SENSITIVITY TO GE ALFALFA REMAINS IMPORTANT FOR ALFALFA EXPORT AND ORGANIC MARKETS

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ABSTRACT

Since the advent of Roundup-Ready (or RR) alfalfa, first in 2005 and fully in 2011, many growers have embraced Genetic Engineering (GE) technology, while others have been concerned about its impacts. There are currently two GE traits: RR and down-regulated lignin (named HarvXtra) commercialized in alfalfa. RR alfalfa is currently the more widely adopated. Two key markets are the most 'sensitive' to the presence of GE crop: organic and export, of which export is largest. While some countries (notably Japan) allow imports of RR alfalfa, other countries do not. Most exporters currently require non-GE alfalfa. China is the most sensitive market, and can reject imported loads tested to contain low-level presence of GE traits. Growers can meet these sensitive market demands, but they must pay attention to a series of steps to assure customers of the non-GE nature of their product. Key among these is the selection of seed tested to be non-GE at the level of sensitivity of their specific market. Coexistence strategies for alfalfa forage require an understanding of the sources of low-level presence, market tolerances of diverse markets, and market assurance processes.

INTRODUCTION

The commercialization of Roundup Ready (RR) alfalfa in 2005 and reduced-lignin alfalfa (HarveXtra) in 2014 as GE options has brought both promises and challenges to the alfalfa industry. Many growers have embraced the RR trait as a method to control weeds and improve quality. While the HarvXtra trait promises to improve quality and harvest flexibiltiy, it is currently in the process of commercialization (see presentations in this symposium). However, there are some markets that do not currently accept GE traits. Domestically, organic markets are foremost, but export markets are undoubtedly more important in terms of hay volume. China, the most sensitive market, has increased US imports from near zero ten years ago to nearly 1.5 million Metric tons in 2016 (see Putnam et al., this proceedings). In this article, the levels of sensitivity are discussed, along with methods to produce hay for sensitive markets.

WHAT MARKETS ARE SENSITIVE TO GE TRAITS?

Approximately 423,000 acres of organic alfalfa hay and silage were produced in 2011 (the lastest available statistics -USDA-ERS), about 2.1% of US production (1.3% considering hay only. Approximately 4.7% of US alfalfa is exported, but this is equivalent to about 15% of the 7 western states' hay production (see related article in this proceedings). These are the most GE-sensitive markets in the US, currently mostly rejecting the technology. Conventional dairies and most horse markets have not been sensitive to date.

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THE CONCEPT OF LOW LEVEL PRESENCE (LLP) AND IMPOSSIBILITY OF 'GE FREE'.

However, the level of sensitivity (or tolerance) of the different GE sensitive markets genuinely differs. Thus, the concept of LLP must be discussed, whether it should be zero or some small number. Low Level GE Presence (LLP) is the level of an unwanted GE trait in an otherwise non-GE crop.

Although all GE sensitive markets demand 'Non-GE' or even 'GE-Free' alfalfa, a moment of reflection should reveal that 'GE-Free' is both a conceptual and practical impossibility. To declare a product as 'GE-Free' (with verifiable data to support), every last morsel of that product must be tested for that trait. For a single container load of hay, for example, consisting of perhaps 25 tons of material this would require over 90 million tests for PCR or ELISA strips! Even if that could be accomplished, PCR is a very sensitive test but has lower limits of detection. So if the required LLP amount was very low, the ability to detect would be still challenging even if all the load was tested. Never mind that there would be nothing less for its intended use!

Issues related to sampling must also be considered, since sampling protocols can have a large influence on the ability to detect a trait at a low level (see Putnam, 2015 for a full discussion of sampling for LLP). Thus 'non-GE' alfalfa must be defined within the practical level of tolerance of a specific market, as well as analytical limits of testing and sampling.

MARKET AND REGULATORY INFLUENCES ON LLP

Organic Sensitivity – GE alfalfa is not permitted in organically-certified hay used for dairy or other livestock production. However, the level of LLP permitted is somewhat undefined and subject to approval of the certifying agency. The Non-GMO Project, a national program which provides a consumer-assurance sticker (Non-GMO verified) for non-GE food products in the US, defines an 'action threshold' of 5% for dairy and livestock feeds and 0.25% for seeds used to produce feeds for dairy production (see Non-GMO Project Standard, http://www.nongmoproject.org/wp-content/uploads/2016/08/Non-GMO-Project-Standard.pdf). Although elsewhere I have suggested a 0.9% threshold (see Putnam et al., 2016, this is similar to the EU standard for labelling of GE traits), it is clear that some organic producers may tolerate a somewhat higher (or lower) threshold. Since organic certification is a process-based certification (not an analytical determination), sometimes grower assurances are sufficient, but some buyers do test. This threshold of tolerance by definition cannot be determined scientifically since it determined by consumer preference—it is a market-driven determination.

Export Sensitivity – GE alfalfa (RR alfalfa) is permitted in some importing countries but not others. Japan allows importation of RR alfalfa at 100%, however, many importers even to Japan currently reject GE alfalfa due to logistical considerations (keeping track of GE and non-GE hay lots) and market concerns from their customers. Some markets, such as China, currently do not allow importation of GE alfalfa since the trait is not approved (although that is expected to change at some point). Thus this is a 'regulatory' threshold, not a market determined threshold. China is currently the most sensitive market; any level detected can lead to rejection. Current testing is generally conducted via Polymerase Chain Reaction (PCR), which is effectively approaching approximately 0.1% limits of detection , although any detection may result in rejection, even below this amount. Other markets may ask for non-GE alfalfa relying on written

assurances alone. Currently, many exporters are routinely testing alfalfa hay for LLP utilizing Polymerase Chain Reaction (PCR).

MEASUREMENT CONSIDERATIONS

We have conducted tests to determine the utility of several measurement techniques for detecting LLP in alfalfa hay samples. We obtained non-RR alfalfa hay and spiked it with different concentrations of RR alfalfa hay and analyzed these samples both by real-time PCR and Eliza strips. Real-time PCR quantifies the presence of the gene itself, while ELISA strips detect the presence of the CP4 EPSPS protein, which is produced by the gene. Results can be seen in Figure 1. Both methods 1) correctly identified the non-RR alfalfa (that is zero RR samples resulted in non-detect zero values). 2) Both methods correctly identified samples as being positive for the trait when they were spiked with approximately 0.1%-100% RR. 3) Both techniques) tended to predict the approximate quantity of the trait at levels below 1% (Figure 1), but the level of variation at these lower levels tended to be greater, especially for PCR. The Envirologix method included use of a 'Quikscan' instrument to detect intensity of signal of the ELISA strips (the reaction of the strips to the protein tends to become fainter and fainter as the protein concentration goes down). Thus detection of the RR trait at low levels is feasible with both methods. However, further work needs to be done to examine the methodology of the ELISA test strips at low levels. Note that detection of the HarvXtra trait directly would require use of PCR, since it does not produce a unique protein (it is a down-regulated trait intrinsic to alfalfa) However, since it will only be sold stacked with the RR trait at this time, detection of the RR trait may be a good proxy for the presence of either GE trait.

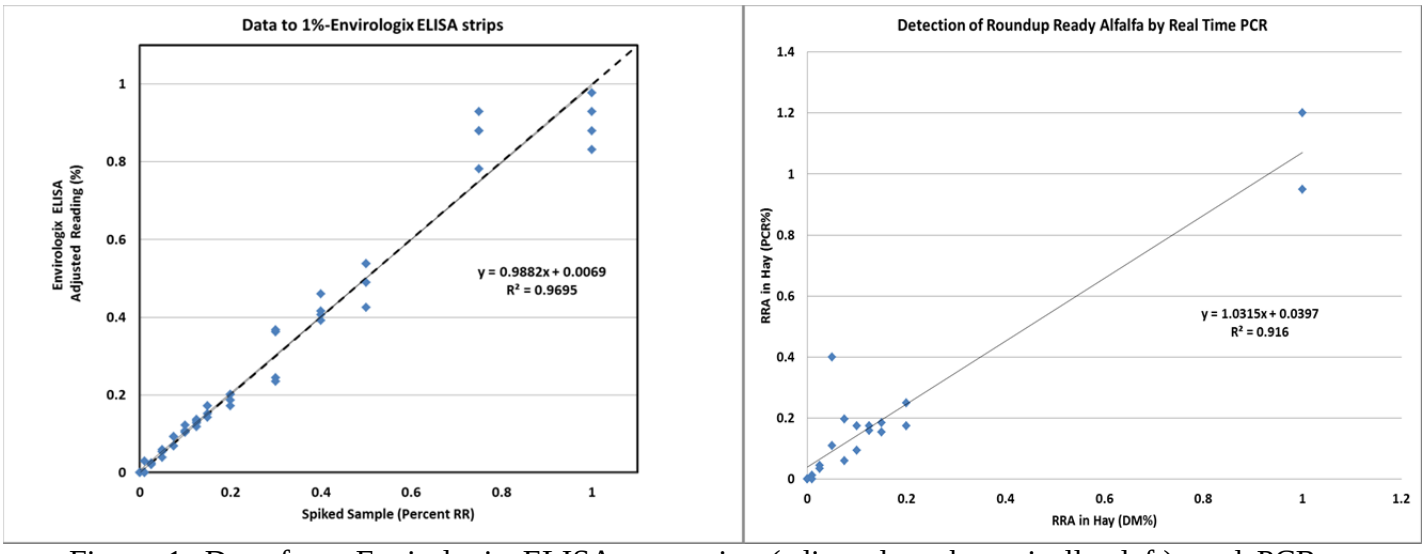

Figure 1. Data from Envirologix ELISA test strips (adjusted mathematically, left), and PCR analysis (right).

LIMITATIONS OF TESTING AND SETTING A PRACTICAL TOLERANCE LEVEL

Although the above data confirm that both PCR and ELISA tests provide useful information about LLP, and should be helpful in common hay trading, we should be cautious about the

limitations of testing. While it is tempting to rely solely upon testing for market assurance, there are reasons that this would not be a good strategy. The reasons are the following:

- Problems with sampling variation alone could produce false negatives with regards to LLP. A full discussion of sampling for LLP was provided at last year's symposium (Putnam, 2015) and recommendations provided. Remember that this sampling protocol is for an evenly distributed source of LLP that can be fairly represented in a ground hay sample, sampled utilizing standard protocols. Some sources of LLP, such as an occasional bale in an otherwise non-GE stack of hay, or mixing of partial lots are very likely not to be detected, or could be non-detect some of the time and detected other times. It all depends whether the GE bales happen to be represented in the sample or not.
- At very low tolerance thresholds (e.g. below about 0.2%) of LLP, minor problems in sample handling (dust in grinders, small amount of contamination in containers or with sampling procedures) could produce false positives. Some of the data reported by exporters show LLP at levels of 0.05% and below.
- Shipping containers full of hay are notoriously difficult to sample – access to bale faces is limited, so that fair sampling methods are constrained. Such methods require (as recommended) access to 30 sites to the butt-ends of bales randomly distributed across the hay mass.
- We should remember that analytical tests, especially PCR require a VERY small sample for analysis. Approximately 0.2 grams of material or less is used – this must represent many tons of material (in a stack up to 150-200 in a lot, or in a container, 20-25 tons). Thus, in addition to sampling error, sub-sampling error is likely to be great. Imagine that a method is meant to test 0.1% of a heterogeneous sample. That's 1 in 1,000 particles that must be fairly represented in a 0.2 Gram sample. How many particles in 0.2 grams, and what are the chances that either none or more than one might be represented? This may be an explanation for the greater variation in the PCR quantitative test at lower levels. The ELISA tests a larger sample, and appears to be less variable at low LLP at least in initial tests (Figure 1).
- Statistically sound testing methods do not absolutely guarantee a zero presence, only a probability of non-detect utilizing a given sampling protocol and at an analytical limit of detection.

Thus, establishment of protocols for producing non-GE alfalfa for both export and organic markets may assist buyers and producers meet specific market requirements, given a specific level of LLP tolerance. Several of the recommendations provided below (Non-GE Protocol) may provide market assurance to buyers of non-GE alfalfa – these are 'process based' steps that are generally not difficult to accomplish. But the first step is to decide upon an acceptable LLP level as the target.

DEFINING NON-GE ALFALFA FOR SPECIFIC MARKETS

The first step in this process is to define the market goal. As discussed above, there are differences in tolerance for LLP in alfalfa hay – and though some would claim a desire for 'GE free' – this is both a technical and practical impossibility, as discussed above. Below, I've suggested definitions for markets which include a definition of the Non-Detect level of tolerance. This guideline may be used to satisfy regulatory sensitive markets (e.g. China at the lower

tolerance level), or market-based sensitivity level (which could range from 0.9 to 5%). The regulatory tolerance level of Non-Detect at 0.1% is based upon an understanding of the lower limits of detection by PCR and the greater variability and uncertainty when very low detections are possible.

PROTOCOL FOR PRODUCING NON-GE ALFALFA BY GROWERS

Alfalfa hay growers who wish to sell into markets sensitive to biotech traits may be concerned with methods to ensure practical co-existence 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 upon a range of practices, beginning with seed production and purity. Since gene flow is limited in hay production, the selection of non-GE seed (that has been tested) is likely the most important of these protocols. For a full discussion of coexistence strategies see Putnam et al. (2016) and the NAFA website (NAFA, 2016).

I. Select Certified Cultivars for Seed Purity and Quality. Recommendation: Request non-GE alfalfa seed which has been determined by a lab test to be non-detect below the level of tolerance demanded for your market (Table 1). **Note:** PCR testing is required for China currently. All major seed production companies have reported availability of non-GE alfalfa seed confirmed through PCR testing to meet low-level presence tolerances described in Table 1.

II. Reduce Possibility of Gene Flow. Recommendation: Determine distances from your field to the closest GE-fields, harvest before excessive flowering, or certainly before ripe seed is formed, prevent synchronous flowering through harvesting schedules, 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.

III. Prevent Inadvertent Transfer of Hay During Harvest. Clean balers and equipment when moving between fields or alternatively reject the first few bales which may contain unwanted genes from the previously harvested field. **Note:** Organic growers already must follow this practice when moving from non-organic fields.

IV. Identification of Non-GE Alfalfa Hay/Prevent Mixing of Lots. Recommendations: 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:** This is commonly practiced already on commercial hay farms (especially organic and export).

V. Understand the Sensitivities and Tolerances of the Market. 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%. Tolerance for organic markets may depend upon the certifying agency and may range from about 0.9 to 5%.

VI. Testing to Confirm non-GE status in hay. Recommendation. Testing for the presence or absence of a GE trait should be used in combination with process-based protocols (1-5) 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 likely necessary. Currently, use of real-time PCR is the standard for export, but the quantitative ELISA strips appear to have considerable promise.

WHAT IS THE EVIDENCE FOR PRODUCTION OF NON-GE ALFALFA AND LLP?

Discussions with hay exporters have indicated that exporters have been very diligent in testing for LLP in alfalfa hay for export. Since China is the major export market for alfalfa currently, often exporters are testing every lot utilizing real-time PCR to indicate the non-GE status. According to some sources, about 0.5% of the loads (approaching 1.5 million tons total) shipped to China have been rejected in the past year due to LLP. This sounds small but is a major problem for those specific companies and companies take a risk that regulators in China will reject their lots. Thus rigorous testing has been implemented.

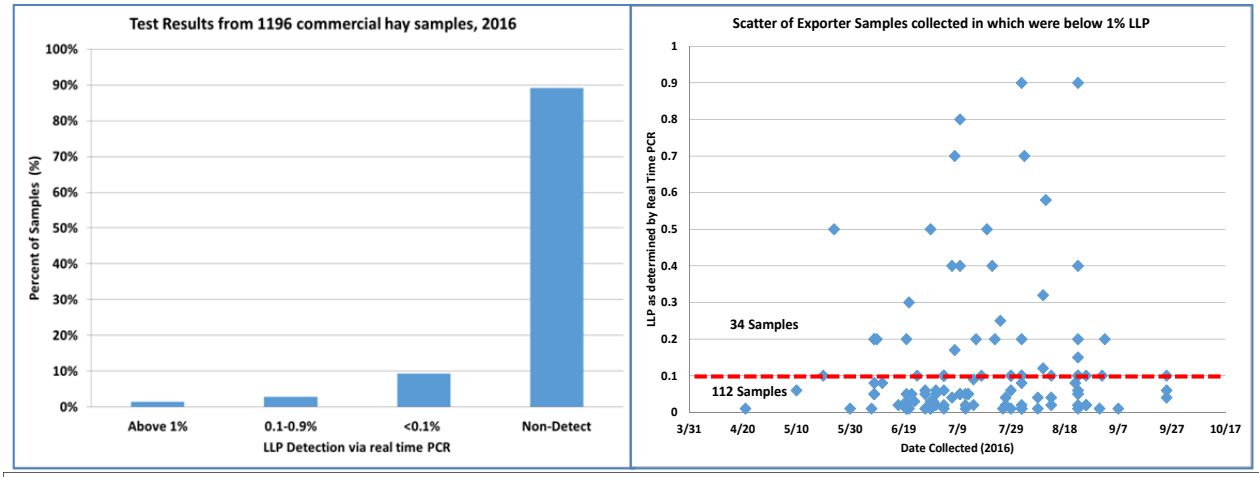

Figure 2. Test Results from a single company testing export hay for LLP in 2016 utilizing real-time PCR. The sources of this hay were primarily from the desert Southwest (Southern California, Arizona). The data on the right were from fields thought to be non-GE fields.

Data from one company shared data on their testing results from 2016 (Figure 2). They tested nearly 1200 samples by October of 2016, and 89% of these samples were non-detect via real-time PCR (Figure 2). 1.4% of these samples were above 1% LLP (5 were at 100% RR), 2.8% of these samples were between 0.1% and 0.9% LLP and 9.4 percent of the samples were at a low level presence below 0.1% (there were 45 samples reported with LLP of 0.01% to 0.05%).

These data indicate both that production of hay with non-detect GE traits is possible, and that testing for LLP is a problem for exporters who must reject loads. Over 10% of these samples came back positive, and as a result were rejected by the exporter. However, it's important to note that most of these were below 0.1% LLP (68 samples or 5.7% of the total). As pointed out above, our level of confidence at these lower levels of LLP are subject to greater variation and uncertainty – thus non-detect at 0.1% is recommended as a regulatory threshold (Table 1).

SUMMARY

Production of non-GE hay to meet market expectations is clearly possible. Alfalfa hay markets sensitive to GE traits in the United States remains a relatively small percentage of the total market but they are still important, particularly in western states. In terms of methods to produce Non-GE hay-special attention must be paid to the purchase of seed. Test seed prior to planting to be certain it meets market standards because this can be a primary cause for LLP. Testing alone is likely to be only one component of a market assurance process to meet the demands of a sensitive market. Following reasonable protocols and market-assurance methods including control of gene flow, prevention of inadvertent mixing are also necessary. For export hay, the expectation is that hay should meet 0.1% standard, similar to a PCR limit of detection, since detection at these levels (and below) by PCR is possible. However, relying on testing alone to meet market expectations is questionable since at very low levels of tolerance (e.g. <0.1%), the reliability of both sampling and analysis becomes more problematic.

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Table 1. Tolerance levels for low-level GE presence in alfalfa hay grown for markets of varying sensitivity (adapted from Putnam et al., 2016).

Name of Crop Product	Type of Market	Non-GE Management Protocol Followed?	Tolerance Level for Non-Detect (% of hay mass)	Method used to confirm
GE-Alfalfa Hay (Roundup Ready (RR) or HarvXtra (RL) Alfalfa	Non GE-Sensitive	No	100%	Not necessary, but ELISA test strips can be used to confirm RR. 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 (ND≤0.9% or ND<5%)	Market-based or Organic Sensitivity (GE trait is not desired by buyer or non-GE required for market certification)	Yes	≤0.9% or ≤ 5% depending upon market demand, and may depend upon certifying agency	ELISA Test Strips or quantitative PCR*
Conventional Non-GE Alfalfa Hay (ND≤0.1)	Regulatory Sensitivity (GE trait is not legally permitted in country)	Yes	≤0.1%	PCR*

*Confirmation of sensitivity of ELISA strips and Polymerase Chain Reaction (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-contact laboratories and company representatives.