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The Ecological Risks of Transgenic Plants

Transgene transfer was reported from engineered crops into plants of the natural environment. Should a "precautionary approach" be adopted? Manuela Giovannetti of the University of Pisa (Italy) stresses the potential risks of plant genetic engineering, particularly warning us against the so-called "Terminator" technology.

Key words. Gene flow, non-target organisms, beneficial microbes, *Bt* toxin, Terminator technology

Abstract. *Biotechnologies have been utilized "ante litteram" for thousands of years to produce food and drink and genetic engineering techniques have been widely applied to produce many compounds for human use, from insulin to other medicines. The debate on genetically modified (GM) organisms broke out all over the world only when GM crops were released into the field. Plant ecologists, microbiologists and population geneticists carried out experiments aimed at evaluating the environmental impact of GM crops.*

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The most significant findings concern: the spread of transgenes through GM pollen diffusion and its environmental impact after hybridisation with closely related wild species or subspecies; horizontal gene transfer from transgenic plants to soil microbes; the impact of insecticide proteins released into the soil by transformed plants on non-target microbial soil communities. Recent developments in genetic engineering produced a technology, dubbed "Terminator", which protects patented genes introduced in transgenic plants by killing the seeds in the second generation. This genetic construct, which interferes so heavily with fundamental life processes, is considered dangerous and should be ex-ante *evaluated taking into account the data on "unexpected events", as here discussed, instead of relying on the "safe until proven otherwise" claim. Awareness that scientists, biotechnologists and genetic engineers cannot answer the fundamental question "how likely is that transgenes will be transferred from cultivated plants into the natural environment?" should foster long-term studies on the ecological risks and benefits of transgenic crops.*

1. INTRODUCTION

The release into the environment of genetically modified organisms has been at the centre of ideological, emotional and political campaigns, which hindered serious discussions on the actual scientific problems involved. The first consequence of the extreme simplification of the debate has been the confusing use of words such as biotechnology, genetic engineering, transgenic organisms, clones.

Actually, biotechnologies have been utilized unawares "ante litteram" for thousands of years to produce food and drink. Imagine a world without biotechnologies and biotechnological products: there would be no wine, no beer, no bread, no cheese, no pizza, but also no antibiotics and many other medicines.

The word "biotechnology" was defined in 1981 by the European Federation of Biotechnology as the integrated utilization of microbiology, biochemistry and engineering for the industrial applications of potential abilities of microorganisms and tissue cultured cells. It was after 1970 that the word "biotechnology" assumed a wider meaning, encompassing also technologies based on genetic engineering, which allow us to add new genes to many

different organisms. Since the genetic code is universal, genes originating from microorganisms or from animals may also work well in plants and viceversa, and the organisms which are thus transformed are called genetically modified organisms, GMO.

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The application of genetic engineering techniques produced many useful compounds for human use; the first example is represented by human insulin, obtained in 1982 after genetic modification of the microbe *Escherichia coli*. The indoor production of many different drugs by pharmaceutical manufacturers did not raise problems; the debate broke out all over the world only when GM plants, such as soybean, corn, cotton, canola, resistant to herbicides and parasites were obtained and cultivated (see Giovannetti [2001]).

After GM crops were released in the field, some scientific journals looked at the potential risks associated with their cultivation and many scientists, in particular plant ecologists, microbiologists and population geneticists, carried out experiments aimed at answering questions such as:

- what is the environmental impact of GM crops?
- is there any possibility of gene flow from GM crops to nearby growing plants?
- are there risks that herbicide-tolerant genes released in the field flow to weeds, and may thus originate "superweeds"?
- do herbicide-resistant transgenic plants contain higher quantities of herbicides?
- how likely is it that transgenes such as antibiotic resistant genes will move into natural microbial populations?
- what is the impact of toxins produced by pathogen-resistant transgenic crops on non-target organisms, such as beneficial insects and microbes?
- and if some of these events do occur, is there any cause for concern?

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Many studies, carried out in diverse countries, reported "unexpected events" related to the cultivation of transgenic plants, *i. e.* the spread of transgenes through plant hybridization with related wild species (Gray and Raybound [1998]; Wolfenbarger and Phifer [2000]), the release of *Bt* insecticide toxin by *Bt* corn roots into the soil environment (Saxena *et al.* [1999]), the transfer of engineered genes from transgenic plants to soil bacteria (Gebhard and Smalla [1998]). The scientific journal *Nature* published a briefing on the subject, advertised on the cover page by the relevant question "GM crops – how safe is 'safe'?" (Butler and Reichardt [1999]), while *Science* published a review on the ecological risks and benefits of GMOs (Wolfenbarger and Phifer [2000]), stressing the need of further experimental work, aimed at evaluating the ecological impact of GM crops (Rissler and Mellon [1996]).

Awareness that little is known of the fate of transgenes after their field release, except that in nature "everything goes everywhere" and genes can flow from one organism to another (Heinemann and Sprague [1989]; Hooykaas [1989]; Doolittle *et al.* [1990]; Courvalin [1995]; Ellstrand *et al.* [1999]; Intrieri and Buiatti [2001]), invites to avoid generalisation and make judgments according to case-by-case studies. In particular, the experimental data collected up to now provide the basis for *ex-ante* evaluations of the risks associated with the cultivation of potentially dangerous transgenic plants, such as those containing genes interfering with fertility and life. In that case, any risk of irreversible genetic events should be evaluated and, in the absence of reliable scientific data, the environmental release of the relevant genes should be excluded (Giovannetti [2003b]).

With the aim of providing examples of *ex-ante* evaluations, here I will discuss available data concerning (a) gene flow through GM

pollen diffusion and its environmental impact after hybridisation with closely related wild species or subspecies (b) horizontal gene transfer from GM plants to microorganisms and among microorganisms, (c) the impact of toxins produced by transgenic crops on non-target organisms.

2. GENE FLOW THROUGH TRANSGENIC POLLEN DIFFUSION

The spread of transgenes through pollen diffusion and hybridisation with closely related wild species or subspecies were demonstrated after the cultivation of oilseed rape (*Brassica napus*) genetically modified for herbicide-tolerance. The work showed both intraspecific and interspecific gene flow with *Brassica rapa*, *Brassica campestris* (=*rapa*) and wild mustard (Wolfenbarger and Phifer [2000]). Further large-scale studies, carried out in the field in Australia, confirmed that pollen of herbicide-resistant GM *Brassica napus* could hybridise with non transgenic crops growing 3 Km apart (Rieger *et al.* [2002]).

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Since a large number of different plant species, ranging from corn and soybean to oilseed rape, sugar beet, cotton, lettuce, tomato, were genetically modified for tolerance to different herbicides, the cultivation of these crops, with the inevitable and unpredictable diffusion of their pollen, could lead to genetic pollution of natural gene pools, the creation of "superweeds" and even of double- or triple-resistant hybrid varieties (Gray and Raybould [1998]; MacArthur [2000]). The most important case of crop-to-crop gene flow is represented by the report of triple herbicide resistance in canola, an event occurred in Alberta, Canada, where volunteer canola plants were resistant to the herbicides Roundup (Monsanto, St. Louis), Liberty (Aventis, Crop Science, Research Triangle Park, NC), and Pursuit (BASF, Research Triangle Park, NC) (MacArthur [2000]).

Other experiments were performed on *Zea mays* (corn) genetically modified to express the *CryIAb* gene from *Bacillus thuringensis* (*Bt*) and producing an insecticide endotoxin able to kill Lepidoptera (butterflies and moths) and Coleoptera (beetles) and in particular to control *Ostrinia nubilalis* (European corn borer), a major pest in Europe and North America. These works reported the ability of *Brassica napus Bt* pollen to hybridize with *Brassica rapa* volunteer plants growing nearby (Halfill *et al.* [2002]).

These data show that it is very difficult to hinder transgene flow through pollen and cross-pollination. Since many important food crops, such as wheat, rice, corn, barley and soybean, are able to hybridise with wild relatives (Gray and Raybould [1998]; Ellstrand *et al.* [1999]), the cultivation of GM crops in segregated areas, surrounded by buffer crops is no solution to the fundamental problem of 'gene escape'. Therefore, when the genetic modification represents a threat, the adoption of a precautionary approach should be adopted and no "segregated cultivation" should be accepted (Giovannetti [1999]; [2003b]).

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3. HORIZONTAL GENE TRANSFER

Little is known about the fate of transgenes after their field release. Recent investigations, carried out by soil microbiologists, showed horizontal gene transfer from GM plants to a bacterium, belonging to the genus *Acinetobacter* and growing in the soil around roots (Gebhard and Smalla [1998]). The authors introduced, together with the engineered gene of interest, a gene conferring antibiotic resistance, which allows the detection of transformed cells incorporating the transgenes. In the experiments, performed under optimized laboratory conditions, 2 μg of transgenic sugar beet DNA yielded bacterial transformants with a transformation fre-

quency of 5.4×10^{-9}. Also plant homogenate of transgenic sugar beet leaves showed transformation capacity, though at lower frequencies, 1.5×10^{-10}. Although such transformation events did not occur under natural conditions, the experimental results suggest that gene transfer from GM crops to competent native soil bacteria carrying homologous sequences is possible. Such data are in contrast with claims that the novel genes transferred to plants could not be incorporated back by bacteria.

When considering the wide use of antibiotic resistance genes as marker genes in GM plants and the resulting large quantities of these genes released in the environment with transgenic crops, we can easily understand the concerns that antibiotic resistance genes may be taken up by indigenous soil bacteria and disseminated into the environment by horizontal transfer, which represents the natural way by which bacteria exchange genetic material (WHO [1993]). When dealing with bacteria, the meaning of "improbable event" must take into account the fact that bacteria, given enough food and space, reproduce very quickly and may reach the number of one billion of billions in about 30 hours and that about one billion of bacteria live in one gram of fertile soil. Thus, even events occurring at very low frequency cannot be considered "improbable" and, in the case of dangerous genes, the low probability of an event has low relevance in the microbial world.

4. IMPACT ON NON-TARGET ORGANISMS

Many studies have been performed on transgenic plants modified to produce insecticide proteins, such as *Bacillus thuringensis* (*Bt*) toxins. A very much discussed paper reported that pollen from corn engineered to produce *Bt* toxin was harmful not only to the target organism, the European corn borer, but also to the non-target monarch butterfly (Losey *et al.* [1999]). Although the data presented in this laboratory based work have been questioned, they fostered further investigations on the effects of GM crops on non-target organisms, such as those obtained on black swallowtail caterpillars (Zangerl *et al.* [2001]).

A very important research reported a peculiar unexpected event:

Bt corn roots were able to release the *Bt* insecticide toxin into the rhizospheric soil, where the toxin was active for at least 234 days (Saxena *et al.* [1999]). Further studies showed that the hybrids of GM corn *Bt*11 and *Bt*176, obtained from different transformation events, were able to release the anti-lepidopteran protein (Saxena *et al.* [2002]), and that the exudates remained active for 180 days or more in the soil, linked to humic acids and clays in plant residues incorporated into soil after crop harvest (Tapp and Stotzky [1998]). The accumulation and persistence of the toxin on surface-active soil particles suggested possible long-term effects on non-target organisms and on the enrichment of toxin-resistant target insects.

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These findings stress the need of further investigations into biogeochemical cycles occurring in the soil environment, where the amount of *Bt* toxin may reach high levels due not only to its release in root exudates during corn growth but also to the incorporation of crop residues after harvest.

The release of insecticide toxins into the soil is an important trait of transformed plants to be taken into account since exudates containing antimicrobial proteins may affect non-target microbial soil communities (Siciliano and Germida [1999]). The effects of transformed *Bt* plants and their exudates on beneficial soil organisms have been investigated in a preliminary study which demonstrated that the *CryIAb* toxin released by *Bt* plants had no apparent toxicity to earthworms, nematodes, protozoa and saprophytic soil fungi, but specific analyses on beneficial fungi were not performed (Saxena and Stotzky [2001]).

We studied the effects of transgenic crops on arbuscular mycorrhizal (AM) fungi, an important group of non-target beneficial microorganisms, fundamental for soil fertility and plant nutrition, which establish mutualistic symbioses with the roots of most plant species. AM fungi are known to be strongly affected by agricultural practices, including treatments with chemical fertilizers and

pesticides, and by changes in soil characteristics, thus representing potential key non-target microorganisms to be monitored in studies on the environmental impact of GM plants (Giovannetti and Avio [2002]). By using an experimental model system the potential effects on AM fungi of two *Bt* corn were investigated. The results showed that root exudates of *Bt* 176 corn plants significantly reduced pre-symbiotic hyphal growth with respect to *Bt* 11, while did not interfere with fungal recognition of the host plant (Turrini et al. [2003a]).

Since transformed plants expressing antimicrobial compounds may release such toxins into the soil, they represent a useful tool for studying their potential impact on non-target organisms. In our laboratory we obtained transformed aubergine plants expressing the antimicrobial protein Dm-AMP1 defensin from *Dahlia merckii* plants and showing resistance against the phytopathogenic fungus *Botrytis cinerea* (Broekaert *et al.* [1995]). Such transgenic aubergines, expressing the defensin in all plant tissues, were also able to release the antimicrobial Dm-AMP1 protein in root exudates, which reduced the growth of the phytopathogenic fungus *Verticillium albo-atrum*. The root exudates containing the antimicrobial protein defensin did not interfere with recognition events and symbiosis establishment of AM fungi, considered as non-target organisms (Turrini *et al.* [2003b]).

The differential behaviour of beneficial symbiotic AM fungi in the presence of different GM plants, such as *Bt* corn and Dm-AMP1 aubergines, confirms the need of case-by-case studies, involving different test organisms and experimental approaches, when evaluating environmental risks of GM plants.

5. STERILE SEED TECHNOLOGIES

Recent developments in genetic engineering technologies produced a system, dubbed "Terminator", which allows the protection of patented genes introduced in transgenic plants. This is clearly stated in the title of the patent, US Patent Number 5.723765 – Control of Plant Gene Expression – obtained in March 1998 by the seed company Delta and Pine Land, together with the United

State Department of Agriculture.

Plants modified through this technology contain the genes which will cause the death of second generation seeds. Thus, any new patented gene introduced in a crop can be protected against further utilisation by introducing "Terminator", a group of genes which will kill the seeds in the second generation: in this way farmers could not save and replant seeds, and should buy new seeds every year. This would have a tremendous impact on the survival of people living in the poorest countries, since FAO estimates that about one billion poor people survives by planting second generation seeds (Giovannetti [2003a]).

Whereas some crops such as corn are not grown from saved seeds, but are planted as hybrid seeds which are bought every year from big seed companies, other important crops, such as rice, wheat, soybeans and cotton, are not always grown from hybrid seeds and farmers, especially poor farmers in developing countries, save and replant the seeds of their crops. This will not be possible any longer if "Terminator" technology will be utilised for engineering crop plants to kill their second generation seeds (Crouch [1998]).

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Here the elegant explanation published by Martha Crouch in 1998 will be followed to describe how Terminator works. She took the example of cotton seeds engineered for herbicide tolerance. Such seeds will develop normally until maturity, when a peculiar toxin will be produced, only in the seeds, which will kill second generation seeds.

Terminator genetic construct is composed of:

1. A promoter activated late in seed development, called LEA (Late Embryogenesis Abundant), fused to the coding sequence for a toxin. In this way the gene will produce the toxic protein only in mature seeds, but it will not kill any other part of the plant. The proposed toxin is a Ribosome Inhibitor Protein

(RIP), originated from *Saponaria officinalis*, which inhibit protein synthesis.

This will allow Terminator plants to grow until maturity and to produce seeds, but since the seeds will not be viable, seed companies would not be able to sell their products. Thus, the toxin coding sequence should be activated only after the production of viable seeds to be sold to farmers, and exactly during the development of second generation seeds. The genetic engineering allowing such event is represented by:

2. A DNA piece inserted in between the LEA promoter and the toxin coding sequence, blocking the production of the toxin. The DNA blocking piece carries at both ends two DNA sequences recognised by a recombinase enzyme, which is capable of cutting and removing the DNA at the outside of the DNA piece, so that the cut ends of the DNA fuse and the LEA promoter is next to the toxin coding sequence. This ingenious genetic construct will obtain the same result as before, *i.e.* the production of the toxin at the end of seed development, and the problem of large production of seeds will remain unsolved. To multiply plants and obtain large seed quantities, another clever piece of genetic engineering has been devised:
3. The recombinase coding sequence is put next to a constitutive promoter, which is repressed, but which can be de-repressed by a chemical compound. Such chemical may be added to the seeds before selling them to the farmers, just before sowing. The antibiotic tetracycline is proposed as the compound controlling the repressible promoter system of recombinase. Thus, without tetracycline, the recombinase gene will be repressed and the toxin will not be produced.

   When the seeds are treated with tetracycline, the cascade of genetic activation of the different constructs placed in the plant genome may be described as follows:

   Tetracycline will interact with the repressor protein which will not interfere with the recombinase constitutive promoter. Recombinase will be produced, which will cut out the DNA piece blocking the toxin gene. Toxin will be produced at seed maturity. In this way, seed companies can treat seeds just before selling them, activating the production of recombinase in

growing plants, the production of the toxin killing second generation seeds, and the protection of any patented gene introduced in the crop.

6. RISKS ASSOCIATED WITH TERMINATOR SEEDS

One major risk associated with the environmental release of Terminator genes is represented by 'gene escape' through transgenic pollen and cross-pollination, which, as already discussed above, cannot be avoided. The potential flow of Terminator genes into and between food crops, such as wheat, rice, corn, barley, sorghum, sugar beet, raises serious concerns, since many experimental and descriptive studies (Ellstrand *et al.* [1999]) "provided ample evidence that spontaneous hybridization with wild relatives appears to be a general feature of most of the world's important crops" (Ellstrand [2001]). Terminator pollen could provoke the death of second generation seeds, but this would become evident too late, after the farmer has planted the saved seeds. This means that Terminator genes would not spread any further, but it means also that an unpredictable number of saved seeds would be dead, representing a threat to the crops cultivated nearby Terminator crop plants.

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It has been widely accepted that gene escape through pollen cannot be hindered, and agrochemical and biotech companies claimed that Terminator technology could prevent the spread of transgenes in the environment. This would not be the case. In fact, the efficiency of tetracyclin treatments on every single seed out of millions of kilograms cannot be guaranteed, and recombinase may remain inactive in some seeds. Such seeds would carry all the Terminator genetic construct, the plants would grow and produce second generation seeds which will not die, but will develop into viable plants producing pollen, and then seeds, carrying both Ter-

minator genes and the patented transgenes to be protected. In this way the genetic construct devised to kill second generation seeds would enter the complex chain of interactions characterising real life: how could we prevent birds and bees from spreading Terminator pollen and seeds, and soil bacteria from undergoing transformation and further horizontal gene transfer?

7. UNEXPECTED EVENTS: SHOULD WE WORRY?

In addition to all the data discussed above on "unexpected events", we must also consider that transformation techniques, based on random insertion of new constructs into genomes, make it impossible to predict the events occurring after the introduction of new genes into an organism, such as the location of transgenes in chromosomes, possible rearrangements of genes, potential switching on of silent genes, unplanned increase or decrease in the content of secondary metabolism compounds and alterations in crop chemistry (Firn and Jones [1999]; Tweedie and Bird [2000]; Buiatti [2003]).

> The "precautionary approach" takes into account the risks and weighs benefits against costs, also considering the irreversibility of potential harmful events.

Awareness that scientists, biotechnologists and genetic engineers cannot answer the fundamental question "how likely is that transgenes will be transferred from cultivated plants into the natural environment?" should foster long-term studies aimed at evaluating the environmental impact of transgenic plants. Moreover, a "precautionary approach", which takes into account the risks and the irreversibility of potential harmful events, and weighs benefits against costs, should be adopted (Foster *et al.* [2000]). This in contrast with the "safe until proven otherwise" claim which supports the view that absence of data on harm equals absence of harm and safety of products and processes: instead, it means simply that experiments still have to be performed (Giovannetti [2002]).

The growing number of experimental findings showing previ-

ously unpredicted outcomes and unforeseen data could represent the basis for future *ex-ante* evaluations of potentially dangerous transgenes such as those interfering with fundamental life processes.

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IMPATTO AMBIENTALE DELLE PIANTE TRANSGENICHE

Riassunto

Le biotecnologie sono state utilizzate *ante litteram* per migliaia di anni per produrre cibi e bevande ed anche le tecniche di ingegneria genetica sono applicate da circa vent'anni dall'industria farmaceutica per ottenere medicine fondamentali per la salute umana. Il dibattito sugli organismi geneticamente modificati (GM) si è scatenato in tutto il mondo solo dopo che le piante GM sono state rilasciate in campo. Ecologi, microbiologi e genetisti hanno condotto esperimenti per valutare l'impatto ambientale delle colture GM. I risultati più significativi riguardano: la diffusione dei transgeni attraverso il polline GM ed il suo impatto ambientale dopo la ibridizzazione con piante spontanee della stessa specie o di specie affini; il trasferimento genetico orizzontale dalle piante transgeniche ai microrganismi del suolo, considerati non-target; l'impatto delle proteine insetticide rilasciate dalle radici delle piante trasformate

sulle comunità microbiche del suolo. Recenti sviluppi dell'ingegneria genetica hanno prodotto una tecnologia, denominata "Terminator", capace di proteggere i geni brevettati introdotti nelle piante GM attraverso l'uccisione dei semi di seconda generazione. Questo costrutto genico, che interferisce pesantemente con i processi fondamentali della vita, è considerato pericoloso e dovrebbe essere valutato *ex-ante*, tenendo presenti tutti i dati sugli "eventi inaspettati", invece di basarsi su dichiarazioni del tipo "innocuo fino a prova contraria". La consapevolezza che gli scienziati non sono in grado di rispondere alla domanda "quale è la probabilità che i transgeni siano trasferiti dalle piante coltivate all'ambiente naturale?" dovrebbe stimolare studi di lungo termine sui rischi ecologici e sui benefici delle colture GM.