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Quantification of Low-level GM Seed Presence in Canadian Commercial Flax Stocks

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Detection and quantification of the prevalence of genetically modified (GM) organism contamination in seed exports is a critical element of regulatory compliance. While the procedures to reliably detect high levels of GM contamination are well understood, no comparable statistical approaches are available for the quantification of levels of GM prevalence below the established detection rate of standard tests. Presented is a simple statistical approach based on simulation modeling for the quantification of low levels of GM contamination. The approach can be modified to match any sampling regime and can account for rates of false positive and negative assay results. The application of this method is demonstrated using the low level of contamination in Canadian commercial flax stocks by the GM flax variety "Triffid." We show that rates of GM contamination in commercial flax stocks ranged between one (1) GM seed per million and one (1) seed per hundred thousand. A simulation model was used to determine whether the observed rates of positive tests are within the range expected from false positive rates of the test. We showed that for the majority of categories of grain or seed, the very low level of GM prevalence still remains outside that which is to be expected based on false positives returned or by chance alone. These results indicate a pervasive low-level presence of GM construct in the Canadian commercial flax system.

Key words: CDC Triffid, flax (*Linum usitatissimum*), GMO, seed purity analysis, seed testing, statistical methods, transgenic seeds.

Introduction

Flax (*Linum usitatissimum*) is grown for seed (linseed) or fiber (isolated from the stem of the plant). Canada is the world's largest producer of flax seed, generating 45% of world production (Food and Agriculture Organization of the United Nations, 2009). Most of Canada's flax is grown in the Provinces of Manitoba, Saskatchewan, and Alberta and is used primarily for seed oil. Western Canadian farmers produced 930,000 tonnes of flax in 2009 (Statistics Canada, n.d.). In April of 2009, transgenic seeds were found in two 5,000-tonne shipments of flax grain during preprocessing in Europe. Further, shipments of Canadian flax have tested positive for a transgene in Japan and Brazil (Flax Council of Canada [FCC], 2009a). The presence of a transgene in flax shipments from Canada relates back to the mid-1990's introduction of the genetically modified (GM) flax variety CDC Triffid (McHughen, Rowland, Holm, Bhatty, & Kenaschuk, 1997). This variety was deregistered in 2001 due to concerns about the effect of production of GM flax on export markets. Approximately 10-12,000 acres of Triffid were grown by Canadian seed growers, and about 5,500 tonnes of Triffid seed were collected during the recall (Canadian Seed Growers Association). Off-shore markets for Canadian flax have not approved GM flax and have no tolerance for its detection in shipments (FCC, 2009b).

The FCC (2009b) has reported detection of widespread, low-level presence of GM flax in commercial flax stocks. Since this detection has been reported, extensive flax seed testing has been instituted in Canada prior to planting, post harvest, at initial receptor sites (elevators, railcars), and at grain terminals prior to export. The majority (>80%) of grain exports to the European Union (EU) take place from Thunder Bay, and in 2011, 5% of flax samples at this location were positive for the presence of the transgene utilized in CDC Triffid. Testing of adventitious presence—a critical element of regulatory compliance—is confounded by the practical level of detection of real-time PCR assays (0.01%, or 1 GM seed in 9,999 conventional seeds) and the large sources of error inherent in taking representative and random samples in large seed lots (Begg, Cullen, Iannetta, & Squire, 2007; Lamb & Booker, 2011). The current testing protocol requires the collection of a 2 kg sample of any flax entering the handling system,

Table 1. Seed categories tested. The p-value is probability of observing a number of positive tests equal to or greater than the observed number of positive tests simply from false positives.

Type	Year	Type of test	# lots tested	# positive tests	P	Mean triffid presence	Lower 95% CI	Upper 95% CI
Farm saved	2010	4 × 60	20	0	1.0000	n/a		
Farm saved	2011	4 × 60	462	23	0.0009	1.3871E-06	1.31E-06	1.46E-06
Pedigree	2010	4 × 60	1	0	1.0000	n/a		
Pedigree	2011	4 × 60	217	4	0.7607	4.7109E-07	4.25E-07	5.17E-07
Production	2009	1 × 60	5,220	150	<0.0001	2.7336E-06	2.64E-06	2.83E-06
Production	2009	4 × 60	246	77	<0.0001	1.06E-05	1.02E-05	1.10E-05
Production	2010	1 × 60	545	21	<0.0001	3.9885E-06	3.86E-06	4.12E-06
Production	2010	4 × 60	3,470	224	<0.0001	1.77E-06	1.67E-06	1.87E-06
Production	2011	4 × 60	801	76	<0.0001	2.7167E-06	2.64E-06	2.8E-06
Rail cars	2010/11	4 × 60	988	50	<0.0001	1.2854E-06	1.23E-06	1.35E-06
Bins	2010/11	4 × 60	55	4	0.0419	2.5736E-06	2.47E-06	2.68E-06

and testing of four 60 g subsamples (4 × 60) for the presence of GM flax (Canadian Grain Commission, 2010). The sampling protocol presumably gives a 95% probability (or 5% error) of detecting 1 GM seed in 9,999 non-GM flax seeds (Remund, Dixon, Wright, & Holden, 2001; Whitaker, Freese, Giesbrecht, & Slate, 2001). However, Lamb and Booker (2011) demonstrated that low levels of presumed contamination (less than 1 in 9,999) are indistinguishable from the number of positive tests expected from a clean seed lot given the observed rates of false positives. This finding has significant implications for the testing of flax seed lots for GM presence and the whole notion of zero tolerance in the grain industry. Continued testing will be required for the foreseeable future to reduce the risk of product rejection.

Why GM flax was found in Canadian flax stocks after removal of GM flax from the commercial system is not known. There are many potential sources of seed-mediated gene flow, including crop volunteers, mixtures during seed multiplication, transport, planting, harvest, post-harvest transport, and handling by intermediates and end-user (Wilkinson, 2010). Seed-mediated gene flow in flax as a result of harvest loss, seed bank longevity, the emergence and persistence of volunteer flax in subsequent crops has been reported by Dexter et al. (2010). Another situation where the GM flax may have been introduced into the commercial seed stocks is via cross pollination (out-crossing). However, the rate of gene flow in flax is low and is estimated to be between 0.0013 and 0.00003, at 3 and 35 m, respectively (Jhala, Bhatt, Topinka, & Hall, 2011). Introduction of GM flax into the commercial seed stocks is most likely to have occurred through seed carryover from farm machinery, storage facilities, and mixtures during seed multiplication.

Flax acreage in Canada declined drastically, going from 623,000 hectares/1,539,466 acres in 2009 to 353,000 hectares/872,281 acres in 2010 and to 281,256 hectares/695,000 acres in 2011 (Statistics Canada, n.d.). Prior to GM detection in Canadian flax stocks, exports to the EU were at 400,000 tonnes (W. Hill, personal communication, 2011). Exports of Canadian flax to the EU have steadily declined to 270,000 tonnes in 2009/10; 220,000 tonnes in 2010/11; and 200,000 tonnes in 2011/12 (W. Hill, personal communication, 2011). Weather conditions in Western Canadian flax production areas over the past two cropping seasons have held back flax plantings and reduced production. However, the declines in flax acreage and exports to the EU is also likely a direct result of the GM issue in the EU.

This study was initiated to determine the GM prevalence in Canadian commercial flax seed stocks. Extensive testing data covering the period since the initial GM detection in Canadian flax were obtained. We determine whether the observed rates of positive tests are within the range expected from false positive rates of the test, and we use a simulation model to estimate GM prevalence in those stocks. We expect that detection of GM flax in commercial seed stocks will largely be a function of testing intensity. Moreover, we anticipate that GM presence detected at extremely low levels in the commercial grain or seed lots will not be significantly different from what can be expected from the number of false positives returned from clean grain or seed lots.

Methods

Observed Canadian Test Results

Data were obtained detailing the number of tests carried out to detect the GM construct found in CDC Triffid between January 2009 and March 2011 from the FCC. Test results on farm-saved sowing seed, pedigreed seed, and production or grain were obtained (Table 1). In total, data on 26,633 individual tests on 10,982 seed lots were obtained. In addition, testing data from 988 rail cars and 55 bins at the terminal in Thunder Bay were obtained from a grain handling company. Initially, the industry testing protocol only required a 1 × 60 g subsample for each 2 kg composite be tested for GM. This protocol was later updated in September 2010 to require 4 × 60 g subsample for each 2 kg composite to be tested for GM. Here we report only the number of positive and negative test results. Some labs report detection below the 0.01% level or (less than 1 GM seed in 9,999 seeds) as "trace," however there was no consistency between labs or years on how trace results were reported. In particular, prior to December 2010, trace results obtained in the lab were reported as negative. Here we have treated all "trace" reports as negative.

Expected Levels of Positive Tests

The test for the GM construct has a specificity of 0.006, indicating that a false positive result can be expected in 0.6% of individual tests (Lamb & Booker, 2011). This low rate of false positives can, however, result in substantial numbers of positive results in tests of clean seed. For example, at this rate of false positives, 9.4% of clean grain lots tested 16 times in a product handling chain will have at least one positive test ($0.0918=1-0.994^{16}$). It is critical to determine if the number of positive tests observed deviates from the expected number of false positives given the observed false positive rate. This question was evaluated by first estimating the probability that the observed or a larger number of positive results could have arisen given the rate of false positives. A probability ≥ 0.05 indicates that the number of observed results is not significantly different than that expected by chance. This probability was calculated using the dbinom function in the R statistical package (R Development Core Team, 2010). The probability was estimated for a particular number of positive tests that could arise given the false positive rate with total number of tests and the false positive rate as arguments. In cases where only a single test of 10,000 seeds was reported per lot (1 × 60 g tests), a false positive rate of 0.006 was used. In cases where four tests per lot were carried out (4 × 60 g tests), a false positive rate of 0.0238 was used, since a false positive rate of 0.006 per test means that, on average, 2.4% of clean lots will have at least one false positive test out of 4 ($0.0238=1-0.994^{4}$). Summing the results of the dbinom function across all numbers of positive tests ≥ the observed number of positives gives the probability that the observed or a larger number of positive results could have arisen given the rate of false positives. In addition, the expected distribution of false positive results was plotted using the dbinom function.

Estimation of GM Prevalence in Contaminated Seed or Grain Lots

In all cases except pedigree seed and terminal bins, the number of positive tests was much higher than that expected given the false positive rates. We used a simulation model to estimate the prevalence of GM contamination for these lots (Lamb & Booker, 2011). This simulation model generates the range of GM prevalence expected to arise, given the number of positive tests observed and the total number of tests done. The model incorporates the rates of false positive and false negative results expected to occur during the testing process and the number of individual seeds used in each test. The simulation was written using the open-source R statistical package (R Development Core Team, 2010); for a full description of the simulation and all code required to reproduce the results described here, see Lamb and Booker (2011). The simulation was used to estimate the mean level of GM contamination and 95% confidence intervals in each type of seed (Table 1). We estimated the contamination level separately for each seed type and year. We also produced separate estimates for the cases where 1 × 60 g and 4 × 60 g tests were carried out on the same seed type. This was done because in many cases the 4 × 60 g tests reported only an aggregate result (positive if one or more of the four tests was positive) and not the results of the four individual tests.

Results and Discussion

Between 2009 and 2011, a total of 12,025 seed or grain lots were tested with 629 lots testing positive (Table 1). No positive results were reported from farm-saved or pedigreed seed in 2010, though the testing rate was low. The number of positive tests on pedigree seed in 2011 (4 out of 217) was not significantly different (p=0.7607) than that expected from the false positive rate, and the number of positives observed for terminal grain bins (4

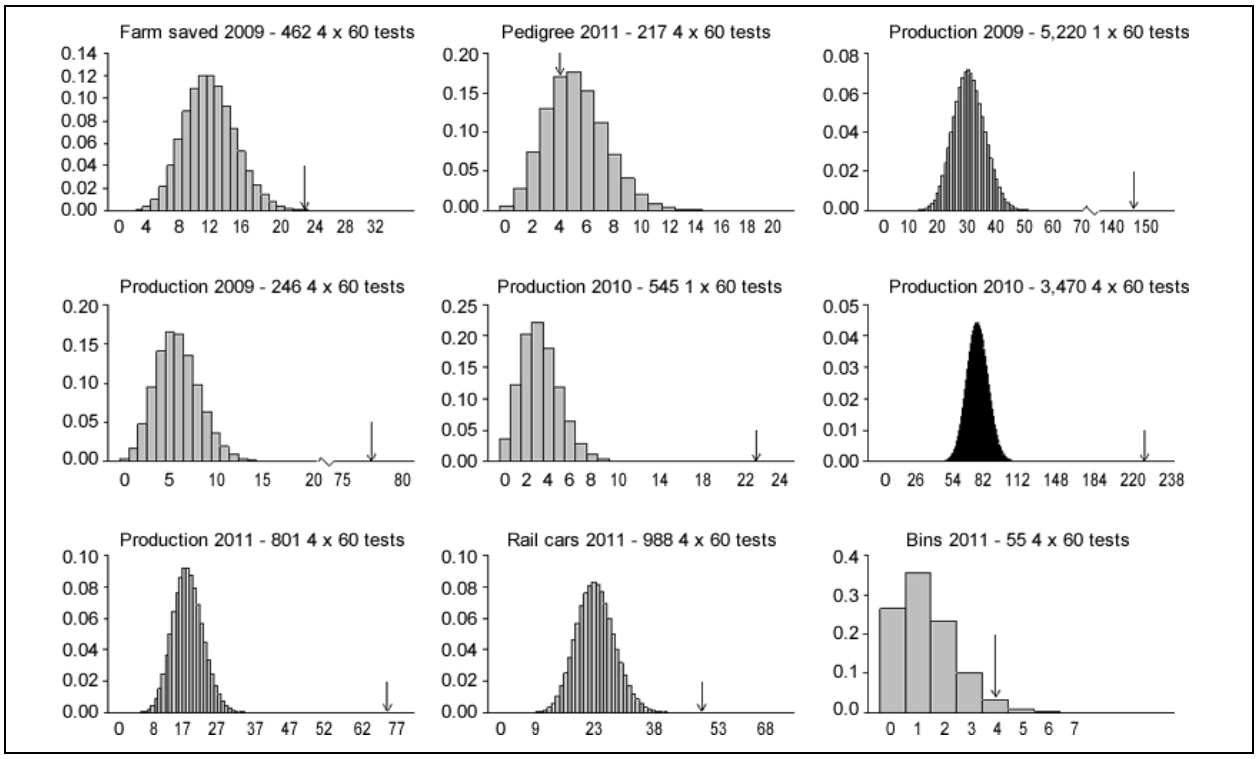

Figure 1. The expected distribution of false positive tests for each series of tests completed. The arrows indicate the observed number of positive tests.

out of 55) was marginally higher (p=0.0419) than that expected from the false positive rate (Figure 1). Rates of positive tests were significantly higher than expected by chance in all other categories of seed or grain (Table 1; Figure 1).

Rates of GM prevalence in contaminated seed or grain categories ranged between 1 in 1,000,000 and 1 in 100,000 seeds, indicating a pervasive low-level presence of the GM construct in the Canadian commercial flax system (Table 1). Trends in prevalence levels between years are difficult to distinguish given the widely varying testing efforts and changing testing protocols. Of the 4,969 lots tested using the 4 × 60 g protocol where the results of the four individual tests are available, 4,642 lots tested clean and 231, 58, 21, and 17 reported 1/4 through 4/4 positive tests, respectively. Only 0.34% of seed lots had 4/4 positive tests, an indicator of contamination likely higher than the 0.01% threshold. Of the remainder, the majority (70.64%) had only one out of four positive tests. One out of four positive tests indicates a GM prevalence between 6.9 and 7.3 seeds per 100,000 (95% CI), well below the 1 in 10,000 threshold, and 2.4% of clean seed lots are expected to test positive given the false positive rate. These results indicate that small pockets of highly contaminated seed are present, but the majority of positive lots are contaminated only at relatively low levels—if they are contaminated at all.

Overall, deployment of testing protocols has resulted in significant reduction in the levels of GM events from 2009 to 2011 (Table 1). In particular, GM levels have dropped substantially over this time in production or grain. However, the very low level of GM prevalence still remains outside that which is to be expected, based on false positives returned (Figure 1). This result indicates that GM flax is still present in the Canadian flax system. Positive tests at the rail-car level are diverted away from export to GM-sensitive markets. When testing is done again at the terminal in Thunder Bay (where European export grain is gathered), the number of positives returned for terminal bins is not substantially different from that expected based on the false positive rate (Figure 1). This result indicates that the testing protocols are working to remove GM flax from this part of the value chain, although false positives likely continue to be a significant problem for the industry.

The level of GM prevalence in pedigree seed is not different from what is expected based on the false positive rate and is therefore not a significant source of contamination (Figure 1). GM prevalence in sowing seed

(farm saved and pedigreed) is not reflected in levels found in the production or grain (Table 1); this lower prevalence is likely due to the less-intensive testing done for sowing seed versus production seed (Table 1).

Repeated testing done through the product handling chain produces substantial numbers of contradictory results, thus increasing both the consumer's risk of accepting a contaminated lot and the producer's risk of having clean seed lots rejected. The technical solution involving testing for the presence of extremely low levels of GM flax (i.e., < 0.01 % level) is limited due to the difficulty of sampling large seed lots for such a rare event and the inherent error rates of the GM polymerase chain reaction (PCR) assay. These factors are further confounded by the typical number of tests done along the value chain for flax. There is an overwhelming need to implement a policy of low-level acceptance between trading nations to prevent the disruption of trade due to a largely inconsequential event.

The Way Forward for the Canadian Flax Industry

Crop Development Centre (CDC) flax varieties accounted for 87% and 83% of the seeded flax acreage in Western Canada in 2009 and 2010, respectively (B. Siemens, personal communication, 2010, 2011). Positive results for GM flax presence in some Canadian pedigree seed lots have been found when testing is done at or below the 0.01% level (FCC, 2009a). Lamb and Booker (2011) estimated that GM flax in breeder seed lots ranged from 2 seeds per million to 6 seeds in 100,000. Given the false positive rates inherent in the current testing system, we cannot not distinguish breeder seed lots reported as contaminated from clean sample.

It is not physically possible to eliminate GM flax from the existing breeder seed lots. Consequently, the CDC has developed and applied a protocol to reconstitute a number of flax varieties and re-release them as "Triffid-free" Breeder Seed. This new breeder seed source is one of our best opportunities to ensure the Canadian flax crop is free of Triffid seed. It is the intent of the Canadian flax industry to flush the system of existing CDC seed stocks by the fall of 2013 so that the portion of the commercial flax crop sown using the reconstituted CDC flax varieties can be planted from this new seed source as early as 2014.

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