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Herbicide Tolerant Biotech Cotton: The Resistance Issue in the USA

Weeds may be annual or perennial, but they are always found in cotton fields everywhere unless proper control measures are taken. Early weed competition in the first few weeks after germination can cause significant yield losses. Weed infestation can deprive the cotton plant of proper growth, change its branching scheme, and change the overall shape of the plant. When cotton is in its flowering stage, weeds compete with the cotton plant for nutrients, and they also harbor pests. Furthermore, weeds growing outside the cotton crop serve as hosts for insects, mites and pathogens. When harvest time comes along, particularly if the cotton is machine picked, weeds interfere with defoliation. While defoliants will cause cotton leaves to dry and fall, weeds may not defoliate or be killed. Machine picking of weed-ridden fields will produce higher trash content in the cotton lint and result in stained cotton. Thus, a cotton field must be free of weeds from sowing until harvesting. Studies carried out in some countries have indicated that the optimum benefits of fertilizer and insecticide applications can only be achieved if there are no weeds in the field.

Weeds can be removed manually with small implements, mechanically through cultivation practices, biologically employing pathogens or chemically by applying herbicides. Manual weeding of fields or with the help of small implements is not feasible in large-scale production systems such as in the USA. The rising cost of labor is making this system expensive in many more countries. Mechanical control is feasible, but has its own limitations, such as the inability to remove weeds close to the plant, possible damage to the crop, soil compaction and the high cost of inter culturing. Biological weed control is the intentional manipulation of natural enemies for the purpose of controlling target weeds. There are three approaches to biological control: Conservation, augmentation and importation of natural enemy populations. Conservation is the preservation and maintenance of the natural enemies that occur in a given area, but it is rare indeed to achieve effective weed control exclusively through this method. Augmentation is the periodic release of microorganisms or agents that do not occur naturally in sufficient numbers to provide pest control. Augmentative releases may be designed to "seed" natural enemies in numbers large enough to overwhelm weed populations. Importation of natural enemies into areas where they do not occur is sometimes called classical biological control. Natural enemies from the native range of the pest are identified, collected, imported, reared and released. In a best-case scenario, the natural enemy will establish permanent populations and provide control of the pest without the need for further releases. Biological control is often the most environmentally friendly method, but unfortunately, without integrated control practices, it is not the most effective method.

The main drawbacks to biological weed control are: the high cost of the research, time and money needed to find suitable organisms, the time that biological agents take, so that when they finally do work the loss may have already occurred, biological agents must have a population of host plants to survive, so weeds cannot be completely eliminated, and finally, it might just be too expensive to produce and maintain bio agents. This is why biological weed control is not used in any country.

Chemical Control

Chemicals are the most effective and efficient way to control weeds. They may have their own consequences, but the weeds are killed in the minimum possible time. The discovery of 2,4-D and MCPA in 1944 marked the beginning of herbicide use in crops. Chlorpropham, dalapon, and diuron were developed between 1947 and 1954 and were among the first herbicides specifically labeled for use in cotton (Buchanan, 1992). Chemicals can be applied before planting (pre-emergence) so that the field is free of weeds during germination, and then applied again a few weeks after germination, or after planting (post-emergence), depending on the weed species found in the field. Growers are generally aware of their field conditions and have a good idea of the kind of weed that will appear. In addition to pre- and post-emergence applications of herbicides, chemicals may also be applied as lay-by applications if weeds tend to grow in patches in the field. Lay-by applications may also be used together with a mechanical method to control both the weeds growing close to the plant and the ones growing in the rows between plants. However, there is a limit to the time after emergence when post-emergence herbicides can be applied safely without affecting the cotton plant. Selective, post-emergence herbicides (sethoxydim and fluazifop) targeting grassy weeds were commercialized for use in cotton in the early 1980's. However, selective, post herbicides targeting broadleaf weed species were not introduced until 1996 (Wilcut *et al*., 1995). Pyrithiobac was registered for pre- and post-emergence application in cotton in 1996 and remains the only selective, post herbicide without a growth stage restriction for application.

Herbicide Resistant Biotech Cottons

BXN™ Biotech Cotton

The first herbicide tolerant biotech cotton was approved for commercial production in the USA as BXN™ in May of 1995. The BXN™ gene that conferred resistance to the herbicide Buctril (bromoxynil) was "nitrilase" from *Klebsiella pneumoniae* subsp. *ozaenae.* The development of herbicide tolerant biotech cotton revolutionized weed control in cotton. Buctril® 4EC (Bromoxynil) herbicide and the patented BXN™ cotton system allowed growers to effectively control commonly occurring broadleaf weeds in cotton from emergence until 75 days before harvest. Nitrilase gives cotton the ability to metabolize the bromoxynil herbicide, and the weeds will normally be killed in 2-3 days. BXN™ may be sprayed together with Buctril® compounds a maximum of three times from emergence up until 75 days before harvest. Glyphosate-tolerant herbicide-resistant biotech cotton (Roundup Ready®) was approved a few days later than BXN™ cotton. Because Buctril® was previously not registered for use on cotton, Buctril® received a three-year conditional registration, while Roundup Ready® was awarded unconditional approval. For various reasons, including its limited weed control spectrum, competition with the Roundup Ready® trait, and stacking of Roundup Ready® with Bollgard and the Bollgard II genes, BXN™ cotton is now obsolete. BXN™ varieties were last sold around 2004/05.

Roundup Ready® Biotech Cotton

Roundup Ready® biotech cotton was approved for commercial cultivation in the USA in the 1997/98 season. According to Stewart (1991), the mode of action of glyphosate lies in the inhibition of an enzyme 5-enolpyruvylshikimate-3phosphate (EPSP) synthase, which is a key catalyst in the production of aromatic amino acids. Since animals do not synthesize amino acids, glyphosate has low toxicity to birds, fish and mammals including humans, but ample toxicity to plants. Resistance to glyphosate has been accomplished by two different routes. In the first, a strong constitutive promoter was placed in front of a natural EPSP synthase gene so that the enzyme was over produced in the transformed plants. In the second, a mutated bacterial EPSP synthase gene that changed one amino acid in the enzyme protein resulted in the enzyme being insensitive to herbicide. With the appropriate promoter, plants transformed with this gene were resistant to glyphosate. The use of Roundup on Roundup Ready® cotton increased broad-spectrum weed control, minimized competition from hard-to-control annual and perennial weeds, and simplified weed management. Glyphosate has proven to be a reliable herbicide treatment for use on transgenic crops and has improved weed management in the short term. The adoption of herbicide-tolerant cotton, expressed in terms of percent of area planted, was approximately 20% in 1998, 68% in 2001, 73% in 2004, 81% in 2005, 85% in 2006 and over 85% since then. Roundup can be sprayed on cotton only up to the four-leaf stage. Weeds emerging thereafter have to be controlled manually, mechanically or with lay-by applications. Weed species shifts and selection for glyphosate-resistant weeds resulting from over use of glyphosate have been confirmed.

LibertyLink® Biotech Cotton

The Bayer CropScience company developed the LibertyLink® herbicide-tolerant cotton system. LibertyLink® cotton varieties were approved for commercial cultivation in 2004. LibertyLink® varieties were resistant to Ignite® herbicide also called Liberty®, Finale® and Rely®. The chemical name for Ignite® is glufosinate ammonium, so any chemical having glufosinate ammonium can be sprayed over the top of the cotton plant until 70 days prior to harvesting. In terms of growth, Ignite® may be sprayed over cotton until early bloom or, more technically, up to the 10-leaf stage. However, the total seasonal application rate should not exceed 1.9 kg a.i./ha (200 ounces/ha formulated) with no more than 0.7 kg a.i. (100 ounces/ha formulated) to be sprayed in one application. The herbicide application rate may be adjusted according to weed types, weed intensity and weed size. These criteria were, in fact, true for all over-the-top-applications of herbicides. Ignite® was most effective against broad leaf weeds, but grassy weeds can also be killed to some extent. Ignite® had no soil activity and translocation within the plant was minimal. Weeds will show chlorosis, and within 3-5 days weeds will show signs of wilting. LibertyLink® carried an enzyme that converted the herbicide into a non-phytotoxic compound. Liberty 200 herbicide is an inhibitor of glutamine synthetase. It is always better to kill weeds at an early stage, when they are only a few inches tall, but that will require successive applications of Ignite®. Ignite® is very effective against morning glory and cocklebur, while pigweed and nut grasses are not perfectly controlled, nor are grassy weeds. The LibertyLink® herbicide-tolerant cotton system is comparatively a new option for weed management.

Roundup Ready® Flex Biotech Cotton

The Roundup Ready® technology was limited by relatively poor expression of the gene in the reproductive parts of the plant, thus conditioning glyphosate applications exclusively to the period prior to the fruiting stage. The problem of poor gene expression was overcome in Roundup Ready® Flex. The

Roundup Ready® gene and the Roundup Ready® Flex gene use the same coding sequence of the EPSPS gene (enol-pyruvyl shikimate phosphate synthase) from Agrobacterium strain CP-4. The Flex gene is more constitutively expressed so that it is active in the fruiting structures. The Roundup Ready® Flex varieties have much higher levels of tolerance to glyphosate in the vegetative stage as well as the reproductive phase, with an extended over-the-top application window. Roundup Ready® Flex was approved for commercial use in the USA for the 2006/07-crop year. The Roundup Ready® Flex varieties used the same metabolic tolerance expressed in the Roundup Ready® trait. The difference between Roundup Ready® and Roundup Ready® Flex is that Flex varieties posses an improved promoter sequence that enables the plant to tolerate glyphosate herbicides in the vegetative as well as the reproductive stages. Glyphosate products may be sprayed on Roundup Ready® Flex varieties until a week before harvesting. The results of many years of experience shows that Roundup sprayed on Roundup Ready® Flex cotton varieties does not produce any damage on subsequent plant growth and development, yield or fiber quality. Different doses and times of application were tested with no negative impact on the cotton plant.

Glyphosate, being a post-emergence chemical herbicide, that is highly biodegradable controls only emerged weeds and does not keep new weeds from emerging. This means that multiple applications of chemicals are required to have season-long weed control. Roundup Ready® biotech cotton limited the use of glyphosate products to only the four-leaf stage, which meant that only a limited number of applications could be made in a single season. A much wider window, in the form of Roundup Ready® Flex, opened the door for multiple applications of glyphosate, which meant more frequent use of the same chemicals in a single season and the ensuing likelihood of faster development of resistance. Extended use of glyphosate could be intermingled with insecticide applications, and the limited studies conducted by Miller *et al*. (2009) showed that producers may be able to combine multiple pest and crop management strategies to reduce application costs with minimal effect on the crop. The negative effects evaluated in this study of co-applications on Roundup Ready® Flex cotton actively growing at the four- to five-leaf growth stage were limited to minor transient visual leaf vein chlorosis burn that lasted no longer than 21 days and did not result in reductions in crop height or yield. Miller *et al*. (2009) cautioned that if the co-applications evaluated in this research were applied to cotton in early growth stages, especially under less-than-optimal environmental conditions, or to cotton under stress, the potential for injury might increase. The studies are limited exclusively to specific insecticides and to early stage applications of herbicides. Different insecticides/micronutrients/plant growth regulators and herbicide co-applications should be tested independently to avoid losses. In 2008, Monsanto recommended the use of Roundup WeatherMAX® and Roundup Original MAX® on Roundup Ready® Flex cotton, thus confirming that even other glyphosate chemicals have to be used on Flex cotton carefully.

The option to use herbicides at any stage of crop development may result in the temptation to delay herbicide use during the early stages, which is not desirable. Early stage control of weeds is recommended even for Roundup Ready® Flex cotton, but this does not mean that non-chemical control measures should be abandoned. It is very important that an integrated approach continue to be followed with minimum reliance on herbicide use.

Effects of Herbicide Resistant Biotech Cottons

Herbicides provide more timely and targeted weed management with the ability to control weeds that emerge together with the crop or soon after. Herbicides, though expensive, provide efficient and complete control compared to other methods, but continuous herbicide use has its own consequences. The following are some of the consequences of heavy reliance on herbicide use, particularly with cottons that allow a longer window in which to use herbicides.

- Reduced use of inter-culturing and hoeing operations to remove weeds,
- Minimum use of pre-emergence herbicides – Pre-emergence herbicides are applied without knowing the kinds of weeds that will emerge nor their intensity after the cotton germinates. Post-emergence use of herbicides discourages the use of pre-emergence herbicides,
- Extensive use of herbicides,
- Heavy reliance on certain chemicals like Roundup and Ignite®,
- Emergence of resistant weed species - multiple applications of Roundup Ready® Flex over extended periods increases the likelihood of developing resistant weeds,
- Herbicide drift, particularly around irrigation structures, facilitates the development of resistant weeds,
- Volunteer herbicide resistant plants from other crops, such as herbicide resistant corn or soybeans, cannot be killed in cotton fields.

Herbicide Resistance and Its Management

The fact that weeds could develop resistance to herbicides was no surprise to researchers. Extensive use of a particular product, either insecticide or herbicide, enhances the ability and likelihood of the development of resistance to that product. The first report of herbicide resistance occurred in 1960 with the discovery of Trazine-resistant common groundsel (*Senecio vulgaris* L.) and since then many weeds have been found to be resistant to various chemicals.

According to the International Survey of Herbicide Resistant Weeds (http://www.weedscience.org/In.asp), 334 resistant biotypes, 190 Species (113 dicots and 77 monocots), have already developed resistance to herbicides. Herbicide resistance is the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. Just as in insects, cross-resistance or multiple-resistance can also develop in weed biotypes. Characteristics, such as annual growth habit, high seed production, relatively rapid turnover of the seed bank due to high percentage of seed germination each year, (i.e., little seed dormancy), several reproductive generations per growing season, extreme susceptibility to a particular herbicide, resulting in over use of that chemical and high growth vigor of the resistant biotype are the factors that help weeds develop resistance to herbicides (Vargas and Wright, 2005). Herbicide characteristics that lead to rapid development of resistance include: action on a single site, broad-spectrum control and long residual activity in the soil. Some cultural practices can also lead to selective pressure for resistant populations.

Development of resistance to Roundup herbicide means development of resistance to all chemicals carrying glyphosate. However, a resistant biotype may be susceptible to other chemicals. MacRea *et al*. (2006) evaluated LibertyLink® cotton for the management of glyphosate resistant Palmer amarant (*Amaranthus palmeri*). They tried many combinations of pre- and post-applications including lay-by application on Palmer amaranth after it had emerged, and was 6, 12, or as much as 25 to 50 centimeters tall. They concluded that Ignite® 280 provided control of Palmer amaranth (resistant to glyphosate) when the herbicide was sprayed on weed plants 5 cm tall or shorter. Later applications reduced the effectiveness of Ignite® 280 to almost zero control on 50 cm tall weed plants.

Horseweed (*Conyza canadensis*) is a common weed in the mid-south region where most cotton is grown in the USA. Glyphosate-resistant horseweed began to appear within a few years after the widespread adoption of Roundup Ready® crops. Horseweed has the ability to produce from 50,000 to 250,000 seeds per plant (Hayes, and Steckel, 2005). The authors also advised in 2005 monitoring cotton areas for glyphosate resistance in common ragweed, goosegrass, nutsedges, tropical spiderwort, prickly sida, giant ragweed, and the pigweeds, especially Palmer amaranth. Palmer amaranth showed signs of resistance to glyphosate as early as 2004. During the next two years, Palmer amaranth, also known as pigweed or careless weed in the USA, developed significant resistance to glyphosate. Experiments conducted showed that in-field control of Palmer amaranth increased with different chemicals as the herbicide rate increased, but even the highest rates of Roundup WeatherMax® and Staple® LX applied singly and in combination were unable to affect more than 92% control (Sosnoskie *et al*., 2009). When resistance has developed, the target weed biotypes should not be allowed to reach the stage of reproductively mature seeds. No new generation of weed seeds can be allowed to issue from resistant populations of any biotype. Horseweed in Tennessee and giant ragweed in Arkansas are also reported to have developed resistance to glyphosate. All efforts should be made to avoid development of resistance. Extensive use of a single group of chemicals should be avoided, otherwise resistance becomes extremely likely. Vargas and Wright (2005) suggested the following strategies to delay the development of resistance to a particular herbicide group.

- Alternate herbicides with different modes of action
- Use the minimum number of applications of any one herbicide per season
- Use tank mixes of different modes of action when possible
- Use short-residual herbicides
- Rotate crops with different seasons of growth
- Plant crops having different registered herbicides
- Do not entirely eliminate tillage from the production system
- Use hand weeding to remove escape weeds and prevent them from going to seed
- Prevent weed seed spread by using clean equipment
- Use certified planting seed

To control resistant species like Palmer amaranth, it is recommended to start with clean fields using a burndown herbicide program or tillage. Pre-emergence residual herbicides recommended particularly for the control of Palmer amaranth or other resistant weeds should be used. The philosophy is to ensure that the need for over-the-crop-use of glyphosate compounds is minimal.

New Technologies

The U.S. Department of Agriculture approved the GlyTol™ glyphosate-tolerant technology for cotton in May 2009. Bayer CropScience developed the GlyTol™ cotton event GHB614 as an alternative herbicide tolerant cotton product. GlyTol™ gives cotton growers the flexibility to use glyphosate herbicides other than Roundup products. Flexibility to use different glyphosate products would delay the development of resistance by weeds. According to Bayer CropScience, the transformation event in GlyTol™ contains the stably integrated gene 2mepsps, which encodes the 2mEPSPS protein. The gene was introduced by Agrobacterium-mediated gene transfer. Southern blot analyses show that the GlyTol™ cotton event GHB614 contains one complete copy of the 2mepsps gene. The 2mepsps gene was generated by introducing mutations into the wild-type epsps (wt epsps) gene from maize, leading to a double mutant EPSPS protein with two amino acid substitutions (2mEPSPS). This modification confers to the protein a decreased binding affinity for glyphosate, allowing it to maintain sufficient enzymatic activity in the presence of the herbicide. Therefore, plants bearing this gene are tolerant to glyphosate herbicides (http://www.aphis.usda.gov/brs/aphisdocs/06_33201p.pdf).

GlyTol™ varieties will be commercially grown in the USA in 2010.

Researchers, particularly in the private sector, are working on a number of other herbicide resistance transgenes. Some of these new transgenes will be used to develop new multiple herbicide-resistant cottons that offer growers more herbicide options to meet their changing weed management needs and to help sustain the efficacy of glyphosate. Personal communications with Monsanto indicate that they are working on a triple gene herbicide resistant cotton. It may be available for commercial production in May of 2012, or perhaps later.

Dow AgroSciences has submitted an application to the USDA for a new family of herbicide resistant traits. The technology will be introduced in corn in 2012, soybeans in 2013 and cotton in 2015, and will cover the glyphosate and glufosinate chemical groups.

GlyTol™ + LibertyLink® Herbicide Resistant Biotech Cotton

Bayer CropScience reported at the 2009 Beltwide Cotton Conferences that they have developed a double gene herbicide resistant biotech cotton called GlyTol™ + LibertyLink®. The glyphosate tolerant technology in the form of GlyTol™ expressing the 2mepsps gene has been stacked with LibertyLink® cotton which is resistant to glufosinate ammonium (Ignite®). GlyTol™ + LibertyLink® is expected to be released for commercial use in 2011. If approved by the USDA, GlyTol™ + LibertyLink® will be the first stacked gene herbicide tolerant variety in cotton. Field-testing is still going on and will continue for the next few years, but the results achieved so far are encouraging. Rinehardt *et al*. (2009) reported results of three trials conducted in three different states in order to: 1) to determine if the herbicide tolerance to glyphosate and glufosinate in GlyTol™ + LibertyLink® cotton is affected when crop protection chemicals are tank-mixed, and 2) to determine if GlyTol™ + LibertyLink® cotton can tolerate glyphosate and glufosinate applications at rates that exceed full label rates. The results showed that the tank-mix treatment of glyphosate, glufosinate, and 2-pyridinesulfonamide at the 6-8-leaf cotton stage reduced plant height 10 days after application. However, plant heights for this treatment were not significantly different from those of the unsprayed as checked at harvest. Application of plant growth regulators tank-mixed with glyphosate and glufosinate did significantly reduce plant heights at harvest. Minor foliar phytotoxicity was also observed with tank-mixes of glyphosate, glufosinate and both 2-pyridinesulfonamide (5%) and Pyrithiobac (1%). However, none of these or any other tank-mixes, had any significant effects on lint yield.

The high herbicide rate trials used treatments with 2X rates of both, glyphosate and glufosinate, and 1X, 2X, 3X, and 4X tank -mixes of glyphosate and glufosinate. The results of these trials indicated that there was no significant effect on plant height regardless of rate or timing of application. A visual phytoxicity rating of 6% was observed with the 3X glyphosate + 3X glufosinate tank-mix applied at the 2-4 leaf growth stage. However, no damage was observed with later applications beyond the 2-4 leaf-stage, and there were no significant effects on lint yield.

In trials conducted in 2007 and 2008 across the cotton belt in the USA, Henniger, *et al*. (2009) also showed that GlyTol™ + LibertyLink® plants produced no adverse effects on plant establishment, maturity, vigor, yield and quality following multiple applications of commercial formulations of glyphosate. Multiple applications of glufosinate ammonium, alone or in combination with glyphosate at full rates, showed no effect on the agronomic or reproductive characteristics of GlyTol™ + LibertyLink® varieties.

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