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# Plant biotechnology: ecological case studies on herbicide resistance

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**The emerging field of molecular ecology aims to improve the ecological predictability of transgenic crop plants. The most widely cultivated lines are Roundup-Ready® plants, which are genetically modified to be resistant to the broad-spectrum herbicide glyphosate. Recent publications demonstrate two ecological effects that were not anticipated: the widespread emergence of glyphosate-resistant weed biotypes and the formation of a metabolic herbicidal residue. Both effects appear to be due to the increased use of glyphosate rather than the genetic modification in the transgenic crop plant. With one prominent exception, opinions collected from the literature point towards a certain degree of resistance mismanagement and an inadequate testing of the ecological effects of extensive glyphosate use.**

## Moving from potential to real ecological effects

The recent 10th Anniversary issue of *Trends in Plant Science* (December 2005) was devoted to plant biotechnology milestones and featured examples of 2nd generation transgenic crop plants containing enhanced levels of provitamin A, vitamin E, pharmaceuticals, essential mineral nutrients or terpenoids. An Editorial article by Marc Van Montagu [1] highlighted the need to tackle the topic of molecular ecology to secure a sustainable and environmentally friendly type of agriculture. In the August 2004 issue of *Trends in Plant Science*, I reviewed the sub-discipline of molecular ecotoxicology to assess the environmental selection pressures potentially associated with 1st generation transgenic crop plants containing bacterial genes for resistance towards antibiotics, insects or herbicides [2]. The main potential effects were the emergence of additional antibiotic-resistant bacteria, toxin-resistant insects or herbicide-resistant weeds. This Opinion article will introduce recent articles that have succeeded in moving from speculation about the potential and hypothetical effects to reporting real ecological effects demonstrated under field conditions: the occurrence of herbicide-resistant weeds is widespread in the USA and an unforeseen herbicidal residue has been found in transgenic soybeans. Both effects appear to be due to the huge amounts of glyphosate released into the environment rather than the genetic modification itself. The lesson for molecular ecology is to take indirect ecological effects into consideration and to generate realistic predictions.

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## New herbicide-resistant weed biotypes

In 1996, the Monsanto Company (http://www.monsanto.com) released Round-up Ready®-soybeans commercially in the USA. This was followed by commercial introductions of Roundup-Ready®-maize, Roundup-Ready®-cotton, Roundup-Ready® canola and Roundup-Ready® alfalfa. Information on these products can be found in the '2006 Technology Use Guide' at the manufacturer's website (http://www.monsanto.com/monsanto/us_ag/layout/stewardship/tug/default.asp). In 2004, the most widely adopted transgenic crop lines were grown under single or repeated applications of glyphosate herbicide on ~80% of the US soybean crop area, ~60% of the US cotton crop area and ~18% of the US maize crop area [3]. After earlier observations of weed biotypes with natural glyphosate resistance [4], Stephen C. Weller and associates at Purdue University (West Lafayette, IN, USA) postulated the herbicide-induced evolution of glyphosate-resistant weed biotypes on the basis of studies on naturally resistant field bindweed (*Convolvulus arvensis*) [5]. Some general definitions and principles of herbicide resistance in weeds are summarized in Box 1. A single weed population was found to contain five biotypes whose response to glyphosate ranged from sensitive to four times more tolerant than the sensitive biotype. These biotypes had developed in the absence of glyphosate while remaining sensitive to two other herbicides. Multiple biochemical resistance mechanisms and underlying genes were demonstrated [5]; by contrast, laboratory studies had produced glyphosate-resistant plants mainly by overproduction or mutation of the target enzyme 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase [6,7]. It was concluded that the natural glyphosate resistance of *C. arvensis* could be enhanced by selection pressure in the form of repeated glyphosate applications [5]. Monsanto scientists subsequently acknowledged the demonstration of naturally resistant biotypes but quoted the safe use history of glyphosate as evidence that resistant weeds were unlikely to evolve [7]. Glyphosate had been classified as low-risk with regard to resistant weeds [8]. Field tests performed by the Monsanto Company at ~300 locations had '…not revealed any potential to increase

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## Glossary

**Allele:** any of the alternative forms of a given gene.
**Gene flow:** the migration of genes (including all alternative forms of the genes) into the gene pool of one population from another, sexually compatible population (also termed 'vertical' gene transfer).
**Gene pool:** all the genes (including all alternative forms of the genes) of a given population.
**Horizontal gene transfer:** non-sexual transfer of genetic material from one organism into the genome of another organism.
**Mutation:** the spontaneous or induced heritable change of a gene or chromosome. Radiation or certain chemicals can act as mutational inducers.
**Pleiotropic effect:** multiple changes in the phenotype of an individual caused by a single genetic change.

the weediness of any other cultivated plant or native wild species' [9]. Thorough field testing of the Roundup Ready® technology was particularly important to clarify the potential risk of evolved weed resistance to glyphosate. However, natural weed resistance to glyphosate has long been known [4,5,7] and can be a basis for evolved resistance (Box 1). The US National Research Council has questioned the quality of industrial risk assessment studies of transgenic plants, particularly with regard to the topics of weediness and weed management [10].

Weed biotypes with evolved glyphosate resistance were first reported in ~1997 (summarized in Table 1). The first case, a rigid ryegrass (*Lolium rigidum*) biotype found in 1996 in Australia, was discussed in a review published in 1997 [11]. It concluded that '…glyphosate resistant crops will need to be used in rotation with conventional cultivars, and in conjunction with non-chemical weed control and other herbicides if the selection of glyphosate resistant weeds is to be avoided…' [11]. Reduced movement of glyphosate to its site of action in the plastid was proposed as a component of resistance in *L. rigidum* [12]. Inheritance of glyphosate resistance in rigid ryegrass from California (USA) appeared to be nuclear, incompletely dominant, multigenic and pollen-transmitted with no indication of maternal inheritance [13]. After 3 years of glyphosate use in a Malaysian orchard, glyphosate resistance in goosegrass (*Eleusine indica*) biotypes had increased 8–12-fold [14]. Monsanto scientists acknowledged the existence of these resistant biotypes, and were able to identify a simple amino acid exchange in EPSP-synthase as an underlying component of evolved resistance [15]. Glyphosate resistance in goosegrass is inherited as a single, nuclear and incompletely dominant gene [16].

Horseweed (*Conyza canadensis*) (Figure 1), which is on a list of the ten most economically important weed species [17], is the weed with the most widespread glyphosate-resistant biotypes in the USA. In 2000, it was predicted that *Conyza* spp. and some other weed species would be poorly controlled by glyphosate [18]. A horseweed biotype with 8- to 13-fold enhanced glyphosate tolerance was first isolated at the University of Delaware (Lewes, DE, USA) after three years of growing Roundup-Ready®-soybeans [19]. Subsequently, resistant horseweed biotypes have been found at hundreds of locations in numerous US states [3,20–24]. It is estimated that ~200 000 hectares in western Tennessee [20] and 4000–40 000 hectares in Delaware are affected [24], and that soon millions of acres will be affected in the USA [25] (1 hectare = 2.47 acres). A single horseweed plant can produce <200 000 seeds a year.

With regard to resistance mechanisms, changes to the EPSP-synthase target enzyme appear to be less frequent than changes in glyphosate translocation processes [3,22,23]. Multiple biochemical factors appear to contribute to resistance [3,22,23] and an incompletely dominant single locus nuclear gene has been linked to glyphosate resistance [23,26]. Biochemical studies point to reduced translocation of glyphosate as a major reason for resistance [27–29]. The recently described field of weed genomics [17] has the potential to elucidate the precise mechanisms of weed biotype adaptation. The first genomic results on horseweed are already available [17,23,26]. At present, the observations on horseweed seem to be consistent with the scenario for resistance development postulated some 20 years ago [5]. The herbicide resistance of weeds is usually accompanied by a growth penalty [4]. This was also observed for glyphosate-resistant biotypes of morning glory (*Ipomoea purpurea*) isolated from a field site in Georgia (USA) [30]. However, under herbicidal selection pressure, the glyphosate-resistant biotypes had a relative growth benefit that was determined and incorporated into a case study of evolutionary genetics [30].

### Box 1. Evolution of herbicide-resistant weed biotypes

#### Within a population

Weed populations are as a rule genetically heterogeneous. Even when there is overall herbicide sensitivity, there might be a few individuals with minor alleles or with mutated genes that confer natural herbicide resistance. Under herbicidal selection pressure, the naturally resistant sub-population can come to dominate the population and, over time, its soil-bound seed bank. Pre-existing natural resistance can thereby lead to apparent evolved resistance. Often, herbicidal selection pressure favors new mutational or gene transfer events that increase the level of resistance. The literature usually does not differentiate between pre-existing and newly acquired traits leading to evolved resistance.

#### By gene transfer between populations or species

Herbicide-sensitive members of a weed population can acquire herbicide resistance by cross pollination and gene flow from a herbicide-resistant (e.g. transgenic) population of the same or a closely related species. Less established mechanisms of gene transfer involve horizontal gene transfer from microorganisms (e.g. *Agrobacterium*) or viruses into plant genomes. Herbicidal selection pressure can also help the genetically modified individuals in the weed population to become dominant and, over time, predominate in the soil-bound seed bank.

#### Single-step versus multi-step mechanisms

In principle, resistance can arise at each step in the action sequence of herbicides (i.e. uptake, translocation, binding to the target site, transduction of the primary effect, or detoxification and disposition). In the cases of atrazine and glyphosate, single target site mutations have led to resistance. Generally, a single mutation can lead to multiple biochemical changes through pleiotropic effects. Multiple mutational or allelic differences that each give only partial resistance can also lead to resistant biotypes.

For further details see Refs [17,42,43].

**Table 1. Discovery of glyphosate-resistant weed biotypes[a]**

| Year of first report | Weed species | Occurrence | Refs |
|---|---|---|---|
| 1984 | Field bindweed (*Convolvulus arvensis*) | Indiana, USA | [5] |
| 1998 | Rigid ryegrass (*Lolium rigidum*) | Australia | [23,44] |
| 2000 | Goosegrass (*Eleusine indica*) | Malaysia | [14,23] |
| 2000 | Common waterhemp (*Amaranthus tuberculatus*) | Iowa and other US states | [23] |
| 2001 | Velvetleaf (*Abutilon theophrasti*) | Widespread occurrence throughout the USA | [23] |
| 2001 | Horseweed (*Conyza canadensis*) | Delaware, Mississippi, Tennessee and many other US states | [3,19,25] |
| 2002 | Chinese foldwing (*Dicliptera chinensis*) | Invasive weed in East Asia | [23] |
| 2003 | Italian ryegrass (*Lolium multiflorum*) | Chile | [23] |
| 2004 | Asiatic dayflower (*Commelina communis*) | Mid-west and other US states | [23] |
| 2004 | Morning glory (*Ipomoea* spp.) | Georgia and other US states | [23,30] |
| 2004 | Common lambsquarter (*Chenopodium album*) | Virginia and other US states | [23] |

[a]Weeds are termed resistant even when they are affected by glyphosate as long as they survive and set seed. Because of space limitations only selected examples are listed and lead references are given. The field bindweed study is a case of natural resistance because no glyphosate had been applied in the field. The other listed biotypes were isolated after field application of glyphosate but it is generally not clear if the evolved resistance was because of pre-existing or newly acquired traits (see Box 1).

Much of the initial risk discussion on herbicide-resistant transgenic plants had focused on the pollen-mediated transfer of resistance transgenes. The significance of this mechanism has been recognized for Roundup-Ready®-canola and glyphosate-resistant rice and taxonomically related weeds [3,23], for Roundup-Ready®-maize and non-transgenic maize [3,23], and for glyphosate-resistant creeping bentgrass and related non-transgenic bentgrasses [31]. Glyphosate-resistant rice and creeping bentgrass have not been commercialized so far. With the possible exception of Roundup-Ready®- and other herbicide-resistant canola lines [32,33], there are apparently no field data documenting widespread pollen-mediated transfer of resistance transgenes [3,23]. This might be because of effective resistance management, or because of the lack of appropriate monitoring systems [10].

## Previous observations of herbicide-resistant weed biotypes

The phenomenon of herbicide-resistant weeds on a worldwide scale started to be documented in the early 1970s for atrazine, paraquat, and a wide range of other herbicides [4]. A 1997 review listed 183 resistant weed biotypes in 42 countries [11], as well as the first observation of evolved glyphosate resistance (see above). When herbicides were classified according to their risk for weed resistance, glyphosate appeared in the group of low-risk herbicides, whereas herbicides belonging to the sulfonylureas and imidazolinones were classified as high-risk [8]. Resistance to imidazolinone herbicides has been introduced into Clearfield® crop plants by conventional breeding and special resistance management plans have been implemented [34].

## A new herbicidal residue

For many years it was believed that glyphosate is not metabolized by plants [6,35]. A comparison of several plant cell cultures [36] then revealed that soybean cells were unique in converting ~50% of the applied glyphosate to the metabolite aminomethylphosphonic acid (AMPA) according to the following reaction: $H_2O_3P\text{-}CH_2\text{-}NH\text{-}CH_2\text{-}COOH$ (glyphosate) → $H_2O_3P\text{-}CH_2\text{-}NH_2$ (AMPA).

However, in a subsequent monograph [37], Monsanto scientists maintained that the metabolism of glyphosate is slow or nonexistent in most whole plants and that degradation to AMPA was only reported for rootless plants and plant cell cultures. No information on herbicidal residues in Roundup-Ready® crop plants was given in the monograph [37] or in the published safety assessment of Roundup-Ready® soybean [9]. The US-Environmental

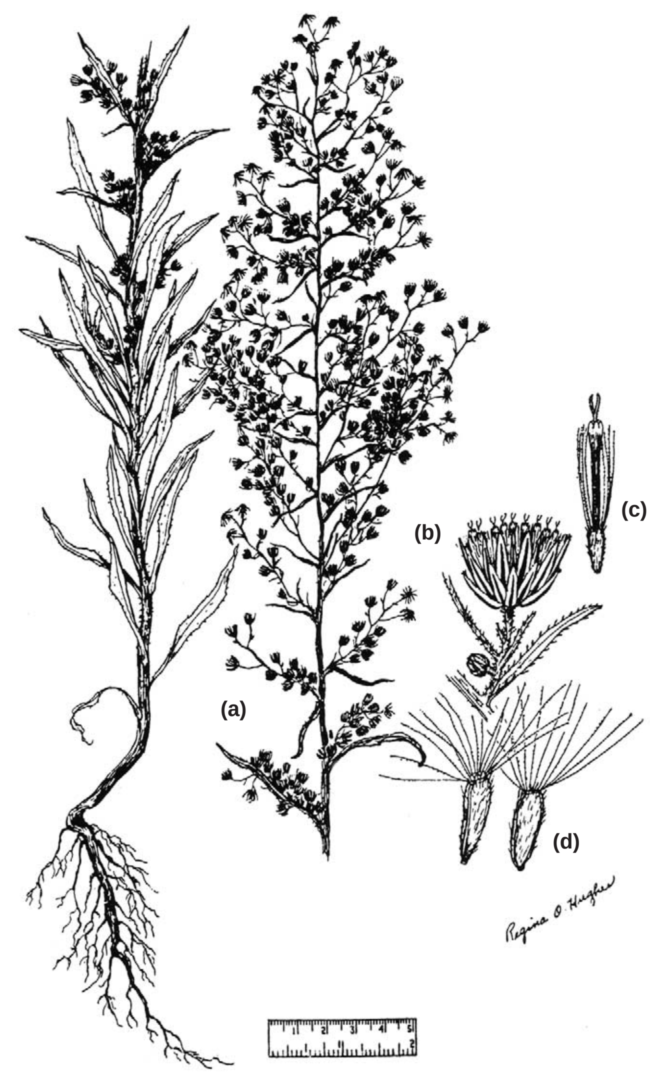


**Figure 1.** Horseweed (*Conyza canadensis*). **(a)** Overall habit. **(b)** Flower head. **(c)** Disk flower. **(d)** Achenes. Reproduced, with permission, from Ref. [45].

Protection Agency set a residue tolerance of 5 mg/kg for glyphosate in soybean seeds without setting a value for AMPA [38]. Recent studies describe high levels of AMPA in Roundup-Ready® soybean leaves, stems and seeds [38–40]. In a study by US-scientists, seeds were found to contain up to 3 mg/kg glyphosate and up to 25 mg/kg AMPA [38]. In a study by Argentinian scientists, up to 1.8 mg/kg glyphosate and 0.9 mg/kg AMPA was found [39]. The presence of high AMPA residues in Roundup Ready® soybean was not anticipated and presents a new type of consumer exposure.

## Opinions on glyphosate-resistant weeds

Do the results summarized above document a case of resistance mismanagement and inadequate testing of the ecological effects of extensive glyphosate use? Before considering some opinions on this question, two possibilities envisioned in a 1994 review on resistance to glyphosate [6] will be considered. With regard to the expected Roundup Ready® technology, it was said: 'Given human nature as it is, farmers may be tempted to rely exclusively on one resistant cultivar for successive years, with the accompanying temptation to apply multiple glyphosate treatments…[which] would surely help set the stage for selecting resistant weeds as has been clearly shown in the case of paraquat-resistant weeds…Conversely, if glyphosate-resistant cultivars are marketed and viewed as an integral component of an integrated weed management system that includes other herbicides, non-resistant crops, and nonchemical control measures, these cultivars may well become incorporated into modern agricultural practices'.

Both possibilities are reflected in published opinions on glyphosate-resistant weeds. The 1962 Nobel Prize winner James D. Watson commented in *DNA. The Secret of Life* [41]: '…let us suppose…that the resistance gene does enter the weed population and is sustained there. It would not actually be the end of the world, or even of agriculture, but rather an instance of something that has occurred frequently in the history of farming…The result is that the scientists have to go back to the drawing board and come up with a new…herbicide…The whole evolutionary cycle will then run its course before culminating once more in the evolution of resistance…'. This relaxed opinion is not shared by the manufacturing company Monsanto. When examining their website http://www.monsanto.com with the search word 'conyza', 29 hits were produced (as of 30 March 2006). The Monsanto '2006 Technology Use Guide' mentioned previously refers specifically to *Conyza canadensis* and other problem weeds. The user is advised to stay with glyphosate herbicides but to also apply other herbicides, such as 2,4-D or atrazine, and to follow some weed management practices. This is stated to assure product stewardship and long-term effectiveness of glyphosate. More rigorous weed and herbicide management measures are proposed in a *Glyphosate White Paper* published by the University of Wisconsin (Madison, WI, USA) at http://ipcm.wisc.edu/uw_weeds/. This publication aims to '…protect the value of Roundup Ready crop technology through proper stewardship of glyphosate use'. Detailed recommendations to maintain the long-term effectiveness of glyphosate herbicides are given, the main points being rotation of crops with non-Roundup-Ready® crops and the use of other herbicides. These recommendations are similar to other opinions on the sustainable use of glyphosate [6,11].

## Conclusions

The results summarized above demonstrate two significant ecological effects of using glyphosate that were not anticipated in publications by Monsanto [7,9,35]. The described ecological effects do not seem to be catastrophic because the resistant weed biotypes can be controlled by using other herbicides and by tilling [3,23,25]. Furthermore, the toxicity of the glyphosate metabolite AMPA might be low [37]. In the context of molecular ecology [1], the observed effects seem to have significance as case studies that were demonstrated under field conditions, albeit only after large-scale commercialization had already occurred.

Currently, seven of the weed species listed in Table 1 can be found in Europe, all have wide-spread distribution (*Convolvulus arvensis*, *Lolium* spp., *Amaranthus* spp., *Conyza canadensis*, *Lolium multiflorum*, *Ipomoea* spp. and *Chenopodium album*). The North American weed species *Conyza canadensis* was introduced into Europe in the 17th century. Almost nothing can be found in the published literature about the existence of European weed biotypes with natural glyphosate resistance, or about the potential of European weed biotypes to evolve glyphosate resistance. If Roundup-Ready® technology is introduced into Europe, it remains to be seen whether European farmers will learn from the experience of US farmers.

In summary, the described results strongly support developing research fields in molecular ecology [1] and molecular ecotoxicology [2]. These scientific disciplines should not remain academic, but help in the predictive ecological assessment and supervision of 1st and 2nd generation transgenic crop plants.

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