



Coexistence and Market Assurance for Production of Non–Genetically Engineered Alfalfa Hay and Forage in a Biotech Era

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Abstract

The introduction of genetically engineered (GE) alfalfa requires a mechanism for producers to successfully grow and market alfalfa (*Medicago sativa* L.) hay destined for GE-sensitive markets such as organic and export. A process of coexistence includes elements of respect for diverse agricultural systems, improved communication, scientific knowledge, and market clarity. A definition for “non-GE alfalfa forage” is proposed, along with suggested production protocols. These protocols include securing non-GE-detect seed, steps to reduce the probability of gene flow in hay fields, equipment sanitation, hay-lot identification, and hay testing for low-level presence. The largest risk for low-level presence in hay is likely to originate from unwanted GE presence in the planting seed. Secondary risks include accidental mixing of hays during harvest or storage, followed by gene flow between forage fields. The tolerance for low-level presence in non-GE hay must meet specific market sensitivities. Promoting absolute zero GE hay (e.g., GMO free) is a practical and analytical impossibility, creates difficulties for farmers, and makes no sense for a nontoxic, unwanted market factor. Regulatory-based tolerances, driven largely by countries that do not permit a GE trait, may require non-GE determination to a limit of detection of approximately 0.1%. Market-based tolerance thresholds may differ greatly depending on the sensitivity of markets. For market purposes, a definition of non-GE alfalfa as having a low-level presence of less than 0.9% of dry matter is suggested. Coexistence strategies for alfalfa forage require an understanding of the sources of low-level presence, market tolerances of diverse markets, and market assurance processes.

GENETICALLY ENGINEERED (GE) traits in crop plants have been commercialized across many crops, including corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L.), canola (*Brassica napus* L.), soybean [*Glycine max* (L.) Merr.], and sugarbeet (*Beta vulgaris* L.) and have very high rates of adoption in North America. The predominant traits are the glyphosate-tolerant trait, or so-called Roundup Ready (RR) trait, which enables broadcast post-emergence applications of glyphosate for weed

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Abbreviations: EIS, environmental impact study; ELISA, enzyme-linked immunosorbent assay; GE, genetically engineered; PCR, polymerase chain reaction; RR, Roundup Ready.

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Table A. Useful conversions.

To convert Column 1 to Column 2, multiply by	Column 1 Suggested Unit	Column 2 SI Unit
0.405	acre	hectare, ha
0.907	ton (2000 lb), ton	megagram, Mg (tonne)

control, and the *Bt* (*Bacillus thuringiensis*) trait, which confers protection from lepidopterous insect pests.

However, not all growers wish to adopt GE crops due to their personal preference or the preference of the markets for their crop. Organic growers are required to use non-GE crops. Additionally, export markets may prefer or require non-GE crop products either because of regulatory barriers or market preference. There may also be other markets (e.g., some retail markets) that demand non-GE hay products. Additionally, some growers object to GE crops out of concern about the technology or because of philosophical opposition to genetic engineering.

It should be pointed out that the vast majority of the markets for alfalfa hay and forage in the United States are not sensitive to the presence of GE traits, since those markets are dominated by dairy and beef, both of which have widely adopted GE feeds (Van Eenennaam and Young, 2014). Research has shown the RR trait in feeds to be safe for animal production. Currently, over 9 billion animals are fed GE crops annually, accounting for 70–90% of the consumption of GE-crop biomass (Flachowsky et al., 2005; Combs and Hartnell, 2008; Van Eenennaam and Young, 2014).

Nevertheless, there exists a diversity of views about how to farm, as well as a diversity of market sensitivities to the presence of GE crops in farm products. To support growers’ choice requires a commitment to coexistence between growers who adopt and those who reject GE crops, a situation that may require technical understanding as well as steps to promote cooperation. Grower organizations have promoted the concept of coexistence to protect the rights of farmers to continue to farm in the manner of their choosing or in ways to successfully meet market demands (USDA-AC21, 2012; Putnam et al., 2012; McCaslin and Van Deynze, 2014; Putnam, 2014; also see National Alfalfa & Forage Alliance website). Additionally, mechanisms that encourage communication and coexistence may be needed as farmers adjust to these technologies (Christensen, 2011).

DEFINING COEXISTENCE

A recent USDA committee defined “coexistence” as “the concurrent cultivation of conventional, organic, Identity Preserved (IP), and Genetically Engineered (GE) crops consistent with underlying consumer preferences and farmer choices” (USDA, AC21, 2012).

Further, we define “successful coexistence” as “the ability of diverse systems (GE, organic, non-GE) to thrive without undue influence of neighbors or resorting to extraordinary protection measures.”

In line with the goal of coexistence, the objective of this paper is to provide guidelines and coping strategies so that growers can continue to successfully produce non-GE alfalfa forage in an environment in which neighbors may be adopting GE-crops. Specifically, the purpose is (i) to offer a definition for “non-GE alfalfa forage”; (ii) to describe and discuss the market factors that may determine the tolerance for low-level presence in non-GE alfalfa; (iii) to describe the primary risks of low-level presence in alfalfa hay; (iv) to describe a protocol for production of non-GE alfalfa that would reduce the risk of low-level presence to satisfy markets; and (v) to discuss the proper use of sampling and testing to determine the GE status of hay. Although RR alfalfa and reduced-lignin alfalfa are currently the only GE traits approved in this crop, this discussion may apply to all GE traits that may be commercialized in the future.

THE ADVENT OF GENETICALLY ENGINEERED ALFALFA

Alfalfa is the fourth-ranked crop in economic value in the USA (USDA-NASS, 2015), behind corn, soybean, and wheat, at 16.7–22.4 million acres and \$7.7–\$11 billion in value annually (2009–2015, USDA-NASS, 2015). Alfalfa is the most important forage crop for dairy farming in the USA, an enterprise worth over \$40 billion in 2014 (NASS).

The RR trait was developed by inserting two *Agrobacterium*-derived genes for the CP4 EPSPS protein, so that the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) necessary for plant growth is not injured by the herbicide glyphosate. This was the first GE trait for alfalfa deregulated and approved for commercial use in 2005 (USDA-APHIS, 2005). Plantings of RR alfalfa were subsequently halted in 2007 due to a lawsuit in which a federal judge ruled that USDA-APHIS must complete an environmental impact study (EIS). After completion of the EIS, the trait was once again deregulated in 2011, after USDA-APHIS concluded that RR alfalfa “will not have a different impact on the physical and biological environment than conventional alfalfa” (USDA-APHIS, 2011). A more complete description of the process of adoption of RR alfalfa and a discussion of the advantages and disadvantages of the trait can be found in Putnam and Orloff, 2013 and Van Deynze et al., 2004.

In November 2014, a second GE trait in alfalfa, the HarvXtra trait, for reduced lignin, was deregulated by USDA-APHIS with a finding of no significant impact (FONSI; USDA-APHIS, 2014). Thus there are now two GE traits that are commercialized in alfalfa.

It is not known whether GE traits in alfalfa will come to dominate planted acreage as they have with other crops.

GE crops occupied 93, 94, and 96% of corn, soybean, and cotton acreage, respectively, in 2014 (USDA-ERS, 2015). Since 2011, there has been significant adoption (i.e., over 50%) of RR alfalfa in some regions but little in others, but adoption is necessarily slow in this crop due to its perennial nature. New plantings to replace existing alfalfa fields typically occur every 3–4 yr, and in some regions every 5–8 yr or longer, so maximum adoption rates are likely to be between about 12 and 20% per year. It is estimated that adoption by growers nationwide may have been in the 30% range in 2014, with major differences between eastern and western states (based on communications from seed companies and cooperative extension sources).

Concerns of GE-sensitive Growers

Whatever the eventual level of adoption of GE traits in alfalfa is, the presence of a commercially available GE trait raises concerns by those who grow alfalfa for GE-sensitive markets. Specifically, there are concerns about the potential for gene flow via bee-mediated pollen transfer from field to field or other sources of unwanted low-level presence of a gene that would serve to negatively affect organic or GE-sensitive alfalfa fields or hay lots—potentially with loss of certification or other market consequences.

GE-Sensitive Markets

GE-sensitive markets are those markets that specifically reject genetically engineered crops. Farmers in these markets may be harmed by rejection of their product if that product contains GE material or has a level of unwanted presence of the gene that is too high to be tolerated by that market. This does not include those who simply choose not to grow GE crops. There are many farmers who do not grow RR (GE) alfalfa because they grow crop mixtures where it has less utility, they do not use herbicides, or they have effective weed-control programs without it. All three groups would be described as conventional growers for non-sensitive markets.

DEFINING “NON-GE ALFALFA FORAGE”

For the purposes of market assurance, “non-GE alfalfa forage” can be defined as alfalfa or alfalfa-grass mixtures that have been produced implementing recommendations for non-GE management practices and the hay has been determined to be “non-detect” using an appropriate sensitivity threshold and level of detection for a specific market.

The recommendations for non-GE management practices are provided below.

Most conventional markets do not require defining “non-GE status,” since those markets are not considered sensitive to the trait. However, a definition of “non-GE alfalfa hay” is likely to be of use to those producing for sensitive markets.

A Process-Based Approach

The management practices suggested here are a process-based approach similar to that taken for other purposes,

such as certified organic production or certified seed production. Typically, this will be production practices for hay which is non-detect for a GE trait below a level of detection determined for specific market purposes. Note that this definition does not include the concept of GE-free hay.

The Impossibility of a “GE-Free” Designation

Some governments have not approved specific GE traits and in so doing have intended a zero tolerance for the importation of that trait in an agricultural product. Likewise, some buyers or consumers wish to have GE-free crops. However, to analytically and practically declare an agricultural product as GE-free, that is, containing no presence of a GE trait is a technical and practical impossibility. To be 100% assured that a hay mass is GE-free, every last gram of that mass must be tested, leaving none for its intended use.

For example, a single stem or a few pounds in a 200-ton hay crop (consisting of 400,000 lbs and billions of stems) would constitute low-level presence in a technical sense. However, it is highly unlikely that any sampling or analytical method would detect this amount, since this level of low-level presence is likely to be much lower than the capability of any sampling or detection method to detect it. There are sampling, sample-handling, and analytical protocols that determine the limits of detection for small amounts of a trait.

“Non-GE” Defined within a Production Protocol and the Limits of Testing

Declaration of non-GE status, therefore, is made within a definition of the threshold of tolerance, implementation of a protocol to prevent unwanted gene presence, and recognition of the analytical limits of detection and the sampling method. A declaration of non-GE-status hay may include other stewardship methods, such as care in labeling, management of inventory, and steps to prevent contamination in the field or during handling. Recognition of the role of testing and sampling variation must also be considered.

DETERMINING TOLERANCES FOR LOW-LEVEL PRESENCE IN NON-GE HAY

The primary GE-sensitive markets for alfalfa hay are export (Fig. 1) and organic (Fig. 2), which have grown to approximately 3.5 and 1.5%, respectively, of US alfalfa production in recent years. These markets differ significantly in their sensitivity to GE traits, since some export markets do accept GE alfalfa. Organic markets do not. Some sensitivity to the GE trait (preference for non-GE) may also come from horse-hay producers or other markets. The total extent of GE-sensitive markets for alfalfa in the United States is unknown but was estimated by Putnam (2006) at 3–5% of hay acreage. The importance of producing non-GE hay may be especially important in high-export states such as Washington and California.

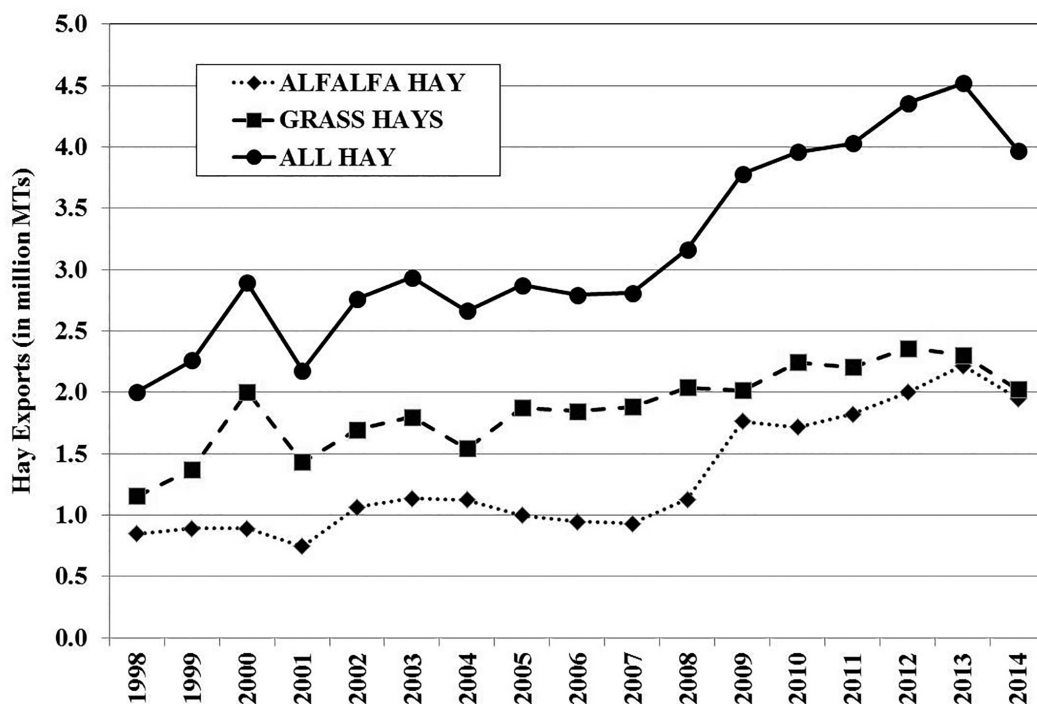


Fig. 1. Exports of alfalfa and grassy hay from United States. The drop in 2014 was partially due to a port strike, lower demand by the United Arab Emirates, changes in the US dollar, and the GE sensitivity of alfalfa in China. In spite of this export reduction, alfalfa exports to China increased by 30% in 2014 vs. 2013. (Source: US Department of Commerce, National Agric. Statistics Services).

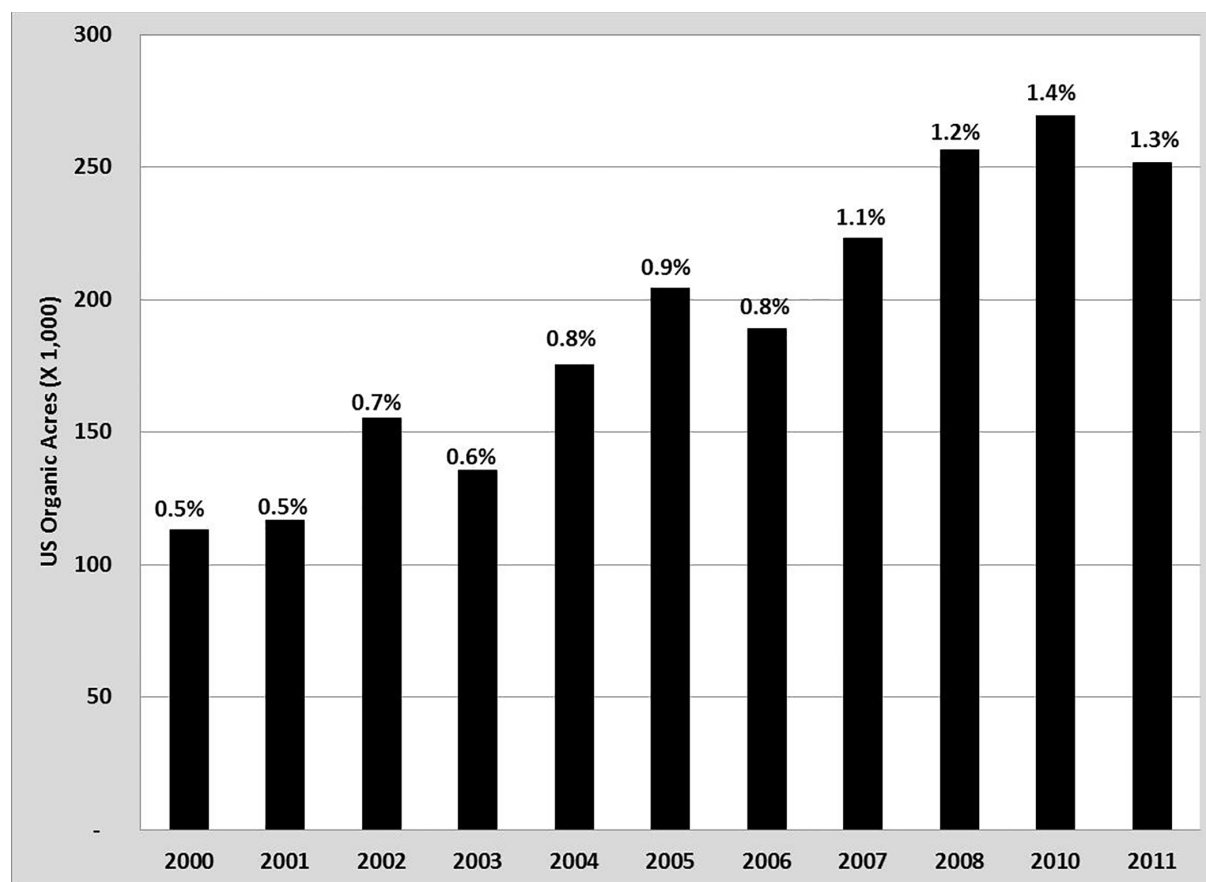


Fig. 2. Acreage of organic alfalfa, 2000–2011. Percentage of US acreage is shown for each year. (Source: USDA Economic Research Service).

In 2014, China began using DNA-based polymerase chain reaction (PCR) testing to detect the presence of the RR trait in conventional alfalfa exported to China. US exporters had been using less-sensitive protein-based enzyme-linked immunosorbent assay (ELISA) test strips for GE detection. China's new testing regime detected the RR trait in hay that was GE non-detect using the ELISA test. In late 2014 there was significant rejection of alfalfa-hay shipments to China because of low-level presence of the RR alfalfa gene, resulting in millions of dollars of losses to exporters and a reported overall loss of market strength (Wheat, 2014). Alfalfa-hay exporters in the United States are now using third-party testing labs for the detection of low-level presence with the PCR method. However, despite these problems, alfalfa exports to China increased by 22% in 2014 from 2013 and increased again in 2015 (Putnam et al., 2015). These events serve to emphasize the importance of producing and assuring non-GE alfalfa for sensitive markets.

GE Sensitivity Varies by Market

It's important to emphasize that the tolerance thresholds for low-level presence may range from non-detect by laboratory analysis to simply written or oral assurances of non-GE status. There are two basic types of threshold tolerance in practice: regulatory and market-based.

Regulatory Tolerances

Requirements originate when a GE trait is not approved in a country, and so a trait cannot be imported or grown. Essentially this is a zero tolerance, but it is circumscribed by the limits to testing. The requirements for non-GE assurances differ by country but could be enforced via testing requirements or written statements of non-GE status depending on the degree of acceptance. In some countries, any detection is considered to be cause for rejection, thus testing by PCR, with a limit of detection of approximately 0.1%, may be required, such as is currently the case with hay and seed exports to China. This does not pertain to US markets, or to Japan, Korea, or Taiwan, where the RR alfalfa trait is approved, but there are still market sensitivities. Middle East export markets generally don't allow GE hay but do not have a regulatory infrastructure or a defined threshold of tolerance.

Market Tolerances

Market-based thresholds of tolerance of low-level presence are determined solely on the basis of customer preference, which includes organic certification. US organic markets must adhere to a process-based market (not regulatory) requirement for organic certification, which requires non-GE hay for organic animal production. However, there is no defined threshold for low-level presence for organic; tolerance levels of 0.9–1.5% have been proposed. For human food markets, GE presence (of approved traits) up to 0.9% is tolerated for food products imported into the EU, and above that amount, the

product must be labeled as containing a GE product. Unapproved traits fall under the regulatory threshold (above), not a market threshold. There are currently no uniform labeling requirements for food products in the USA, although this may change (see Van Eenennaam et al., 2014). In Japan, food must be labeled if more than 5% of it contains a GE trait.

In terms of markets, the degree of sensitivity of a small amount of unwanted low-level presence is likely to differ between individual buyers. In the case of exports, some growers must sign contracts assuring non-GE status of hay crops, and organic buyers must source organic hay, part of which is defined by the non-GE status of the crop. However, in neither case is a tolerance threshold fully described. Some exports of RR alfalfa have occurred in the last 3 yr to Japan and Korea. Additionally, for some markets, the degree of sensitivity may change over time; for example, if China approves the RR alfalfa trait, sensitivity to GE alfalfa in that country will likely diminish or change from regulatory to a market-based sensitivity.

Market Tolerances to Low-Level Presence Cannot Be Derived Scientifically

A tolerance standard based on animal or human health considerations for low-level presence of the RR gene in alfalfa is not possible. This is because peer-reviewed research has confirmed the safety of the current traits contained in alfalfa (USDA-APHIS, 2011; Van Eenennaam, 2014; USDA-APHIS, 2014). Therefore, standards can be determined only by market factors, unlike some contaminant factors in hays, such as toxic weeds or nitrates (Puschner, 2008), which may have negative effects on livestock health at a given concentration and for which a level of safety can be derived from toxicity studies.

The market tolerance threshold for a GE crop is similar to other unwanted market-determined contaminants in agricultural products, such as a small amount of barley present in a wheat crop or the amount of forage grass allowed in an otherwise pure alfalfa-hay product. Zero tolerances are rare in agricultural products, even for toxic factors, and they are not practical for nontoxic factors. Market threshold tolerances for unwanted product contaminants are determined by industry habit, the degree of impact on product utilization, and a practical understanding of agricultural practices. Market tolerances may also include the low-level presence of an otherwise safe gene product (e.g., RR alfalfa) in a hay product that is produced for a GE-sensitive market.

Proposed Tolerances

We propose tolerances for low-level presence as defined and described in Table 1. Tolerance levels for unapproved traits in a country (regulatory tolerance levels) are based on practical limits of detection and sampling utilizing PCR technology. Proposed tolerance levels for marketing purposes are made on the basis of the current tolerances of the most sensitive markets (e.g., European market for food products). Additionally, for market tolerances, successful

Table 1. Tolerance levels for low-level presence of genetically engineered (GE) genes in alfalfa hay grown for markets of varying sensitivity.

Name of crop product	Type of market	Non-GE management protocol followed?	Tolerance level for non-detect	Method used to confirm
			% of hay mass	
GE-alfalfa hay: Roundup Ready (RR) or HarvXtra (RL) alfalfa	Non GE-sensitive	No	100%	Not necessary, but enzyme-linked immunosorbent assay (ELISA) test strips can be used to confirm RR. Polymerase chain reaction (PCR) must be used to confirm RL
Conventional	Non GE-sensitive (may include GE traits or not)	No	N/A	Not necessary
Conventional non-GE alfalfa hay (Non-detect \leq 0.9%)	Market-based or organic sensitivity (GE trait is not desired by buyer or non-GE is required for market certification)	Yes	\leq 0.9%	ELISA test strips or quantitative PCR†
Conventional non-GE alfalfa hay (Non-detect \leq 0.1)	Regulatory sensitivity (GE trait is not legally permitted in country)	Yes	\leq 0.1%	PCR†

† Confirmation of sensitivity of ELISA strips and PCR at different levels should be confirmed at desired level of detection. There may be ELISA tests with sensitivity at 0.1% and below available in the market, but generally PCR is preferred.

production of seed below the low-level presence of 0.9% appears to be practical, and this is probably the most important source of low-level presence in hay production.

Detection of the Low-Lignin GE trait

Table 1 shows that for the RR trait, either protein-based test strips or PCR can be used for detection, depending on the desired tolerance for low-level presence. HarvXtra is a GE trait that relies on suppression of one of the genes in the lignin biosynthetic pathway, thus no novel GE protein is produced and only genetics-based PCR methods can be used for detection. Forage Genetics International, the developer of the low-lignin trait, has indicated that the HarvXtra trait will be sold in combination with the RR trait in all HarvXtra varieties (thus, it is a stacked trait), with a trait purity of about 90% for both traits. In bags of commercial HarvXtra seed, about 10% of the seed will contain the HarvXtra trait but not the RR trait, but this will not be the case for plants in hay fields. Glyphosate spraying during stand establishment will eliminate RR nulls and any plants that contain the HarvXtra trait but not the RR trait will be eliminated. Thus detection of the RR trait with PCR or ELISA strips at low levels in stacked HarvXtra/RR hay fields should accurately predict the level of GE presence including both traits. However, if separate detection of the HarvXtra trait is desired, PCR techniques must be used at any level.

SOURCES OF UNWANTED GENE PRESENCE IN ALFALFA HAY

There are four potential sources of the unwanted presence of a GE trait in alfalfa hay. Each of these sources of low-level presence represents different levels of risk.

Risk of Low-Level Presence in the Seed Source

The most important source of low-level presence is likely to be the planting of seed that already has some amount of the gene present. Seed companies have reported that they produce zero low-level presence in most conventional seed, but there is low-level presence in some seed lots, generally $< 0.5\%$, which is sufficient for most insensitive and market-based requirements. However, these levels are less likely to satisfy regulatory-based tolerance thresholds. The low-level presence of GE traits in conventional seed may trace to the pollination period if the conventional seed crop was grown in proximity to a RR alfalfa field, or it could have occurred during harvesting, seed cleaning, and processing. Alfalfa is an obligate outcrossing plant, and insect-mediated cross-pollination (and gene flow) is necessary for full seed production (Van Deynze et al., 2008). Unless there is selection pressure, which shifts the genetic makeup in hay fields (not probable), whatever low-level presence exists in seed will be expressed in the hay crop. Thus the most important first step in assuring non-GE hay is the selection of non-detect seed at planting.

Risk of Equipment Movement between Fields

Since balers move from field to field, partial bales or foliage may be present in the baler, and that material is sometimes combined with forage from other fields. When GE alfalfa is present in balers or is carried by swathers or rakes, these become another source of its movement into conventional GE-sensitive fields.

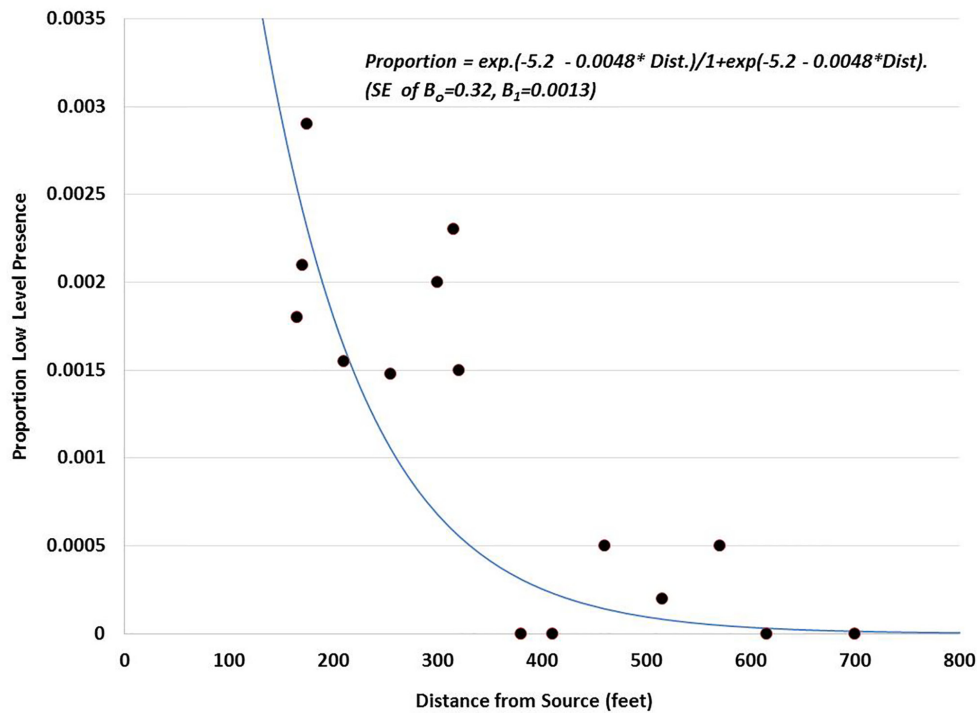


Fig. 3. Gene flow from hay to seed, UC Davis, 2008. Roundup Ready hay harvested at 50% bloom was grown 165 ft from seed-producing fields, which were allowed to produce fully mature seed. This represents a starting point to estimate hay-to-hay gene flow (Data from L. Teuber, UC Davis).

Risk of Misidentification after Harvest

After harvest, if inventories are not managed carefully, bales, partial stacks, or entire stacks can be mistakenly included in non-GE alfalfa-hay lots, resulting in unwanted presence of the gene.

Risk of Gene Flow in Hay

Last, gene flow between neighboring fields poses a risk for low-level presence in otherwise non-GE alfalfa forage. While much has been made of this source of low-level presence, in practice, it may be the least probable source of low-level presence.

UNDERSTANDING AND MINIMIZING RISKS OF GENE FLOW IN HAY

Gene flow of biotech traits in alfalfa has been thoroughly reviewed elsewhere (Van Deynze et al., 2008). To date, we are not aware of any field research quantifying the potential for gene flow between alfalfa-hay fields. However, the *upper limits* for the possibility of gene flow in hay fields can be understood by examining evidence from seed experiments. A field experiment was conducted on the campus of the University of California, Davis from 2008 to 2010 to examine the risk of hay-to-seed gene flow. This study was conducted with four replicate “receptor” seed fields (conventional) and a single GE “source” field (100% RR) managed as a hay crop. Total sizes of the source fields were equal to the receptor fields, and the receptor seed fields were oriented in four blocks in an X pattern to account for wind effects on the movement of pollinators. Receptor fields began at 165 ft from the source field, and were 30 ft wide and 400 ft

long. Active pollinators (honeybees) were placed close to the center of the trial, and the source hay field was allowed to reach 50% bloom before each hay harvest. There were five hay harvests per year. The receptor seed fields were brought to seed maturity, and seed was harvested at regular intervals to detect the effect of distance on gene movement.

Upper Limits of Gene Flow in Hay

Gene transfer between these closely spaced plots were in the range of 0.15–0.25% at the closest distance from the source to the recipient plots, and decayed exponentially from that point to near zero levels at 300–600 ft (90–200 m; Fig. 3). These numbers are approximately the same as or greater than the levels of low-level presence reported by seed companies between commercial hay fields and commercial seed-production fields, where size of field may have an effect on the percentages of gene transfer.

This research quantifies the gene flow from hay to seed fields, but there is a range of environmental barriers to such transfer between neighboring hay fields. These barriers would make significant movement from a biotech crop to a neighboring non-biotech alfalfa field much less probable than that in Fig. 3.

Determining the Probability of Hay-to-Hay Gene Flow

The potential for a trait to be transferred from one alfalfa hay field to another in quantities sufficient to result in detectable low-level presence in hay is dependent on a series of steps, each of which has a particular probability of occurring (Fig. 4). While the exact probability of each step

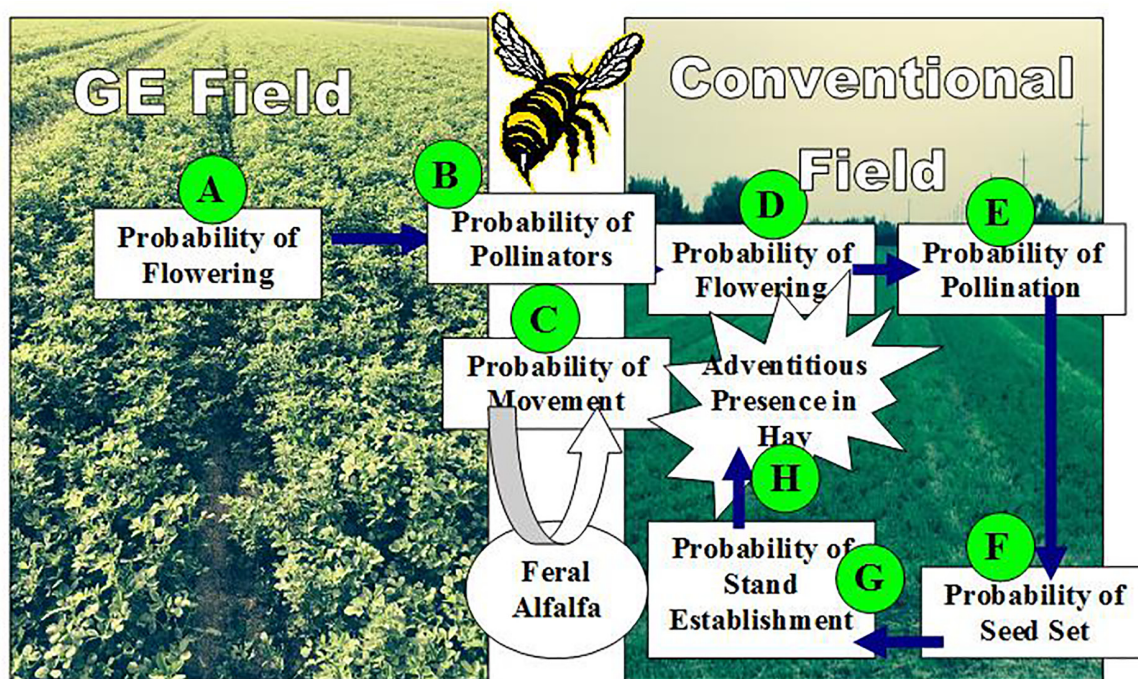


Fig. 4. Steps necessary for gene flow to occur between biotech and conventional alfalfa forage production fields sufficient to cause adventitious presence in hay.

is not known, an analysis of the potential for unwanted low-level presence in hay begins with an estimation of the probability of these steps occurring in practice.

- **Probability of Simultaneous Flowering:** In order for gene transfer to occur, flowering must be simultaneous between two fields (Fig. 4 A and D). Most alfalfa fields harvested for dairy production are harvested in the bud or vegetative stages, before any appreciable bloom has occurred to attain high quality, but depending on the intended market, some fields may have 10% or more flowering. Since pollen has a very short life (hours or days), two fields must flower at roughly the same time to allow gene transfer. Flowering is greater under hot midsummer conditions and with delayed harvest.
- **Probability of the Presence of Pollinators:** For pollen to flow between fields, pollinators must be present and active (Fig. 4 B). These include honeybees (*Aphis mellifera* L.), alkali bees (*Nomia melanderi* Cockerell), and leafcutter bees (*Megachile rotundata* Frabricius). Alfalfa is not a preferred nectar or pollen source for pollinators, and other flowering plants are usually preferred, so alfalfa seed growers must place hives and manage pollination carefully in seed fields to accomplish cross pollination (Rincker et al., 1988). Pollinators are typically not as plentiful in hay fields as in seed fields, since beehives are deliberately placed near seed fields, not hay fields.
- **Distance-Probability of Pollen Movement:** The probability that pollen will move from one field to another is primarily a function of distance (Fig. 3; Van

Deynze et al., 2008). Pollen movement is *not* the same as gene transfer (Mueller, 2004), which requires the additional steps of fertilization and seed production (Fig. 4 E and F). Most pollen movement occurs within fields given the close proximity of neighboring plants to the pollinators. The most effective pollination is accomplished within a 90-m radius of a hive (Rincker et al., 1988). The probability of pollen movement will depend on factors such as wind direction, pollinator activity, and nearby feral (wild) alfalfa, which may act as a “bridge” between fields (Fig. 4).

- **Probability of Fertilization:** Once pollinators have transferred pollen from one field to another, they must land on and trip flowers to deliver the pollen grains. Alfalfa requires floret tripping by insects for delivery of pollen from another plant. The pollen must embed on the stigma, grow a pollen tube through the style, and fertilize the ovule (Viands et al., 1988). This process takes 24–32 h. Fertilization of the ovule completes gene transfer, but not all pollen transferred results in gene transfer, because fertilization of the ovule fails or the pollen cells die.
- **Probability of Seed Maturation and Production:** For low-level presence to occur to a significant degree in hay crops, several additional steps are required. Fertilized embryos must then mature into seed (Fig. 4 E). Flowering typically occurs about 35 d after re-growth, and seed maturation generally occurs an additional 20–40 d after mid-bloom flowering (so 60–70 d total; Rincker et al., 1988). Therefore, seed maturation is not a normal consequence of forage production, since forage harvests are generally spaced 28–35

d apart. However, seed production is feasible on the very small number of plants that have unharvested stems, on hay that is harvested very late, on hay that is grown under intense heat conditions, and on plants located on the edges of fields and ditches that have not been harvested (feral alfalfa). However seed maturation is not a common outcome of hay production and occurs only under specific conditions.

- **Probability that Seed Falls to the Ground and Germinates:** For low-level presence to occur in a neighboring hay crop from gene flow, any seed produced must fall to the ground, germinate, and produce a plant. The seed must have sufficient moisture and coverage on the soil surface to be able to germinate and emerge in a previously established alfalfa stand. Deliberate efforts at overseeding alfalfa to increase the density of existing alfalfa stands via grain drills are typically unsuccessful (Canevari et al., 2001) due to competition, an inadequate seedbed, and allelopathy of the existing plants in the stand. Thus, seed that falls onto an unprepared seedbed with established alfalfa plants would have a reduced chance of success.
- **Probability of Survival of New Alfalfa Plant Recruits:** A seedling germinating in an existing alfalfa stand must survive intense competition from neighboring alfalfa plants and weeds to become established and contribute to yield. In order for gene presence to be detected, the forage containing that gene must contribute to the forage yield of the crop. Alfalfa is a very weak seedling during early growth and does not survive well under intense competition from either weeds or existing alfalfa plants. Growers who have attempted to overseed alfalfa into existing alfalfa stands have frequently been disappointed at not only the low germination, but also the low survival and productivity of the plants that do somehow manage to germinate and grow (Canevari et al., 2001; Canevari and Putnam, 2007).

Environmental Filters Limit Gene Flow in Alfalfa Hay

The above probabilities, to the extent that they are less than 100%, serve as environmental filters that limit gene flow in hay crops. Thus, if little simultaneous flowering occurs, pollinators are few, the crop is harvested before significant flowering, the few seeds produced are removed during harvest, and the conditions for seed germination and survival are limited, gene flow will be greatly reduced in hay-to-hay compared with seed-to-seed or hay-to-seed gene flow. Furthermore, if the probability of an event is zero, this effectively halts any chance of gene flow; for example, if zero flowering occurs and all hay is harvested and removed, then gene flow between hay crops drops to zero.

The observed level of about 0.25% low-level presence in close-proximity, hay-to-seed gene flow found in Teuber's experiment (Fig. 3) represents a worst-case scenario for gene flow between hay crops. Observed levels of

hay-to-seed gene flow reported by seed companies have been at this level or below. In hay-to-hay gene flow, this level is then reduced significantly because of these environmental filters. For example, if 1% of the stems in an alfalfa hay field set seed due to a few plants that remain unharvested (in actual practice, 1% is a very large number of remaining stems), and of those, 1% of the seeds germinate and survive in the stand, the contribution to low-level presence would be predicted to be 0.000025% at the closest distance (0.25% at 165 ft \times 0.01 \times 0.01 = 0.000025%). The gene flow would be diluted even more at larger distances within fields and between fields, since this estimate is at 165 ft. Gene flow may not be zero between neighboring alfalfa hay fields, but it is likely to be much lower than the capability for detection by normal sampling and analysis methods, which are normally in the range of 0.1% and above, depending on level of tolerance (Table 1). The potential for gene flow is probably higher in the very hot regions, such as the deserts of California and Arizona, where alfalfa flowers profusely at 28 d, or in situations where alfalfa is harvested very late. Typically, though, 4–6 wk after flowering is required to produce viable seed.

Although these probabilities are not known with precision, it is clear that the probability for gene transfer in hay fields under normal conditions would be very small, far less than the 0.1% low-level presence that is important for sensitive markets under most conditions.

The highest probability of low-level presence in hay fields most probably originates from low-level presence in seed, not transfer between hay fields. Thus, excess care should be taken to purchase certified seed for those growers interested in sensitive markets.

TESTING FOR THE DETECTION OF PRESENCE IN HAY

There is strong interest in testing hay for the presence of an unwanted GE product at a low level (Fig. 5). However, such testing should be considered within the context of the process-based series of steps that reduced unwanted low-level presence.

Field Trial

A trial was conducted in 2005–2006 in Meridian, ID to determine the ability to detect low-level presence in hay harvested from seed that was deliberately spiked with a low-level presence of the RR trait. Roundup Ready seed was added on a weight basis to non-RR seed at the rates of 0, 1, 5, and 10% and planted at 25 lbs/acre in a farmer's field on four one-acre plots side-by-side in 2006 (Fig. 6). Hay was harvested from each of these blocks in 2006 from cutting 2 and cutting 3 and stacked in separate blocks. A standard hay-coring device (Penn State Sampler) was used to sample each stack with the standardized sampling method (Putnam and Orloff, 2002) with 20 individual cores composited to a single sample, each sample replicated five times to create five composited samples for each of two cuttings from these four fields. Samples were brought to Davis, CA, split carefully with

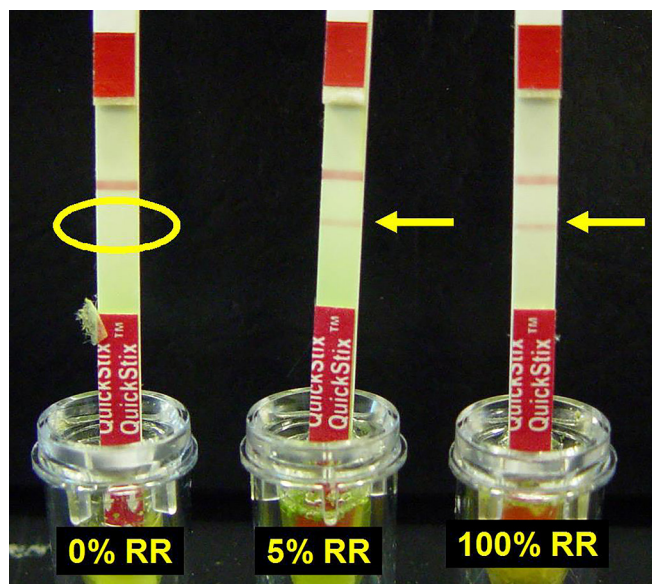


Fig. 5. Test strips have the potential to detect adventitious presence of Roundup Ready (RR) alfalfa in hay and could assist in confirming non-biotech hay for sensitive markets. Arrows show positive reading for 5% RR and 100% RR samples (right), and circle shows negative reading for the control sample (left). However, the limits of detection determined by the method must be considered.



Fig. 6. Field study on low-level gene presence with varying levels of low-level presence (0%, 1%, 5%, 10%) of Roundup Ready (RR) planted in Meridian, ID, 2005–6. Hay was harvested twice from these fields, sampled and low-level presence determined.

a riffle-splitting device (Fischer Labs), and one-half of each sample was ground with a 1-mil laboratory cyclone grinder. Each sample was then again split and the samples coded and sent to each of three laboratories in a double-blind test of the ability to detect the RR trait in the hay samples, using ELISA test strips (Fig. 5).

Test Strips Identify GE and non-GE Hay

The results are shown in Table 2. All of the samples (5 replications each \times 2 cuts \times 3 laboratory measurements \times 2 laboratory test strips \times ground and unground = 120 samples for each percentage level) for the control plots resulted in non-detect in the sample (Table 2). All of the samples at 5 and 10% resulted in detection (Table 2). At the 1% level of GE in the seed, more than 90% of the

Table 2. Detection of the CP4-EPSPS gene in alfalfa hay harvested from seed grown with various known levels of Roundup Ready alfalfa.†

Treat- ment	Repli- cation	Assay operator					
		Idaho		Washington		California	
		Test Strip					
		EL6285	RUR	EL6285	RUR	EL6285	RUR
Control	1	—	—	—	—	—	—
	2	—	—	—	—	—	—
	3	—	—	—	—	—	—
	4	—	—	—	—	—	—
	5	—	—	—	—	—	—
1 %	1	+	+	++	+	++	+
	2	+	+	+++	++	+++	++
	3	++	+	+++	+	+++	+
	4	+	+	+	++	+	++
	5	+	+	+++	++	+++	++
5 %	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
	4	+	+	+	+	+	+
	5	+	+	+	+	+	+
10 %	1	+	+	+	+	+	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	+
	4	+	+	+	+	+	+
	5	+	+	+	+	+	+

† EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase. Two commercial test strips were used (Envirologix and RUR). Five replicates of composited 20 core samples were used, and three different labs (Washington, Idaho, and California) independently measured split blind samples. Strips had an advertised detection limit of 5%. Asterisks (*) indicate that the signal was faintly detected by the operator. Multiple asterisks indicate increased faintness. These are results from ground samples cut 1. Similar results were found in cut 2 and in unground samples from both cuts (four sets total).

observations resulted in a positive reading, but the readings were often faint (indicated by *). The same results were found for the second cutting and for unground samples (data not shown). It should be noted that the test strips were designed to meet a 5% detection level but were largely (but not always) successful at the 1% level. Currently, ELISA test strips are available to detect low-level presence at the 0.1% level (Envirologix) but have not been widely tested at that level of sensitivity. Currently, it is recommended to use PCR for low levels of detection in alfalfa hay destined for highly sensitive markets such as the regulated market in China.

These results serve to confirm that (i) low-level presence in the hay can be detected with standard ELISA methodology in hay that is planted at the 1, 5, and 10% level; (ii) that controls with no presence resulted in non-detect results; and (iii) that standard hay-sampling methods are capable of detecting low-level presence at these levels, provided the trait is evenly distributed throughout the hay mass.

PROTOCOL FOR PRODUCING NON-GE ALFALFA

Alfalfa hay growers who wish to sell in markets sensitive to biotech traits may be concerned with methods to ensure practical coexistence of these genetically diverse systems (biotech origin and non-biotech origin). The stewardship of both non-biotech and biotech traits within a region will depend on a range of practices, beginning with seed production and purity.

Selecting Certified Cultivars for Seed Purity and Quality

It is difficult to overemphasize the importance of cultivar and seed choice for those hay growers who are selling in sensitive markets and are concerned with the purity of their alfalfa stands. This is probably the most crucial step to assure trait purity in a hay product. Alfalfa seed is produced on small acreage in the Pacific Northwest, California, and a few other Western states. Seed production is an exacting enterprise, and seed growers take pride in their ability to manage a complex system to produce high-quality seed (Mueller, 1995; Mueller, 2008). Industry standards for isolation have been developed, and crop inspection and certification services are available in each seed-producing state to assure seed purity, which includes variety identity, seed quality, and lack of contamination with weeds or foreign matter (CCIA, 2015). The incorporation of biotech traits in alfalfa seed production has been considered in detail elsewhere (Mueller, 2004). Thus, the first step in the process of stewardship is selection of certified varieties backed by a company with high standards for seed production.

Recommendation

Request non-GE alfalfa seed that has been determined by a lab test to be non-detect below the level of tolerance demanded for the particular market (Table 1). **Note:** PCR testing is required for China currently. All major seed-production companies have reported that they have available non-GE alfalfa seed that has been confirmed through PCR testing to meet the low-level presence tolerances described in Table 1.

Reducing the Possibility of Gene Flow

Reducing the possibility of excessive gene flow involves understanding the distances of GE-sensitive fields to GE-containing fields and minimizing the possibility of simultaneous flowering. Harvest management can assure that flowering of a GE-sensitive field does not occur at the same time as a neighboring GE-containing field. Harvesting before excessive flowering is a key management factor and is useful for reducing the risks of gene flow. Feral (wild) alfalfa may occur along field edges, ditch banks, or roadsides and remain unharvested. Its origin is not known, but it could be from older plantings by highway departments, spilled seed, or seed transferred by hay trucks or moved by birds. Since feral alfalfa is more likely to flower and set seed and feral may act as a “bridge” for

pollinators between distant fields, control of feral alfalfa is a prudent method to prevent movement of genes between hay fields. As described earlier, because effective hay-to-hay gene flow requires a sequence of events, eliminating any one of those factors will eliminate hay-to-hay gene flow. The easiest step is to harvest before ripe seed set.

Recommendation

Determine the distances to GE-fields, harvest before excessive flowering (or certainly before ripe seed is formed), prevent synchronous flowering, and remove feral alfalfa from areas in close proximity to GE-sensitive alfalfa fields to reduce the possibility of gene flow. **Note:** Several farmers are currently successfully producing GE and non-GE hay on closely situated fields (Simon, 2011).

Preventing Inadvertent Transfer of Hay during Harvest

In regions where both GE and non-GE alfalfa is grown, it is possible for equipment to move hay from field to field, especially with balers (where partial bales may be contained in a baler) but also with rakes or swathers.

Recommendation

Clean balers and equipment when moving between fields or alternatively reject the first few bales, which may contain unwanted genes. **Note:** Organic growers already must follow this practice when moving from nonorganic fields.

Identifying Non-GE Alfalfa Hay and Preventing Mixing of Lots

The coexistence of GE and non-GE alfalfa will require a higher level of awareness of crop identity for products destined for sensitive markets. This process may require some simple identification steps for hay lots to assure that the lots are not mixed and also keeping records during stand establishment (seed tags) that indicate that conventional varieties were sown. A “lot” in the hay industry is defined as a stack that is from the same field and cutting, comprises less than 200 tons, and is identified as to farm, field, and cutting (Putnam, 2002).

Recommendation

Prevent the mixing of hay lots, maintain identity, and assure customers of that identity through record keeping for the planting, harvesting, storage, and transport process for either GE-containing or non-GE alfalfa hay. **Note:** These practices are common on commercial hay farms (especially organic and export).

Understanding the Sensitivities and Tolerances of the Market

Non-GE hay must be produced to meet market demand, and as discussed above, the threshold of market or regulatory sensitivity must be determined (Table 1). A practical solution to coexistence requires a respect for sensitive markets, a determination of the level of tolerance, and recognition that a zero threshold of tolerance is both

nearly impossible to confirm scientifically and difficult to obtain practically. Given that the food crops in some of the most sensitive markets are 0.9% low-level presence in foods (Europe) and 5% in Japan, a market-based threshold of 0.9% may serve the purposes for most markets, with the exception of the regulatory, which currently requires non-detect at a limit of detection of approximately 0.1% (Table 1).

Recommendation

Determine the level of non-detect tolerance of specific markets (organic, export market, or export regulatory).

Note: Although there is no market tolerance for regulated GE traits in China, the level of detection based on their proscribed methods is approximately 0.1%.

Testing to Confirm Non-GE Status in Hay

Testing of hay lots is the final step to confirm that hay lots either contain or do not contain biotech traits. Testing may or may not be required for specific markets, and in some cases a written or oral assurance of non-GE status may be adequate. ELISA test strips (Fig. 5) have been developed (e.g., <http://envirologix.com>) that identify the presence of the CP4 EPSPS protein in alfalfa hay, alfalfa seed, and other crops. Additionally, PCR techniques have been developed that identify the gene at low levels. Protocols for fresh leaf material, unground hay, and ground hay (for testing of routine cored hay samples by commercial laboratories) are available. Reported limits to detection and scientific evidence should be used to determine which method is adequate to meet the needs of specific markets (e.g., 0.9 or 0.1%).

Regardless of the method, sampling procedures are important for detection, and the detection of a gene may be limited by sampling method and type of low-level presence (whether randomly dispersed throughout a hay lot or just a few segments of a bale or an occasional bale). See Putnam 2014 for a full discussion of sampling methods for GE traits.

Recommendation

Testing for the presence or absence of a GE trait should be used in combination with all of process-based protocols above. The limits of detection of each specific method and the limitations of sampling should be considered when interpreting laboratory GE tests. **Note:** Due to the limitations of sampling and analysis at low-level tolerance levels, a combination of process-based and testing protocols are probably necessary.

SUMMARY

Coexistence strategies are a necessary and important component of successful production of both GE and non-GE alfalfa hay, consistent with consumer preferences and a farmer's right to farm. The majority of markets for alfalfa in the United States are not likely to be sensitive to the presence of a biotech trait, but both organic and export markets currently demand non-biotech alfalfa.

There is no reason to think that the coexistence of GE crops in regions where non-GE crops are desired cannot be successfully managed under most situations. A number of farmers have successfully produced both Non-GE hay for sensitive markets (organic or export) and GE alfalfa hay on nearby farms or adjacent fields. Communication and cooperation between farmers are obvious components of any coexistence strategy within a region. Common-sense steps for the production of non-GE alfalfa include primarily securing of non-detect (tested) seed, preventing accidental mixing of hay lots, and taking steps to prevent gene-flow between alfalfa fields. Market assurance can be further assured by hay-lot sampling and testing with a method (PCR or ELISA strips) appropriate for a given level of market or regulatory tolerance, but the limits of testing and sampling must be considered.

The degrees of sensitivity of different markets are likely to differ, thus, non-GE alfalfa produced for sensitive market purposes may have detection limits of 0.9%, whereas non-GE alfalfa produced for regions where the trait is not approved may have a limit of 0.1% detection. The latter is based on limits of detection of the most sensitive method available (PCR). Coexistence strategies for alfalfa hay require an understanding of the tolerances of diverse markets, the mechanisms for unwanted gene presence, and market assurance processes.

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