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Low Level Presence vs. Coexistence: Vestigial presence of stacked-transgenic events and consequences of unintentional releases into crop fields

Ana Chegão

Plant Health and GMO Laboratory Instituto Nacional de Investigação Agrária e Veterinária, I.P. ana.chegao@iniav.pt

Mónica Rodrigues

Plant Health and GMO Laboratory Instituto Nacional de Investigação Agrária e Veterinária, I.P. isabel.rodrigues@iniav.pt

Fátima Quedas

Instituto Politécnico de Santarém Escola Superior Agrária de Santarém maria.quedas@gmail.com

Eugénia de Andrade

Plant Health and GMO Laboratory Instituto Nacional de Investigação Agrária e Veterinária, I.P. eugenia.andrade@iniav.pt

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Abstract:

Maize is one of the three most important crops in the world. It is used as feed, food, food ingredient and additive, and for industrial processing. Recently, its use as raw material for biofuels reaches 50% of the USA production. In addition to its economic importance, several maize lines were genetically engineered to express agriculturally desirable traits, including tolerance to pests and to herbicides, to increase crops productivity.

Although, worldwide, several transgenic maize events may be cultivated, in European Union (EU), only one maize event is authorized for cultivation, the MON 810 event. Therefore, according to the European farmers' freedom of choice between conventional, organic and GM crop cultivation, European guidelines were developed to help those Member States who intended to cultivate MON 810 maize, to develop either national laws or best practices to ensure coexistence between GM crops and conventional and organic farming.

Although, in 2009, fifteen countries had already developed coexistence rules, the uncertain regulatory system concerning both plant-breeding methods, namely those creating GM crops bearing more than one transgene (stacked traits), and thresholds for the presence of adventitious GM seeds in conventional seed lots complicated maize seed trade. In EU, a maize variety having stacked events cannot be cultivated as coexistence only applies to GM crops bearing the single event authorized for cultivation. Besides, the adventitious presence of GM seed in conventional seed lots must meet the requirements of conventional maize growers and their customers

The most used methods to screen for the presence of unauthorized maize events in seeds, based on real-time PCR, show many severe impediments. There is no certified reference material for the quantification of screening elements as for the P35S promoter or T-nos terminator; the detection limit of screening methods do not provide the information needed to ensure the absence of non-unauthorized GM seeds in conventional maize seed lots; there is no means to known if an event is stacked or not in ground seeds.

We made a review of the situation in Portugal, both at the seed importation level and at the national grain production. We found that Nk603 maize is being stacked together with MON 810 as adventitious presences in conventional seed lots. Additionally, we found that analytical results may vary substantially among laboratories as many

producers request the quantification of GM material despite of the transgene

identification. We compared results obtained with commercial Kits for the

quantification of P35S with event-specific methods.

Keywords: GMO, MON 810, NK603, stacked-transgenes, maize, coexistence, real-

time PCR quantification methods.

JEL codes: Q18

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1. Coexistence and thresholds

Coexistence is a set of legally established rules having the overarching aim of ensuring the presence of GM and non-GM crops simultaneously in contiguous fields and, at the same time, the absence of unintentional presence of GM material from authorised events in non-GM products (Devos *et al.*, 2009). One can say that coexistence arose to solve the problems of farmers, in first order, but also of general consumers when demonstrating the wish to choose products originated and distinctive from different modes of crop production. Producers and consumers of all European countries have highly similar behaviour concerning the freedom of choice. In all points of the food chain production, consumers, in general, may be able to choose between conventional, organic or GM crops.

This was one of the earliest concerns of the European Commission (EC) which led to the establishment of labelling procedures for GM food and feed. Labelling is mandatory except when adventitious or technically unavoidable presence of a GMO in a given ingredient remains below 0.9% of that ingredient (Regulation 1829/2003).

The need of labelling and a concomitant threshold together with the knowledge that agriculture itself and the subsequent food chain production are vulnerable to GMO contaminations presupposes a certain level of tolerance for the presence of GM materials in non-GM ones. Consequently, conventional and organic crops may not be completely free from contaminations with GM matters. Indeed, in reality it is impossible to ensure a GM-free production when different modes of agriculture are coexisting in the neighbourhood. According to recently launched EC Guidelines, coexistence means that it must be ensured that crops "have the lowest possible presence of GMOs" (European Commission Guidelines, 2010).

Additionally and very relevant is that the GMOs must be authorized for food and feed purposes. The perception of coexistence relies only on the use of authorized GMOs, *i.e.*, events that have been considered health and environmentally safe at the European evaluation committees. Although this seems not to be controversial it may cause some discussion as only one GM event is authorized for cultivation in European Union (Maize event MON 810).

To summarize, to address coexistence in Europe, it must be clearly stated that reasonable efforts have to be made to prevent traces of maize MON 810, only, and that

events non-authorized for agriculture purposes, although they can be authorized for food and feed, are not covered by the EC coexistence policy framework.

Taken the referred presupposes, coexistence came to increase the level of segregation in the field and throughout the entire food supply chain. Definitely, the gold standard of coexistence is to minimize the admixture of maize MON 810 and non-GM products.

Due to the ongoing tendency for further increase the cultivation area of GMOs worldwide associated with the need to develop crop varieties tolerant to different biotic and abiotic stress factors led to increasing the production of GM seeds having several transgenes, the so called stacked varieties. Such varieties offer enhanced agronomic traits allowing farmers to better meet their production quantity and quality under constantly evolving farming conditions. A biotech crop variety that bears stacked traits/transgenes is called a biotech stack or simply stack. An example of a stack is a plant transformed with two or more transgenes coding for *Bacillus thuringiensis* (*Bt*) *pro-toxins* having different modes of action and therefore targeting different insects and herbicide tolerance genes, for instance.

In 2013, the United States (US) maize GM varieties already represented 90% of the total and the acreage of GM crops increased by 1 million to 70 million hectares (http://www.gmo-compass.org/eng/agri biotechnology/gmo planting/). Just following the tendency observed in recent years, US GM maize varieties are expected to be 92% in 2015 and the adoption of stacked varieties, herbicide-tolerant and insect-resistant, by now reached maize plantings in 2015 (http://www.ers.usda.gov/dataproducts/adoption-of-genetically-engineered-crops-in-the-us/recent-trends-in-geadoption.aspx). This reality brings concerns to the European GM maize producers as the regulatory principles for approval and deliberate release of such varieties differs between United States and Europe. In European Union Countries a stacked variety is a new event that must pass through an individual approval process as it has to be ensured the absence of additional risks arising from the combination of transgenes (Stein and Rodriguez-Cerezo, 2009). The increasing presence of stacked varieties will provide three major concerns:

1) The need of new technological procedures to perform unequivocal identification of a stack allowing for the differentiation with two mono-trait crop varieties;

- 2) Updating the labelling tolerance threshold rules, either the 0.9% for authorised events or the 0.1% for non-authorised, considering the particular cases of stacks bearing at least one authorised GM event for cultivation (*e.g* MON 810 x event X) (Stakholders summary, 2009) and enabling minimal discrepancies between analytical results produced by different laboratories.
- 3) Unavoidable presence in non-GM seed lots (beyond coexistence) but also in authorised GM-seed lots (coexistence between authorised GM with non-authorised GM events) (Stakholders summary, 2009).

Although some reports clearly demonstrate that coexistence is possible under a certain set of technical agricultural practices, technical segregation measures and liability measures (European Commission, 2003), taking the current scenarios, a recent report (Verrière, P., 2013) shows that interdiction of GMOs is the most effective way to prevent unavoidable GMOs' presence in non-GM products.

1.1.Thresholds in conventional seeds

Since 2003 that thresholds for the technically unavoidable present of GM seeds in conventional seed lots have being discussed (ENGL *ad hoc*-group for the "interpretation and implementation of thresholds for GMOs authorised in the European Union). Two years later, the Commission made the first attempt to set contamination thresholds in seeds. In 2006, the Council of European Ministers of Agriculture required the EC to decide on the labelling threshold for seeds. However, due to reasons mainly related with the lack of knowledge about the correlation between the pollination mechanism of the different crop species and the need to ensure the labelling threshold of 0.9% in food and feed, risk assessment process, opinion of environmental Non-Governmental Organizations, among others, the EC never set thresholds in conventional seeds for the presence either of authorized or for non-authorized events (Devos *et al.*, 2009).

In 2009, the Commission evaluated the impact of the establishment of labelling thresholds for seeds and, later, it was established that Member States (MS) have flexibility to decide upon their national measures (European Commission Guidelines, 2010), which must rely on the specifications of particular products such as organic and conventional crops. Additionally, they had to take into consideration that the food chain does not routinely work with the 0.9% labelling threshold but with the technical zero threshold

which corresponds to 0.1% (Commission Regulation (EU) No 619/2011.) as a preventive measure to ensure the 0.9% value on the end product only.

In Portugal, GM maize is also grown and increasing but on a small scale (Quedas & de Carvalho, 2012). GM maize production is still a niche production in Portugal, representing approximately 6% of the total maize production area. It made significant progress during the early years stabilizing after 2012 when it reached the highest area (9278 ha).

Portugal was one of the first MS implementing a national law regulating the cultivation of GM varieties (Decree Law 160/2005), in particular, of MON 810 maize varieties.

1.2. Thresholds of non-authorized events

European Regulation no. 619/2011 introduced the concept of low level presence (LLP) for EU non-authorized GM events present in imported feed material. Despite of the absence of orientation for the seed sector, national governments may use the level of 0.1% for non-authorized/unapproved GMOs for agriculture purposes, such as food/feed authorized stacked events found in shipments of non-GM seeds or in shipments of approved GM seeds (MON 810 maize). This would mean that LLP guidelines may be used to authorize and inadvertently to disseminate GM contaminations with GMO authorized for food/feed but not for cultivation (GMO-FREE EUROPE 2015 - NGO AND SCIENTISTS). Besides the fact that these GM contaminations would not have undergone a risk or safety assessment in Europe (destiny of import), many farmers may have their production contaminated with other GMOs besides MON 810.

Seed lots labelling and the consequent threshold value for the maximum presence of either GM seed in non-GM seed lots or in authorised-GM seed lots is not only a current requirement, but also decisive to ensure coexistence. Furthermore, without labelling of seeds at the detection threshold (0.1% or even 0.01%), it will be impossible, even after 14 years of implementation of the 2001/18/EC directive, to ensure the proper execution of the old traceability requirements (European Commission, 2001).

2. Description of state-of-the actual situation in Portugal

The aim of this data assembling exercise was to enable producers and consumers of being informed of the real state of the national context. An important part of the GMO laboratory activities is to perform analysis on the demand of private clients and to elaborate appropriate reports with reference to the used method (reference validated method), EU legislation and recommendations.

Yearly, the National Competent Authority carries out analytical controls to seed imports and to maize grain domestic production in the context of coexistence. Field inspections are conducted to ensure the fulfilment of the confinement measures, communication with the neighbours and training among other parameters as dictated by the National Coexistence Law (Decree-Law n°. 160/2005). Post-harvesting controls are led to verify traceability and labelling of both GM and non-GM maize production. For the latter, this is applicable only to non-GM maize produced into zones of production of GM varieties (Carvalho & Mourão, 2013).

We assembled the analytical data available in the National Reference Laboratory (NRL) for GMOs.

2.1.Controls to seed imports

To summarize the data presented in Table 1, throughout the years, sowed seeds were gradually being more contaminated by GMOs, in particular NK603, which has been approved in third countries but not approved in the EU for agriculture purposes. The presence of NK603, either as a single trait or as a stacked event, increased from 32% in analysed samples in 2009 to 71% in 2010. This observation seems to be a consequence of the new position of the EC that reflects more relaxed rules for food and seeds imports, under the umbrella of the 'low level presence' regulation (GMO-FREE EUROPE 2015 - NGO AND SCIENTISTS). The expansion of the LLP approach to the seeds is not taking advantage of the Coexistence law which is laborious and was perfectly fitting the needs of the maize grain producers.

2.2.Post-harvesting control

Graph 1 represents the evolution of the analytical results on samples collected for the post-harvesting control and submitted for analysis to the NRL There is some fluctuation which depends on the location of the sample collection relatively to the GMO field (border/refuge lines, neighbour fields) but also relatively to the coordinates on the field (downwind from the transgene donor plants). However, the direct observation of the high of the bars, clearly indicates the increase of the percentage of samples testing positive or

<LOD. Taken the data from Table 2, between 2009 and 2013 samples showed high variability on the GMO percentage whereas in 2014 the values were all below 0,5% (m/m). This observation might be related with the location of origin of the sample. Higher values are associated with the borders.</p>

In 2014, eight maize grain samples submitted by several different clients, (national maize producers) showed the presence of MON 810 and NK603 simultaneously (Table 3).

Both MON 810 and NK603 events are authorised for food and feed but NK603 is unauthorized for agriculture purposes in the European Union. As these grains were from the national production, we decided to re-analyse all DNA extracts still available in our premises for the presence of other events that can be stacked with MON 810. DNA extracts obtained during the post-harvesting campaign of 2009 and 2010 exhibiting high concentrations of MON 810 were taken for the detection and, when appropriate, for the quantification of NK603. Those DNA extracts having very low quantities of MON 810 were not reanalysed as results could be biased by some DNA degradation occurred during the conservation time (five and six years at -20 °C). For 2014, all samples were reanalysed (Graph 2). Samples from 2009 tested negative for the presence of NK603; one sample from 2010 tested positive (0.07% \pm 0.01) and one tested < LOD; one samples from 2014 tested negative, 10 tested <LOD and five tested positive for NK603.

Our results (Table 4) show that the most probable situation is that contaminations are due to events staked with MON 810. The quantification values in each line of the Table 4 correspond to one sample. The registered variations can be attributed to the PCR methods used as all methods based on the amplification of DNA are strongly variable. Therefore, the values are equivalent. For samples with very low amounts of GMO, the coefficient of variation associated with the result is approximately 18.11%, in the most favourable in-laboratory sampling and analytical conditions (de Andrade *et al.*, 2013, personal communication at GMCC-13).

MON 810 is always below the labelling threshold and NK603 is below the LLP threshold avoiding the need of application of control measures. However, it turns out that on the one hand, the presence of an unauthorized GMO is to be a reality and, on the other hand, its presence is increasing in domestic production with all the undesired economic impacts for those farmers who want to harvest a "GM-free" production.

We tried to correlate these observations with the quantification values obtained for the seed lots analysed during the national controls to imported seeds (Table 1). Data on the presence of MON 810 and NK603 are only available for grain samples from the 2014 production campaign. However, one can see that the presence of putative stacked varieties corresponds to 78% which of the same order of magnitude that the value observed at the seed level.

3. Analytical results vs. applied methodologies

Quantification of elements of the transgene construction, such as promotor 35S (P35S) or terminator *nos* (T-nos) is not possible as there is no reference material certified for the mass fraction of such elements. The Institute for Reference Materials and Measurements (IRMM) from the Joint Research Centre, European Commission, develops and certifies reference materials (CRM) for GMP detection and quantification. These CRMs are certified for the mass fraction of a specific genetic modification event (Trapmann, S., 2006). For instance, the ERM®BF-413-series is "certified for different mass fractions of MON 810 maize seed powder in non-GM maize seed powder at nominal levels of 5, 20 and 100 g/kg". The non-GM fraction is known to be non-MON810 but its real identity is unknown. There is some probability for the non-MON 810 fraction to have the unavoidable presence of other GM seeds (Trapman, S., 2006). Therefore, all available CRMs can be used solely together with the correspondent GM event-specific method. If the non-MON 810 fraction has transgenic elements as the P35S promotor or T-nos these will also be detected/quantified by PCR, overestimating or underestimating the quantity of GM material.

We decided to compare the analytical results obtained with a normalized validated method with a commercial kit which was developed for the quantification of P35S promoter. In Table 3 it is shown the expected overestimated quantification results as expected by the kit manufacturer (Generon, Technical note). This method is too simplistic to support, considering the huge efforts that were necessary to develop event-specific molecular diagnostic tests for the many transgenes authorized so far, to validate through international coordinated validation exercises and to assess putative risks during the deliberated release into the environment. Before evaluating the impact of the use of such method, the analytical results obtained thereafter should not support producers and food/feed industries on their decisions concerning the commercial value of the batch of

grain. According to the results obtained with this commercial kit, samples 7 and 8 clearly would have to be labelled as they overpass the labelling threshold of 0.9% which clearly does not correspond to the presence of a GM fraction.

4. Conclusions

Member States, in general, and Portugal, in particular, need thresholds for adventitious presence of GM seeds in all type of seed lots and more stringent controls and monitoring of data provided by the economic operators trading on the seed sector, as it was already reported during the Congress of Berlin.

The description of the detection/quantification methods is also needed to properly identify the transgenic events. The use of unsuitable reference materials for the routine GMO quantification by means of P35S promoter, without the identification of the transgene, is a fact. The consequences should be evaluated and, if appropriate, measures should be taken in order to turn mandatory the use of event-specific methods for unequivocal identification of all transgenes present in seeds, grains and food/feed matrices.

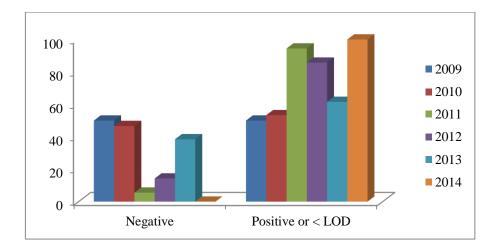
Acknowledgements

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Tables and Figures

Table 1: Number of analysed **seed** samples testing positive or <LOD for MON 810 and/or NK603 during the national control of seed imports and respective quantification results.

	2009	2010	2014	2015
Total of samples	20	28	16	24
MON 810/NK603 Positive or <lod (%="" 810="" <lod<="" m="" m)="" mon="" or="" positive="" td=""><td>• 0.06 • 0.39</td><td>• 5 samples: <lod 3="" <="" <lod="" lod<="" samples:="" td="" •=""><td><lod <b="">0.02</lod>4 samples:<lod <lod<="" li=""></lod></td><td>7 samples: <lod <lod<br="">7 samples: <lod 0.05</lod </lod></td></lod></td></lod>	• 0.06 • 0.39	• 5 samples: <lod 3="" <="" <lod="" lod<="" samples:="" td="" •=""><td><lod <b="">0.02</lod>4 samples:<lod <lod<="" li=""></lod></td><td>7 samples: <lod <lod<br="">7 samples: <lod 0.05</lod </lod></td></lod>	<lod <b="">0.02</lod>4 samples:<lod <lod<="" li=""></lod>	7 samples: <lod <lod<br="">7 samples: <lod 0.05</lod </lod>
(% m/m)				• 0.03 • 0.08
NK603 Positive or <lod (% m/m)</lod 		• 0.07	• 0.02 • 2 samples : 0.01 • 4 samples: <lod< td=""><td></td></lod<>	
NK603/Other events			• 4 samples	-



Graph 1: Percentage of negative and positive maize grain samples from the postharvesting control.

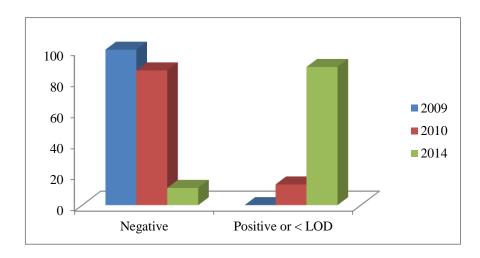
Table 2: MON 810 quantification results [% (m/m)] obtained for the analysed maize **grain** samples during post-harvesting control from 2009 until 2014.

2009	2010	2011	2012	2013	2014
(average±SD)	(average±SD)	(average±SD)	(average±SD)	(average±SD)	(average±SD)
0.30±0.10	0.09±0.02	0.03±0.003	0.54	0.63±0.31	4 samples: <lod< th=""></lod<>

0.56±0.11	0.10±0.03	0.09±0.04	1.83	0.63±0.30	0.01±0.002
2.3±0.52	0.18±0.06	0.23 ± 0.02	2 samples> 5.0	1.93±0.31	0.04 ± 0.02
3.31±0.2	0.24±0.07	0.27 ± 0.06		1.94±0.36	0.02±0.01
17.24±2.44	0.49±0.18	0.41 ± 0.07		1 sample> 5.0	0.03±0.01
	2.56±0.40	1.65±0.23			0.07±0.01
	13.35±0.04	2.13±0.28			0.10±0.02
		4.28±0.06			0.11±0.07
		5.09±0.56			0.17±0.04
		5.27±0.98			0.19±0.03
					0.23±0.07
					0.27±0.09
					0.35±0.09
					0.45±0.09
					0.50±0.03

Table 3: MON 810 and NK603 quantification results [% (m/m)] obtained for the analysed maize grain samples from the production campaign of 2014 submitted by private clients.

	EU-GMFF Reference method		Commercial kit for the quantification of P35S promotor	
gomnleg	% MON 810±SD	% NK603 ±SD	P35S promoter	
samples	(m/m)	(m/m)	(m / m)	
1	0.01±0.003	0.01±0.003	_*	
2	< 0.01	0.02±0.03	0.49	
3	< 0.01	0.42±0.72	_*	
4	< 0.01	0.04±0.05	0.14	
5	< 0.01	0.10±0.02	_*	
6	0.08±0.04	0.04±0.02	0.03	
<u>7</u>	0.05±0.02	0.05±0.02	<u>1.08</u>	
8	0.03±0.01	0.04±0.01	<u>1.76</u>	
* data not available due to insufficient sample.				



Graph 2: Percentage of negative and positive samples analysed for the presence of the transgene NK603 from the post-harvesting control.

Table 4: MON 810 and NK603 quantities is samples tested for both events [% (m/m)].

Samples	MON 810	NK603
	(LOD average = 0.02)	(LOD average = 0.03)
1	<lod< th=""><th>0.32±0.001</th></lod<>	0.32±0.001
2	<lod< th=""><th>0.04±0.002</th></lod<>	0.04±0.002
3	<lod< th=""><th><lod< th=""></lod<></th></lod<>	<lod< th=""></lod<>
4	<lod< th=""><th><lod< th=""></lod<></th></lod<>	<lod< th=""></lod<>
5	<lod< th=""><th><lod< th=""></lod<></th></lod<>	<lod< th=""></lod<>
6	0.01±0.002	<lod< th=""></lod<>
7	0.02±0.01	0.01±0.003
8	0.03±0.01	<lod< th=""></lod<>
9	0.07±0.01	<u>Neg</u> .
10	0.10±0.02	<lod< th=""></lod<>
11	0.11±0.07	<lod< th=""></lod<>
12	0.17±0.04	Neg.
13	0.19±0.03	0.01±0.001
14	0.23±0.07	<lod< th=""></lod<>
15	0.27c0.09	<lod< th=""></lod<>
16	0.35±0.09	Neg.
17	0.45±0.09	0.01±0.001
18	0.50±0.03	<lod< th=""></lod<>
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