had no effect on *F. candida* and *Xenylla grisea* (102).

Perhaps of greater value in terms of risk assessment information are the few field studies in which density and diversity of Collembola species have been monitored. Preliminary results from a survey of Collembola populations present in field-grown crops of Bt silage corn and the isogenic parental line suggest that population density and species diversity were not significantly affected by the Bt corn (13; M. Brownbridge, personal communication). Similarly, there was no effect of maize expressing the coleopteran-active Bt toxin Cry3Bb1 on field populations of Collembola and soil mites over two seasons (1).

GM potatoes expressing cysteine PI had no effect on the abundance of collembolans during two field seasons (25). However, when tobacco leaves expressing PI I were buried in the field, there were significantly lower collembolan numbers in the surrounding soil than in soil from control plots (32). The high mobility of Collembola made it difficult to determine whether the depressed numbers in soil surrounding the GM plant litterbags were due to mortality and/or decreased reproduction or merely to increased migration. As in other studies, the two forms of litter varied in C content, which may have affected the nutritional value of the plant material.

Mite populations in GM crops have been examined infrequently. There was no effect on the abundance of mites in field soil collected from around the roots of GM potatoes expressing PIs (24). Total numbers of orbatid mite *Oppia nitens* adults

and nymphs feeding on leaves of GM Bt cotton and Bt potatoes were unaffected compared with those feeding on non-GM plants (110).

Nematodes and Protozoa

Soil nematodes are a diverse group of organisms that are found in all soils. Some forms have a high colonization potential, so they respond quickly to perturbation and are therefore potentially useful tools for assessing soil ecosystem change (8). Nematodes that feed on rhizosphere bacteria, fungi, protozoa, rotifers, or other nematodes may conceivably be affected by the presence of GM-plant litter or root exudates in the soil. Responses of nematode populations to GM plants varied between studies. Increased nematode populations were found in soil surrounding PI-GM litter compared with soil surrounding non-GM litter buried in the field (32). The nematode populations also varied in their trophic structure, with a higher ratio of fungus-feeding nematodes to bacteria-feeding nematodes. GM plant material contained a lower carbon content than did non-GM plant litter throughout the experiment, possibly resulting in differences in the microbiota developing on the substrates, which may in turn have influenced nematode populations developing on the microflora.

In laboratory experiments carried out as part of a study on the effects of GM potatoes expressing lectins (concanavalin A and GNA), the host-finding response of a bacteria-feeding nematode was significantly inhibited when either lectin was present at a range of concentrations (47). In pot trials and decomposition experiments carried out in the same study, there was no effect on nematode populations. After two field seasons, there were no differences in the abundance of free-living nematodes around the roots of potato plants modified to express cysteine PIs (cystatins), even though the target of this modification was the potato cyst nematode (24). Similarly, there were no differences in nematode populations in soil close to the roots of maize expressing the coleopteran-active Bt toxin Cry3Bb1 and of non-GM plants (1).

In a pot experiment, flagellate protozoan populations in the rhizosphere of one GM line expressing the lectin concanavalin A were significantly lower than that of control plant and GNA plant rhizosphere. In addition, significantly fewer protozoa and amoebae were found in the presence of leaves from lectin-expressing GM lines than in the control lines (47). When a single concanavalin A–producing line was grown in the field, there was a transient reduction of about 40% in soil protozoan population. As protozoa are key components of the soil food web and play an important role in soil nutrient cycling, the effects of concanavalin A on soil microbial processes should be studied further. Few other studies have considered effects on protozoa, so judging the significance of these results is difficult.

Soil and Plant-Associated Microorganisms

Soil and plant-associated microbial populations (e.g., rhizosphere bacteria important in plant growth) come into contact with transgene-derived proteins released from GM plant roots via natural wounding, senescence, and sloughing of cells.

Although some of the protein's activity may be lost by proteolysis in the rhizosphere, some may be retained owing to adsorption to clay minerals or humic components. Early studies, in which the microbial populations associated with Bt plants were estimated by viable plating, failed to detect any impact that could be attributed to the genetic modification (30, 31, 86).

Studies on the effect of GM plants on soil microorganisms have been hampered by the inherent difficulties associated with researching these populations, in particular the inability to culture or identify most microorganisms in soil. However, recent methodological advances, in particular new molecular biological techniques, are beginning to provide insight into the hitherto "black box" of soil microbial communities (58). Studies in which these techniques have been used to examine the effect of GM plants on plant-associated soil communities have been reviewed recently (14). Most of these studies have examined the effects of plants genetically modified for resistance to microbial attack (73, 105), which could be expected to pose a threat to the natural soil microflora that is more serious than that posed by plants modified for insect resistance.

Brusetti et al. (15) characterized the rhizosphere bacterial community associated with Bt maize and unmodified maize using several techniques, including viable counts, community-level catabolic profiling, and PCR-based fingerprinting, that targeted the 16S-23S intergenic transcribed spacers. The culturing techniques did not detect any differences between the Bt maize and the non-GM plants, but the molecular profiling technique found that the community structure differed between the two treatments. Experiments in which root growth solutions were added to soil indicated that exudate of the Bt plant led to the development of a bacterial community that differed from that of the non-GM plants. It was suggested by the authors of the study that the Bt maize exudate may differ from the non-GM plant in several ways, not only in the Cry protein. However, careful chemical analysis of the exudate of Bt maize and its unmodified counterpart has not been carried out.

Several other methods have been used to measure the effects of GM plants on the soil microflora. Community-level physiological profiling of soil surrounding lectin-expressing potato roots found that, although GM potato lines consistently altered the physiological profile of the rhizosphere microbial community at harvest, the effect did not persist from one season to the next over a trial period of two field seasons (47). In their study on the effects of GM nematode resistance (based on the PI cystatin) on nontarget organisms in the potato rhizosphere, Cowgill et al. (25) used phospholipid fatty acid analysis as a measure of the abundance, evenness, or metabolic activity of the soil microbial community. In the first year, two of the GM lines altered the structure of the community; one favored fungal growth relative to bacterial growth in the later part of the season, and the second line appeared to suppress fungal growth. In the second year, phospholipid fatty acid analysis suggested that microbial abundance was reduced by 23%, with suppression of both bacterial and fungal communities. It is not known how cystatins from GM plants affect microorganisms. Free cystatin is likely to be rapidly inactivated by enzyme activity or by adsorption to solid surfaces, and the observed changes in

the microbial community may have reflected changes in the composition of the root exudates of the GM plants and not inhibition of microbial proteinase activity.

Arbuscular mycorrhizal (AM) fungi establish mutualistic symbioses with roots of most plant species, playing an important role in soil fertility and plant nutrition. AM fungi are strongly affected by agricultural practices and changes in soil characteristics and thus are key nontarget microorganisms worthy of monitoring in environmental impact assessments of GM plants (14). Few studies have examined the impact of GM plants on AM fungi to date, but a model experimental system was recently developed to measure the impact of Bt maize on the life cycle of the AM fungal species *Glomus mosseae* (101). Root exudates of one line of Bt maize (Event 176) significantly reduced presymbiotic hyphal growth, compared with another line (Event-11) and control plant root exudates. Development of appressoria was also affected by Bt Event 176, with 36% of appressoria failing to produce viable infection pegs. Event 176 had a higher level of expression of the Bt toxin than did Event 11 (80.63 and <0.55 μg Cry1Ab g^{-1} protein, respectively). The development of AM fungi on Bt crops in the field has not yet been studied.

Measurement of Soil Processes

Most of the studies examining effects on soil biota have examined effects on biomass, diversity, or composition. A limited number of studies have measured effects of GM plants on soil processes; plant litter decomposition has most often been chosen as a key indicator of soil ecosystem function. Several studies have found little difference between the decomposition rates of IR GM plants and non-GM plants. Rate of decomposition of potato (25) and tobacco (32) leaves from plants modified to express a PI were similar to those of comparative non-GM plants, as measured by weight loss in litterbag studies. Similarly, Hopkins & Gregorich (51) found no detectable difference between the decomposition rates of Bt maize and non-GM maize, as determined by CO_2 production. This contrasts with the findings of Stotzky (96, 97), who reported that biomass of Bt maize decomposed less in soil than biomass of near-isogenic, non-GM maize. Stotzky (97) also reported that biomass of Bt canola, Bt cotton, Bt potato, Bt rice, and Bt tobacco decomposed less than the biomass of the respective non-GM plants.

A recent study by Dinel et al. (29) measured respiration in soil samples to which Bt maize and non-GM maize shoots had been added and in soils collected when field-grown crops were harvested. The study used the same plant varieties as those used by Hopkins & Gregorich (51), but in contrast with that study, Bt maize appeared to decompose more slowly than non-GM maize and the cumulative CO_2-C evolved from soils was significantly lower under Bt crops than under non-GM crops. However, this study contained anomalous results; the net rate of CO_2 production increased throughout the duration of the experiment, whereas this rate usually declines over time as less organic matter is available in soil. Bt maize and non-GM shoots were reported to differ in the amount and type of fatty acids they contained, as did soils from beneath field crops. The authors suggested that the

cultivation of Bt maize significantly increased the saturated-to-unsaturated lipid ratios in soils, which appeared to affect microbial activity negatively. However, further investigation is required before this conclusion can be verified.

Further detailed studies on the decomposition of IR GM plants are warranted, as results from the various studies to date are contradictory. The inclusion of appropriate control plants is important in these experiments. Changes in plant characteristics resulting from the process of genetic manipulation or tissue culturing, rather than the presence of the transgene-derived protein, may be a contributing factor in observed differences between the decomposition rates of GM and non-GM plant tissue, and studies should include chemical analysis of plant material. Most studies to date have measured decomposition of Bt plants; decomposition of GM plants expressing other IR transgene-derived proteins that may be less specific in their spectrum of activity must also be studied.

FUTURE DIRECTIONS AND RESEARCH NEEDS

Major knowledge gaps remain in the field of nontarget testing of IR GM plants. For example, it is difficult to assess the effects of any new technology on nontarget organisms of uncertain taxonomic status, with poorly understood biology and unknown population dynamics, which is often the case for little-studied, native species. Although this is a problem with nontarget species above-ground, the issue is compounded when assessing effects on soil ecosystems, as the soil fauna and microflora are diverse and by no means fully described (54). Often, species chosen for risk assessment testing are those that are easily maintained in laboratory culture, and these are not always representative of the environment in which the GM plants will be grown. Studies that are proving most useful in terms of assessing the impact of IR GM plants on soil ecosystems are typically comprehensive studies that measure impacts on several components of soil biota and/or soil processes. Studies by Griffiths et al. (47) and Cowgill et al. (25) are examples of well-conducted studies in which a systematic and logical progression of nontarget testing was undertaken.

Most studies to date have used unmodified plants as their control for comparison. The use of appropriate control plants that have been transformed and regenerated by the same process used in the construction of the GM plants but do not contain the transgene allows more precise determination of the effects of the transgene itself. Many studies have concluded that the differences observed between GM and non-GM plant lines resulted from changes unrelated to the transgene (somaclonal variation). Conner et al. (20) recommended the use of null-segregants, transgenics that have lost the transgene following outcrossing and chromosome segregation, as the most appropriate control for whether expression of a transgene causes unexpected impacts. Studies should also include an unrelated plant cultivar as an additional control for comparison, as many studies have found that even when differences between GM and unmodified plant lines were observed, these were not greater than those observed between different cultivars.

The extent of exposure of nontarget organisms to transgene-derived proteins depends on the persistence of the protein in the environment. Techniques to determine the fate of transgene-derived proteins in soil are needed. Methods such as immunological detection of proteins need to be coupled with bioassays that provide information on the ecological impact of any transgene-derived protein remaining in the environment. Similarly, studies on the impacts on nontarget invertebrates resulting from consumption of pollen from GM plants should be supported by data on the accumulation of transgene-derived protein in the pollen.

Major knowledge gaps with respect to assessment of the effects of GM plants on soil microbial communities remain; for example, little is known about the effects of "normal" variation (i.e., effects of season, cultivar, or cropping practices) on microbial communities. These baseline data are necessary because they provide the context in which the ecological significance of population shifts sometimes observed in response to GM plants must be assessed. Transient changes in populations have frequently been reported in response to GM plants, but the ecological significance of these shifts can be determined only by conducting trials at several sites and over several years. The need to sample over extended periods has been demonstrated in several studies examining the effects of GM plants on microbial communities; for example, environmental factors related to season, field site, or year caused greater differences in microbial populations than did the differences between non-GM plants and potato plants genetically modified for resistance to bacterial disease (50). The detailed study by Heuer et al. (50) provides a good example of how studies on impacts of GM plants on soil microorganisms should be conducted, with sampling carried out at several sites and over many seasons.

Methods that better access key ecological processes in soil are also required. Soil respiration studies (measuring the rate of CO_2 evolution) may give an index of the biomass, but high levels of functional redundancy within soil microbial communities may buffer significant, but highly specific, effects (103). Greater test specificity could be obtained by adding degradable substrates such as lignin or cellulose (54), which would measure the activity of specific groups of decomposer microorganisms in soil. The importance of including a measure of soil function in studies on impacts on soil biota was demonstrated by Cowgill et al. (24), who showed that, despite changes in the microbial community structure resulting from cultivation of GM plant lines, there was no impact on the key ecosystem functions of C mineralization and decomposition.

More research is needed to determine the effect of IR GM plants on whole ecosystems, rather than on individual test species. Ecosystems are complex, multidimensional environments, and the research required at the field level is expensive and multidisciplinary. However, it is necessary to measure impacts on a sufficiently large scale and over a sufficiently long period to allow for environmental variability. It must also be realized that all studies, no matter what scale, have limitations. For example, the recent farm-scale field trials in the United Kingdom examined sugar beet, rape, and maize expressing herbicide resistance genes on over 60 sites

each (94). In these studies, information was gathered on the effect of the farming practice of using herbicide-resistant crops on wildlife. However, it was not a study on the direct effects of transgene expression on wildlife and results must be interpreted accordingly. If the steady increase in global areas planted in commercial GM crops over recent seasons continues in the future, we will be better able to judge the accuracy of environmental biosafety predictions made using the current risk assessment approach.

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