Review

New multiple-herbicide crop resistance and formulation technology to augment the utility of glyphosate†

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Abstract: Glyphosate has performed long and well, but now some weed communities are shifting to populations that survive glyphosate, and growers need new weed management technologies to augment glyphosate performance in glyphosate-resistant crops. Unfortunately, most companies are not developing any new selective herbicides with new modes of action to fill this need. Fortunately, companies are developing new herbicide-resistant crop technologies to combine with glyphosate resistance and expand the utility of existing herbicides. One of the first multiple-herbicide-resistant crops will have a molecular stack of a new metabolically based glyphosate resistance mechanism with an active-site-based resistance to a broad spectrum of ALS-inhibiting herbicides. Additionally, new formulation technology called homogeneous blends will be used in conjunction with glyphosate and ALS-resistant crops. This formulation technology satisfies governmental regulations, so that new herbicide mixture offerings with diverse modes of action can be commercialized more rapidly and less expensively. Together, homogeneous blends and multiple-herbicide-resistant crops can offer growers a wider choice of herbicide mixtures at rates and ratios to augment glyphosate and satisfy changing weed management needs.

Keywords: glyphosate; crop; weed; ALS; sulfonylurea; imidazolinone; resistance; tolerance; homogeneous blends

1 INTRODUCTION

Even after 30 years of sales, glyphosate is still the largest selling and fastest growing agrochemical.[1] Glyphosate controls over 300 annual, perennial and biennial herbaceous grasses, sedges and broadleaves as well as woody brush and trees at a wide range of application timings without any residual soil activity and recropping restrictions.[2] In 1996, the introduction of glyphosate-resistant soybeans [*Glycine max* (L.) Merr.] (Roundup®Ready®; Monsanto Company, St Louis, MO) promised a simple, inexpensive and flexible total weed management system without crop injury and mechanical tillage. This system had convenience, performance and economics. These advantages helped drive the almost universal adoption of glyphosate-resistant crops by growers in large areas of North and South America.[3] Today, herbicide-resistant corn (*Zea mays* L.), canola (*Brassica napa* L.), cotton (*Gossypium hirsutum* L.), alfalfa (*Medicago sativa* L.) and soybeans are on about 82% of the 102 million hectares of biotech crops.[4,5] The use of glyphosate and glyphosate-resistant crops is still growing rapidly at rates greater than 10% per year.

Researchers had a number of technical successes in developing herbicide-resistant crops with non-transgenic methods such as traditional selection, seed mutagenesis and cell selection.[6] However, large commercial success with herbicide-resistant crops did not come until 1996 with transgenic glyphosate-resistant soybeans. These soybeans, and the other glyphosate-resistant crops that quickly followed, transformed the way many growers managed weeds. Growers rapidly adopted glyphosate-resistant crops and, at least initially, did not have to rely on preventive soil-applied herbicides. Growers could wait to treat weeds until they emerged and still be certain to get control. Many growers waited until the weeds were large in the hope that all the weeds had emerged and only one application would be needed. Today, experts are challenging this practice from both an economic and a sustainability perspective.[7]

Growers often do not fully appreciate the economic impact of herbicide-resistant weeds, particularly glyphosate-resistant weeds.[8] In spite of warnings from experts about the sustainability of weed management relying solely on glyphosate, many growers continue

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†Paper presented at the symposium 'Glyphosate resistant crops and weeds: present and future' held by the Agrochemicals Division of the American Chemical Society at the 233rd National Meeting, Chicago, IL, 25–26 March 2007.
(*Received 13 March 2007; revised version received 20 May 2007; accepted 8 June 2007*)
Published online 10 December 2007; DOI: 10.1002/ps.1486

to rely totally on glyphosate for weed management. Consequently, many growers now have glyphosate-resistant and tolerant weed populations and can no longer rely only on glyphosate for weed management. This paper discusses new technologies that can make it easier for growers to manage resistant weed populations by increasing the number of herbicide options with different modes of action.

1.1 Glyphosate sustainability

The intensive use of glyphosate in glyphosate-resistant crops across wide areas has resulted in extremely high selection pressure, for the evolution of glyphosate-resistant weeds and prompted concerns about sustainability.[9] Weed control methods cannot stay static and remain effective. Weeds will eventually circumvent any single control method.[10] Indeed, the composition and density of weed populations inherently difficult for glyphosate to control are changing rapidly.[7] These weeds include resistant and naturally tolerant weeds such as yellow nutsedge (*Cyperus esculentus* L.), *Amaranthus* spp., tropical spiderwort (*Commelina benghalensis* L.), morningglory species (*Ipomoea* spp.), wild buckwheat, *Acalypha* spp., common and giant ragweed (*Ambrosia artemisiifolia* L. and *A. trifida* L.), common lambsquarters (*Chenopodium album* L.) and a number of winter annuals.

For two decades, genetic and biochemical evidence suggested that the evolution of glyphosate-resistant weeds was unlikely.[11] Overproduction of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.26) did not increase glyphosate resistance enough for plants to survive.[12] EPSPS modifications that conferred glyphosate resistance modified the active site, EPSPS, and reduced fitness.[13] Furthermore, no higher plants could significantly metabolically inactivate glyphosate.[14] The use pattern of glyphosate also did not favor the evolution of resistant weeds, i.e. low rates in mixtures and targeted control of perennial weeds whose seed production and other adaptive strategies were slower than annuals.[11]

Expectations changed in 1996 with the discovery of glyphosate-resistant rigid ryegrass (*Lolium rigidum* Gaudin).[15] The next year, researchers identified glyphosate-resistant goosegrass [*Eleusine indica* (L.) Gaertn.].[16] In spite of the frequency of mutations that impart glyphosate resistance being lower than for other herbicides,[17] the intensive use of glyphosate across widespread areas has resulted in 12 reports of glyphosate-resistant weed species.[18] Experts now agree that glyphosate-resistant weeds are widespread and will continue to increase, but do not agree how close these infestations are to the 'tipping point' that will force the average grower to make fundamental changes in weed management practices.

A herbicide does not lose all its value when weeds evolve resistance. In practice, herbicide resistance often evolves locally and in only one or two weed species. Often the grower just needs to add another herbicide to the spray mixture. For most weed species, resistance is not a serious problem for the grower because the resistant weeds are not very competitive, or a number of other economical control options exist, e.g. tank mixtures of 2,4-D or dicamba with glyphosate to control glyphosate-resistant marestail.[19] For other species, evolved resistance can cause serious crop production problems without any attractive management options. Even so, economically stressed growers have been reluctant to take proactive measures to prevent resistant weeds when past efforts have been largely ineffective.[19,20] Growers want rapid return on their investment, and any financial return from proactively applying herbicides with overlapping weed spectrums, i.e. 'double knockdown', to manage resistant weeds does not accrue in the year of application. A number of economic studies are under way to help growers justify long-term, proactive weed management practices.[8,17,19]

In principle, all experts support preserving herbicide utility, but disagree as to what are the best tactics to preserve utility. One controversial issue is over how much to control the weeds.[21–23] A common paradigm is to manage weeds to economic thresholds by using the lowest herbicide rates needed to protect yields. This approach has generally not taken into account the long-term problems created by allowing weeds to escape control and produce seeds. Application of this type of economic threshold can be disastrous if it discourages a grower from controlling difficult-to-control weeds until after they establish a seedbank. This paradigm needs to be changed to 'what is the optimum level required for long-term sustainable weed management'.[22,23] Multiple-herbicide-resistant crops may enable new highly effective herbicide options that will make preventing weed seed production more practical.

1.2 Mixtures and sequential application of herbicides with multiple modes of action

Herbicide-resistant weeds tend to be a problem when growers rely on a single herbicide mode of action over several years. To delay the evolution of herbicide-resistant weeds, experts recommend using mixtures and sequential applications of herbicides with overlapping weed spectrums and different modes of action and metabolic pathways.[24–26] In theory, mixtures and sequential applications reduce the selection pressure for weeds to evolve resistance to any single herbicide mode of action because the herbicide with a different mode of action controls any weed escapes from the other herbicide and thus prevents propagation.

To control or delay the evolution of resistant weeds effectively, the weed spectrums of the herbicide components of the mixtures need to overlap. Currently, corn and soybean growers in the USA have many naturally selective options among 48 registered herbicide actives with 12 different modes of action. Typically, growers use most of these naturally selective herbicide active ingredients to control more than a dozen weeds in their fields. However, most commonly used mixtures do not provide sufficient redundant control of key weed species adequately to delay the evolution of resistant weeds. An important issue today is to understand which weed management programs can provide this overlap and thus be more sustainable.

Best weed management practices have traditionally encouraged growers to use diverse physical, chemical and biological weed control methods in an integrated fashion without excessive reliance on any one method.[9] Indeed, weeds have adapted, and a single application of glyphosate is no longer effective for many growers. Experts agree that proactive measures to manage herbicide-resistant weeds are better than reactive measures, but the basic biological principles for controlling and preventing resistant weeds are similar. Both focus on managing selection pressure.

Most growers do not seriously manage for resistant weeds until they are problems in their fields.[27] Part of the reason growers do not manage proactively is because most know other options are still available and expect companies to continue to provide new technology. Unfortunately, companies are not being as successful in discovering selective herbicides with new modes of action as they have been in the past. Therefore, it is more important than ever to find ways to utilize currently available herbicide technologies. One way to utilize current herbicide technology more fully is with multiple-herbicide-resistant crops. Multiple-resistant crops give growers more options to use mixtures of currently available herbicides with different modes of action.

2 GLYPHOSATE AND ALS HERBICIDE RESISTANCE TECHNOLOGY

2.1 Glyphosate resistance

Glyphosate competes with the substrate phosphoenolpyruvate (PEP) at the EPSPS enzyme-binding site in the chloroplast to inhibit the shikimate pathway and ultimately cause plant death. Interest among breeders to improve crop safety biologically to herbicides increased when scientists reported finding weeds with high levels of herbicide resistance.[28] Attempts to discover glyphosate resistance in crops with conventional breeding failed, and the discovery of a glyphosate-resistant EPSPS with sufficient catalytic activity was very difficult.[29] However, researchers eventually identified a number of glyphosate-resistant genes that provided commercially acceptable glyphosate resistance. A *CP4* gene from *Agrobacterium* was the most efficient and is now in most glyphosate-resistant crops.

Recently, two different systems have been identified in soil microorganisms to inactivate glyphosate, a decarboxylase enzyme from a soil fungus[30,31] and three weakly active *N*-acetyltransferase isozymes from the soil bacterium *Bacillus licheniformis* (Weigmann) Chester.[32] Metabolic inactivation should eliminate any concerns about residual glyphosate in sensitive plant tissues.[33]

Glyphosate acetyltransferase deactivates glyphosate by acetylating glyphosate into non-phytotoxic *N*-acetylglyphosate (NAG) (Figs 1 and 2). To increase the glyphosate acetylation activity, a collection of recombined glyphosate acetyltransferase genes (*gat*) were expressed in *E. coli* and screened for glyphosate acetylation. Recombinants with the highest glyphosate acetylation then went through iterative rounds of gene shuffling. At two points during the shuffling process, site-directed mutagenesis introduced additional amino acid substitutions to augment the naturally occurring 12 amino acid differences among the natural isozymes. Eleven rounds of gene shuffling introduced over 20 amino acid substitutions and increased glyphosate acetyltransferase activity more than 5000-fold.[34,35]

Figures 3 and 4 illustrate the high level of glyphosate resistance with glyphosate acetyltransferase in corn and soybeans. On a dose–response basis, glyphosate resistance increases more than 100-fold in both experimental lines. Analogous dose–response studies in soybeans show that the commercial target site based system increases resistance to glyphosate 49-fold.[36]

2.2 ALS-inhibiting herbicide resistance

Acetolactate synthase (ALS; EC 4.2.1.6) is an enzyme required for the production of the branched-chain amino acids valine, leucine and isoleucine. ALS is a nuclear encoded enzyme that moves to its active site in the chloroplast using a transit peptide. Soon after the discovery of ALS-inhibiting herbicides, tissue culture with tobacco (*Nicotiana tabacum* L.) and corn cell callus facilitated discovery of ALS-resistant genes.[37,38] Varieties with non-transgenic ALS-resistant genes are commercially available in canola, corn, lentils (*Lens culinaris* Medik.), sunflowers (*Helianthus annuus* L.), soybeans and wheat (*Triticum aestivum* L.).

The five chemical classes of commercially used ALS-inhibiting herbicides [sulfonylureas (SUs), imidazolinones (IMIs), triazolopyrimidines (TPs), pyrimidinylthiobenzoates (PTBs) and sulfonylamino-carbonyl-triazolinones] all bind to the ALS enzyme,

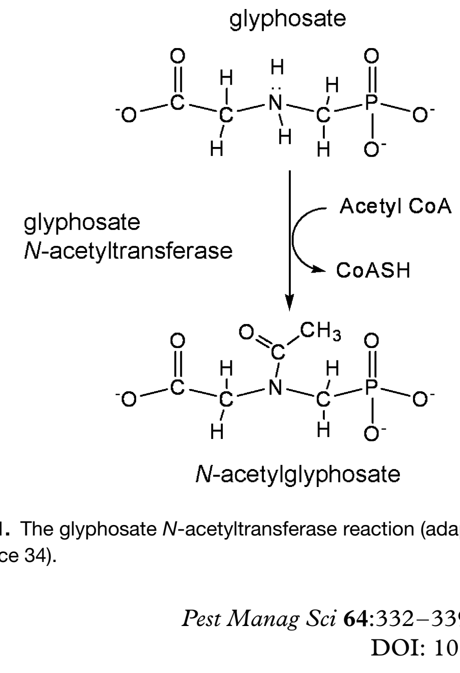
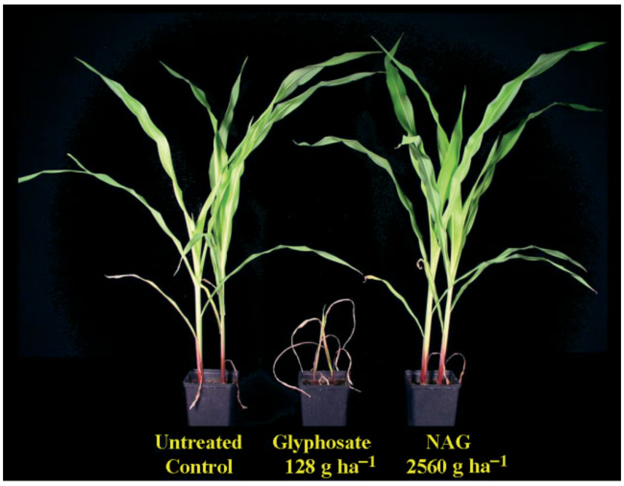


Figure 1. The glyphosate *N*-acetyltransferase reaction (adapted from Reference 34).

Pest Manag Sci **64**:332–339 (2008)
DOI: 10.1002/ps



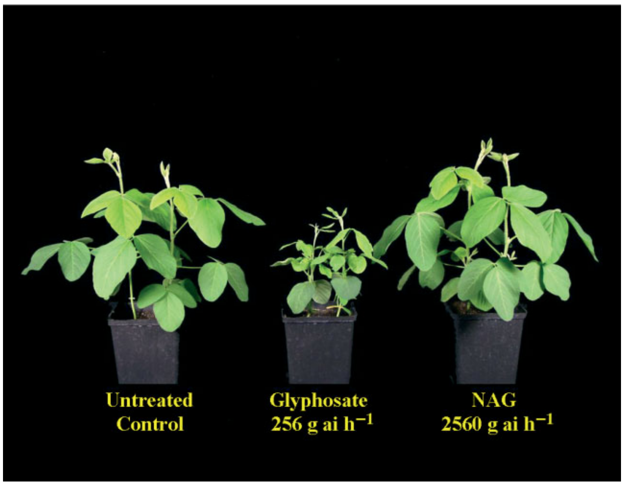


Figure 2. Relative postemergence activity of unformulated glyphosate acid and *N*-acetyl glyphosate (NAG) on non-transgenic varieties of corn, 'P3394', and soybeans, 'P94B53'. All treatments had 0.25% (v/v) tallow amine ethoxylate surfactant.

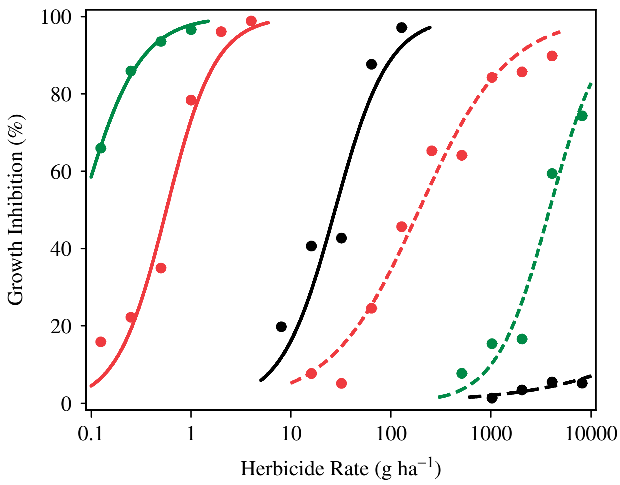


Figure 3. Comparison of the postemergence response in the greenhouse of chlorimuron (green), metsulfuron (red) and glyphosate (black) on experimental positive (- - - -) and null (——) hemizygous glyphosate and ALS-resistant corn isolines at the 2–3-leaf growth stage. The *gat* gene had 11 rounds of gene shuffling.

although not all at the same attachment points. Resistance to ALS-inhibiting herbicides has predominantly

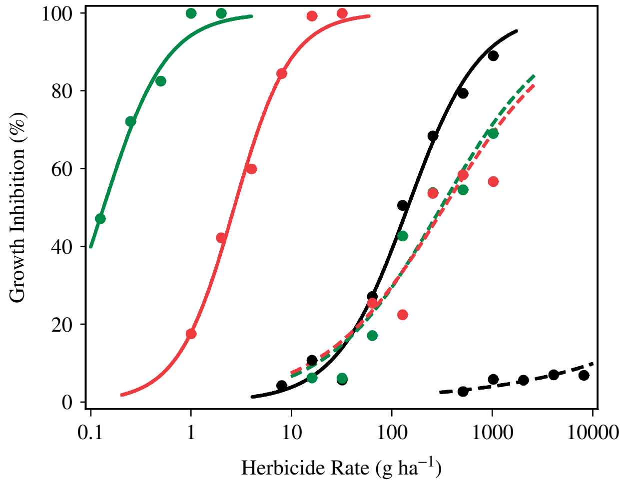


Figure 4. Comparison of the postemergence response in the greenhouse of tribenuron (green), nicosulfuron (red) and glyphosate (black) on experimental positive (- - - -) and null (——) homozygous glyphosate and ALS-resistant 'Jack' soybean isolines at the first trifoliate growth stage. The *gat* gene had seven rounds of gene shuffling.

occurred by point mutations in genes encoding ALS, but resistance based on enhanced metabolism also occurs.[39,40] ALS mutations are not always cross-resistant to all classes of ALS-inhibiting herbicides.[39] ALS mutations can be broadly grouped into three phenotypes: (i) broad cross-resistance to SUs, IMIs, TPs and PTBs; (ii) resistance to IMIs and PTBs only; and (iii) resistance to SUs and TPs only.

The large number of ALS-resistant weeds is partly due to the large number of permutations in the ALS amino acid sequence that can cause resistance and still allow the enzyme to retain functionality. Resistance to ALS-inhibiting herbicide does not usually cause any noticeable reduction in fitness. Consequently, some ALS-resistant weed populations are widespread now, and growers must use other types of herbicide or mechanical tillage to control those weeds. Even so, ALS-inhibiting herbicides are widely used and account for about 17.5% of the global herbicide market (M Phillips, personal communication).

A highly herbicide-resistant ALS gene (*hra*) has two point mutations, proline to alanine at position 197 and tryptophan to leucine at position 574.[41] The tryptophan to leucine substitution is similar to the XA-17 corn mutation and gives high levels of resistance to all ALS-inhibiting herbicides, while the proline substitution gives a tenfold increase in resistance to specific sulfonylurea and triazolopyrimidine herbicides.[39] Ongoing experimentation in corn indicates that the two mutations together give slightly more resistance than the stronger single mutation.

Figure 3 illustrates the high level of resistance that the *hra* gene gives in an experimental hemizygous corn line to chlorimuron and metsulfuron. The increased corn resistance to chlorimuron is over 10 000-fold. Soybeans are not as resistant to all ALS-inhibiting herbicides as corn, but Fig. 4 illustrates a very high increased resistance to tribenuron and nicosulfuron.

Corn and soybeans express the *hra* gene in the shoots and roots, and thus also have a high level of resistance to ALS-inhibiting herbicides applied to the soil. The yield advantage from early-season weed control with herbicides that have soil residual should help motivate growers to implement more diverse weed management practices.[20,42]

3 MULTIPLE-RESISTANT CROPS

Multiple-herbicide-resistant crops can be developed by stacking genes with conventional breeding or molecular techniques. A multiple-herbicide-resistant breeding stack is a cross between two crop lines containing different genes and then selecting for the presence of both. Breeding stacks can combine transgenes or cross-transgenes with non-transgenes, e.g. glyphosate resistance combined with sulfonylurea resistance in soybeans (STS®; Pioneer Hi-Bred International, Johnston, IA) or with imidazolinone resistance in corn (Clearfield®; BASF Corporation, Research Park Triangle, NC). A molecular stack combines the herbicide-resistant genes in the same genetic construct and thus makes it easier for breeders to work with the multiple traits.

One of the first multiple-herbicide-resistant crops will have a molecular stack of the *gat* gene for a high level of metabolic resistance to glyphosate with the *hra* gene for broad-spectrum ALS-herbicide resistance. This molecular stack will provide growers with a wide range of foliar-active and soil-residual herbicide options in corn, soybeans and other crops (Optimum™ GAT™; Pioneer Hi-Bred International). Work is under way to obtain necessary approvals from government regulatory agencies. Growers will still be able to use all currently registered selective herbicides because these multiple-herbicide-resistant crops will maintain their natural tolerance to selective herbicides.

Glufosinate crop resistance (Liberty® Link®; Bayer CropScience, Research Triangle Park, NC) is the most common commercial non-glyphosate herbicide-resistant trait, partly because it is widely used as a selectable marker for other transgenic traits such as insect resistance. In these crops, an acetyltransferase enzyme (PAT, EC 2.3.1.X) metabolically inactivates glufosinate. Glufosinate controls a broad spectrum of weeds and is an alternative to glyphosate. Currently, there are no reports of any weeds being resistant to glufosinate, so the use of glufosinate to manage glyphosate-resistant weeds will almost certainly increase. Breeding stacks of glufosinate and glyphosate resistance are already commercial, and glufosinate resistance will likely be combined with other herbicide resistance systems in the near future.

Efforts are also under way to commercialize dicamba resistance in combination with glyphosate resistance in soybeans.[43,44] Dicamba is a synthetic auxin herbicide that controls a number of important broadleaf weeds in corn. Soybeans are usually very sensitive to dicamba, but a bacterial *O*-demethylase enzyme can metabolically inactivate dicamba and allow soybeans to withstand rates 5 times the labeled use rate in corn.[45] The auxin class of herbicides is not highly prone to weed resistance. Currently, only three weed species are reported to be dicamba resistant: common hempnettle (*Galeopsis tetrahit* L.), Kochia (*Kochia scoparia* L.) and wild mustard (*Sinapis arvensis* L.).[18]

Multiple-herbicide-resistant crops can increase the number of herbicide options with different modes of action and thus potentially help growers manage resistant weeds better.[46] If growers know which weeds are likely to be herbicide resistant in their fields, selecting the best weed management options will be easier. To make those selections, growers need resistant weed surveys such as those currently done in Canada.[27] Additionally, these herbicide resistance mechanisms should eliminate all herbicide crop phytotoxicity and make the crop more competitive for light, water and nutrients with any surviving weeds. Multiple-resistant crops should establish a canopy more rapidly, and that can be a quite effective trait to help manage weeds.[47]

To satisfy future weed management needs successfully, seed companies that are developing herbicide resistance must work closely with chemical companies that are registering new herbicide uses. To date, five herbicide resistance transgenes for three herbicides have been used in commercial crops: the genes for CP4 EPSPS, a mutated corn EPSPS, glyphosate oxidase (GOX, EC 2.5.1.19) metabolic system, a nitrilase for bromoxynil (EC 3.5.5.6) and the *bar* or *pat* glufosinate acetylation system.[3] Government regulators have also approved field tests of resistant transgenes for protox-inhibiting herbicides, 2,4-D, isoxazole, dalapon and chloroacetamides.

Transgenes also exist to make crops resistant to other herbicides (Table 1).[45,48–58] Some can confer resistance to more than one herbicide mode of action, such as the P450[54] and aryloxyalkanoate dioxygenase transgenes.[50] Other enzymes that metabolize herbicides are potential candidates to make transgenes for herbicide resistance. These metabolism-based resistance mechanisms do not give resistance to every herbicide with a specific mode of action and thus offer the opportunity to use herbicides with the same mode of action to control weeds in the herbicide-resistant crops and to control any feral crop the next year. At present, scientists have the knowledge and skills to find, improve, and rapidly transfer genes that confer herbicide resistance.

4 HOMOGENEOUS BLENDS OF HERBICIDE GRANULES

A key obstacle for proactive resistant weed management has been the extra effort needed for growers to select and apply mixtures of herbicides with multiple modes of action. To make mixtures easier to use, companies often premix two or more herbicide active ingredients into a single product formulation.

Table 1. Non-glyphosate resistant transgenes that are not currently commercial (adapted from Reference 48)

Herbicide/herbicide class	Characteristics	Reference
2,4-D	Microbial degradation enzyme	49
Aryloxyphenoxyoxypropionate ACCase inhibitor	Microbial aryloxyalkanoate dioxygenase	50
Asulam	Microbial dihydropteroate synthase	51
Dalapon	Microbial degradation enzyme	52
Dicamba	*Pseudomonas maltophilia,* O-demethylase	45
Hydroxyphenylpyruvate dioxidase (HPPD) inhibitors	Overexpression, alternate pathway, and increasing flux of pathway	53
Phenylurea	*Helianthus tuberosus*, P450	54
Paraquat	Chloroplast superoxide dismutase	55
Phenmedipham	Microbial degradation enzyme	56
Phenoxy acid (auxin)	Microbial, aryloxyalkanoate dioxygenase	50
Phytoene desaturase (PDS) inhibitors	Resistant microbial and Hydrilla PDS	57
Protoporphyrinogen oxidase (PPO) inhibitors	Resistant microbial and *Arabidopsis thaliana* PPO	58

Such premixed formulations deliver a defined ratio of herbicide ingredients, but the process to develop and register traditional premixtures is slow and costly. Premixed formulations typically require 6–9 months of laboratory development followed by 1–2 years of regulatory field tests and extensive environmental and toxicological testing. A different ratio of the same herbicides requires another set of expensive and time-consuming tests. Thus, the composition of these premixed formulations tends to stay fixed and not keep pace with changing weed management needs.

One new technology that can reduce the time and cost to introduce new mixture offerings in the marketplace is homogeneous blending of dry herbicide granules.[59] Homogeneous blends do not segregate during shipping and handling and thus eliminate the need for unit-area-package mixtures of granules. To eliminate the segregation of herbicide mixtures, the key properties of granule size, density and shape must be carefully controlled.[60,61] The control of size is the most important and must be precise, as differences as small as 10% can result in significant segregation.[62]

Homogeneous blends of granular products provide an alternative to formulating premixed herbicides. Ensuring the homogeneity of each subsample made by a grower helps meet governmental regulations and allows companies to develop new mixtures more rapidly. The regulatory requirements for granule blends are similar to the requirements of the component products and include the standard physical and chemical tests. Each component in the blend is a separate formulation with its own registered assay tolerance.

Multiple-herbicide-resistant crops alone are not a weed management strategy. New formulation technologies are necessary to allow growers to utilize multiple weed management tactics more easily. Homogeneous dry blending is one of these new formulation technologies that can supply growers with a single mixture product to meet individual grower weed management needs. For the first time, blends of dry herbicide granules will remain homogeneous during shipping and handling and thus require the user only to purchase a single product rather than separately measure and inventory multiple products.

Managing herbicide-resistant weeds is getting complex. New computer-based technologies will help growers select the optimum herbicide mixtures with diverse modes of action. Syngenta recently developed a website where growers can either ask weed management experts questions or use a computer module to define their weed management needs by crop, weed spectrum, location, preferred management methods and resistance history and get a customized management recommendation (www.resistancefighter.com [January 2007]). This approach or other types of computer model[63,64] can be linked with formulation blending technology to prescribe most effective herbicide mixtures in an easy-to-use way to help growers manage weeds.

5 SUMMARY

The first generation of glyphosate-resistant crops transformed weed control by giving growers a simple, flexible, effective and economical way to control weeds. However, weeds are adapting to single-mode-of-action herbicide-resistant crop systems, and glyphosate applied alone is rapidly losing its effectiveness. Growers must use more diverse weed management programs to maintain the effectiveness of glyphosate. Multiple-herbicide-resistant crops will enable more weed management options and thus should help sustain glyphosate.

A new molecularly stacked multiple-herbicide-resistant crop technology combines a metabolic system to inactivate glyphosate with a modified ALS enzyme that is insensitive to a wide range of foliar and soil-residual ALS-inhibiting herbicides. These crops will maintain their natural tolerance to selective herbicides and thus will increase the number of herbicide mixture options with multiple modes of action.

Additionally, new formulation technology called homogeneous blends can enable faster registration and less costly commercialization of new mixtures. This technology will allow growers to select a single product with the optimum rates and ratios of herbicides

DOI: 10.1002/ps

for their weed management needs. The anticipated shorter commercialization time lines will allow mixture offerings to evolve quickly to meet changing weed management needs. Given the lack of development of new selective herbicides with new modes of action, new multiple-herbicide-resistant crops and formulation technology can enable easier and more efficacious use of mixtures and offer a practical way to help preserve glyphosate utility with existing herbicides.

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