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COEXISTENCE IN CULTIVATION OF GENETICALLY MODIFIED MAIZE (MON810) WITH CONVENTIONAL MAIZE

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ABSTRACT

In many countries, including Romania, the coexistence of genetically modified maize and conventional maize is regulated by law. The study of cross-pollination plays an important role in ensuring this coexistence. As a result, at the Agricultural Research and Development Station Şimnic - Craiova, several experiences (spaced from 0 to 100 m of MON 810) were established, where the rates of cross-pollination were monitored by the effect of Xenia and quantifying the impurity gene level by analysing the DNA of MON 810. The highest levels of cross-pollination were recorded in experiences with coincidence at flowering. Based on the data obtained we recommend to farmers delayed sowing, the use of protective borders and the fulfilment of minimum insulation distances of 20 m for consumption maize and 100 m for lots of hybridisation (distance in case of which the cross-pollination does not exceed the minimum threshold of 0.9%).

Key words: GMO coexistence, cross-pollination, insulation distances, percentage of Xenia.

INTRODUCTION

Genetically modified plants (GMP) represent the agricultural technology with the fastest spreading in history (Raney, 2006). Thus, since 1996, the overall surface cultivated with GMP has grown continuously, reaching in 2014 the value of 181.5 million hectares. At EU level, only two plants are approved for GMP cultivation, namely maize MON 810 and the Amflora potato. In 2014, the Bt maize was grown only in five EU countries occupying 143,016 hectares, the largest surface being cultivated in Spain, namely 131,538 hectares (James, 2014). In Romania, to the year 2014, maize MON 810 (Bt) resistant to the attack of the European corn borer (*Ostrinia nubilalis*) was grown on 770.7 hectares (MADR, 2014).

Although the GMPs have experienced a spectacular spread, their cultivation and marketing of these has generated controversy with numerous implications in several areas (Mocuţa et al., 2011). In order to ensure the free choice of consumers, the EU has adopted several normative acts including Regulation (EC) No. 1830 (2003), which provides for mandatory labelling and Regulation (EC) No. 1829 (2003), which defines the limit of 0.9% GMPs for labelling.

Maize (*Zea mays* L.) is a species with predominantly anemophile allogamous pollination, which makes it an ideal plant for studying and modelling the spreading of pollen (Loos et al., 2003). Moreover, maize has demonstrated the Xenia effect i.e. the pollen from the male parent affects the colour of the seeds (Watanabe et al., 2006), being thus possible the study of cross-pollination rate, between two types of corn with different colours of seeds.

Isolation distance is one of the most important safety measures relating to coexistence, a measure that could reduce the flow of genes. Isolation distances vary considerably across the EU, from a minimum of 25 m in Sweden, to a maximum of 800 m in Luxembourg, for conventional grain maize (Devos et al., 2009).

In Romania, the isolation distance for genetically modified maize is 200 m according to Law No. 266 (2002). These isolation distances established by national authorities are not always scientifically

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substantiated, therefore, remain a controversial subject.

Another important strategy for coexistence in maize, is temporal isolation. Difference between sowing dates can result in a difference between flowering periods, limiting the cross-pollination (Devos et al., 2007). The synchronization between the donor and the receiving plants, and the conditions for local wind are other major factors of influencing the coexistence of plants (Hüsken et al., 2007; Warwick et al., 2009; Langhof et al., 2010). Pollen-mediated gene flow is the major biological source of the "impureness" of conventional maize with transgenic maize. Because of local climatic conditions which may influence the extent of this phenomenon, the rate of cross fecundation should be evaluated in every agricultural region (Devos et al., 2009).

The paper presents information on the influence of distances, of protection curtains and of seeding period on the percentage of xenia; quantifying the level of gene "impureness" at conventional maize with MON 810 through DNA analysis and phenotypic aspects of transgenic genotypes derived from Xenia.

MATERIAL AND METHODS

Biological material studied was represented by:

- conventional maize: inbred line with white grain (A 188); local populations with yellow-orange grain (P 32 and P 285); sweet hybrid ”Deliciul verii”; F 376 hybrid; parental forms ♀ F 376 and ♂ F 376;

- genetically modified maize for resistance to European corn borer (*Ostrinia nubilalis*): (MON 810) and DKC 5784 YG hybrid.

The experiments were performed at the Agricultural Research and Development Station Şimnic – Craiova, Romania (44°19' latitude North and 23°48' longitude East) in climatic conditions of the years 2012-2014.

In 2012 the sowing was done simultaneously, both for donors and for the receivers of the pollen. The gap between the donor's flowering (MON 810) and the apparition of silk at the receiver (conventional maize) was 1 up to 13 days.

In 2013 sowing was delayed (based on observations of the previous year) in such a way that the donor's flowering (MON 810) corresponded to the apparition of female flowers at the receptor of pollen (sweet maize hybrid ”Deliciul verii”).

In 2014 a micro-culture with xenia was set up, where the quantifying of gene pollution level was made with MON 810 and the phenotypic aspects of transgenic genotypes derived from Xenia were noted. For quantification, the method of DNA extraction with “Wizard® Magnetic DNA Purification System for Food” was used.

Sowing diagrams are presented in Figures 1 to 7.

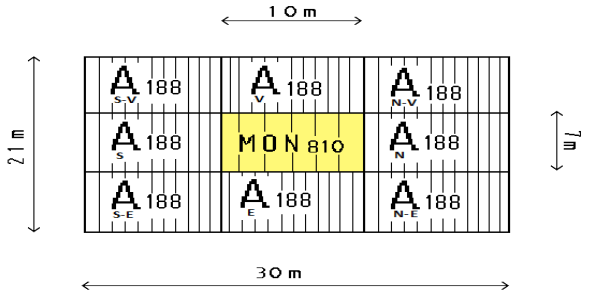

Figure 1. I[st] Experiment – 2012 [in 2013 the inbred line of A 188 was replaced with sweet hybrid ”Deliciul verii” (due to lack of seed) and the orientation towards the cardinal points was changed]

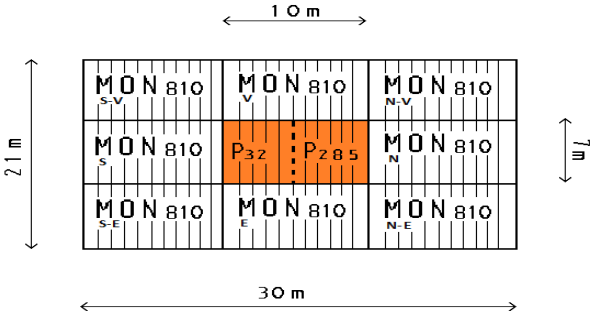

Figure 2. II[nd] Experiment – 2012 [in 2013 – local populations P32 and P 285 were replaced with sweet hybrid ”Deliciul verii” (due to lack of seed) and the orientation towards the cardinal points was changed]

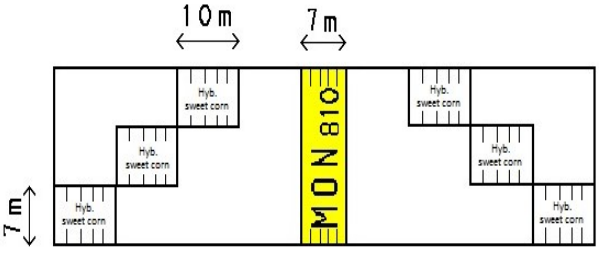

Figure 3. III[rd] Experiment – 2012 [in 2013 – the orientation towards the cardinal points was changed (E – W)

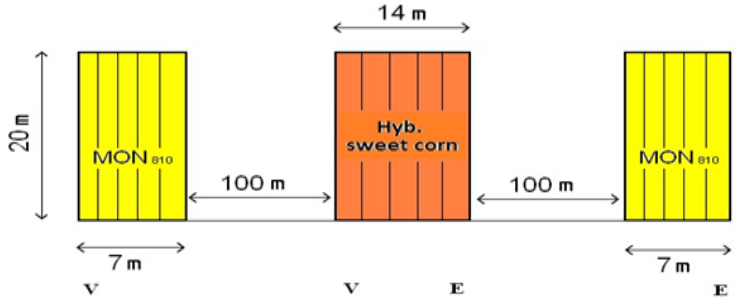

Figure 4. IV[th] Experiment – 2012 [in 2013 – the orientation towards the cardinal points was changed (S – N)]

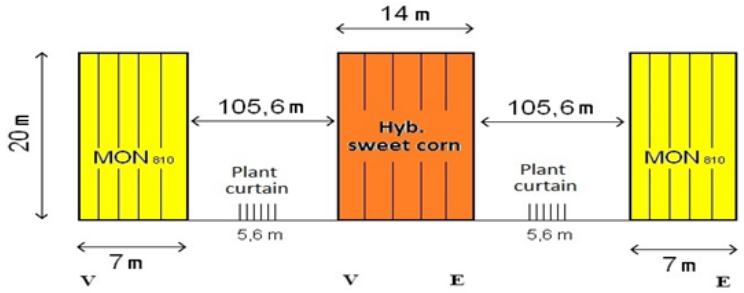

Figure 5. V[th] Experiment – 2012 [in 2013 – the orientation towards the cardinal points was changed (S – N) and sunflower curtains were replaced with Sudan grass]

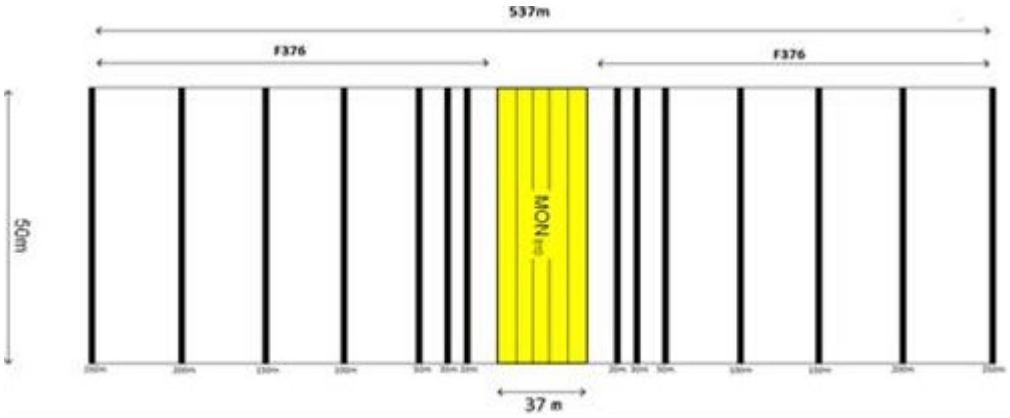

Figure 6. VI[th] Experiment – 2014

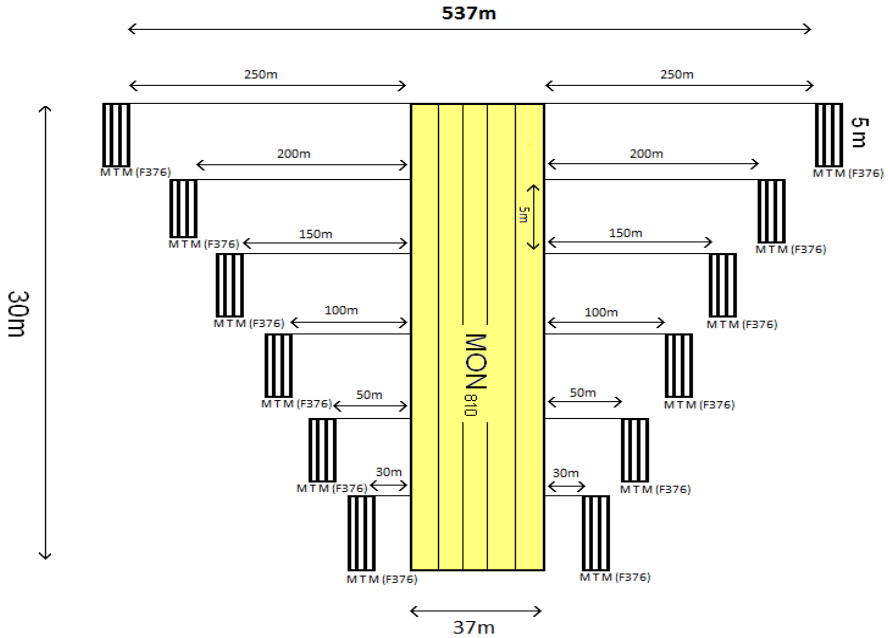

Figure 7. VII[th] Experiment – 2014

RESULTS AND DISCUSSION

Percentage of Xenia

Comparing the two years of experimentation (Table 1), the average percentage of Xenia was markedly greater in 2013, when there was coincidence in flowering. It is also very clear that Xenia percentage decreased as the distance from the pollen donor (MON 810) increased.

Table 1. Average percentage of Xenia for conventional maize depending on sowing period and distance from MON 810

Genotype	Year	Wind direction	Percentage of Xenia
Ist Experiment			
A188	2012	S-E	11.9
Deliciul verii	2013	S-E	0.86
A188	2012	E	17.2
Deliciul verii	2013	E	2.15
A188	2012	-	-
Deliciul verii	2013	N-E	0.83
A188	2012	-	-
Deliciul verii	2013	N	1.95
A188	2012	-	-
Deliciul verii	2013	S-W	1.38
A188	2012	-	-
Deliciul verii	2013	S	2.44
A188	2012	-	-
Deliciul verii	2013	N-W	1.30
A188	2012	-	-
Deliciul verii	2013	W	3.72
IInd Experiment			
P 32; P 285	2012	-	-
Deliciul verii	2013	-	27.62
IIIrd Experiment			
Deliciul verii	2012	N 30 m	0.35
	2013	W 30 m	0.94
	2012	N 20 m	0.42
	2013	W 20 m	0.77
	2012	N 10 m	0.52
	2013	W 10 m	1.74
	2012	S 10 m	0.32
	2013	E 10 m	1.40
	2012	S 20 m	0.29
	2013	E 20 m	0.94
	2012	S 30 m	0.26
	2013	E 30 m	0.90
IVth Experiment			
Deliciul verii	2012	W 100 m	0.42
	2013	S 100 m	0.41
	2012	E 100 m	0.42
	2013	N 100 m	0.44
Vth Experiment			
Deliciul verii	2012	W 100 m	0.26
	2013	S 100 m	0.34
	2012	E 100 m	0.24
	2013	N 100 m	0.34

Plot area or, more precisely, the number of pollen donor plants, based on the number of receiver plants influenced the number of Xenia on the cob and the percentage of Xenia of the respective plot. This was the case of Ist Experiment (2012) when the plants belonging to the inbred line A188 disappeared because of drought, so that the quantity of pollen emitted by MON 810 plot was received by a small number of plants, and the percentage reached 11.9 and 17.2%, respectively. The same situation was registered in the case of IInd Experiment in 2013.

An important role-played the protection bands between the donor and the receiver of pollen, these diminishing the degree of contamination (Vth Experiment).

Identifying the level of gene contamination

To determine the percentage of gene contamination ("impureness") determinations have been made to quantify the DNA from MON 810 of VIth and VIIth Experiments (Table 2).

From VIth Experiment (F 376 consumption maize) we analysed samples 1-7, which were collected from various distances from MON 810.

In the VIIth Experiment (hybridization lot F 376), for lack of seed, we determined percent DNA - MON 810, only for 8-10 variants:

To these analyses we added (Table 2):

- Variant 11 = Version 78/2014 - which in came from seed obtained from IInd Experiment, Row 1, in 2013 (the row on which we found the highest percentage of Xenia – 44.9%);

- Variant 12 = Pop 285 (local population x MON 810), from IInd Experiment, 2012 (which was located in the middle of a plot with MON 810 and even flourished and silk together with MON 810 – where we did not registered Xenia visible in the grain – difference of colour). Then it was assumed that it might be the case of "gametophyte incompatibility".

The results of these analyses showed that in a culture with consumption maize (F 376) next to one with genetically modified maize

(VIth Experiment), the maximum level of gene contamination was 0.33% at 20 m distance from the MON 810. Given that the maximum admitted level of contamination for consumption maize is 0.9%, we can say that even a distance below 20 m could be acceptable.

The percentages decreased progressively with increasing distance from MON 810 (at 30 m – 0.26%; at 50 m – 0.12%). At 100 m the percentage of DNA - MON 810 was already 0% (below the limit of quantification of the kit). The same was recorded at 150 m and 200 m.

Some traces, respectively of 0.005%, appeared at 250 m distance from the source MON 810, but the quantification limit of the kit is 0.1% and below this limit of quantification errors may occur. Even if these percentages were real, it is clear that from 50 m on the level of contamination is below 0.1%, which is insignificant and does not present any risk.

Table 2. Quantification of the percentage of DNA MON 810 in the F1 and F2 seed resulting from conventional maize pollination with genetically modified maize

Sample No.	Name of the variant	Provenance of seed	Percentage of DNA MON 810
VIth Experiment – 2014			
1	Variant 1 – 20 m (East + West)	(F 376 x MON 810) – F_1	0.33
2	Variant 2 – 30 m (East + West)	(F 376 x MON 810) – F_1	0.26
3	Variant 3 – 50 m (East + West)	(F 376 x MON 810) – F_1	0.12
4	Variant 4 – 100 m (East + West)	(F 376 x MON 810) – F_1	0 (Below the quantification limit of the kit)
5	Variant 5 – 150 m (East + West)	(F 376 x MON 810) – F_1	0 (Below the quantification limit of the kit)
6	Variant 6 – 200 m (East + West)	(F 376 x MON 810) – F_1	0 (Below the quantification limit of the kit)
7	Variant 7 – 250 m (East + West)	(F 376 x MON 810) – F_1	0.005
VIIth Experiment – 2014			
8	Variant 8 – 30 m (East R6+ West R1)	(♀ F 376 x MON 810) – F_1	9.77
9	Variant 9 – 50 m (East R6+ West R1)	(♀ F 376 x MON 810) – F_1	1.46
10	Variant 10 – 100 m (East R6+ West R1)	(♀ F 376 x MON 810) – F_1	0.19
Xenia micro-culture – 2014			
11	Variant 11 = Var. 78/2014	IInd Experiment R1 – 2013 (sweet hybrid x MON 810) – F2 (2014 – free pollination in a plot with transgenic plants)	50
IInd Experiment – 2012			
12	Variant 12 = Pop 285	(local population x MON 810) – F_1	38,31%

Note: The quantification limit of the kit is 0.1%. Errors of quantification may occur below this limit.

As the plot terminated (row next to row) next to the MON 810 plot, each row acted as a barrier (curtain protection) in the way of MON 810 pollen. Until the first row for which quantification was made (at 20 m) there were other 29 lines which took over part of MON 810 pollen, and this was more than enough, the percentage of contamination being of nearly 1/3 of the admissible one. Perhaps the distance that could be admitted can go below 20 m, provided there is no free space, but row next to row.

When this protection curtain disappeared, the percentage of contamination was very high, such as 9.77% at 30 m (variant 8), which is unacceptable, especially in the case of a lot of

hybridization (where the permitted level is 0.3%).

At a distance of 50 m, the contamination level was already of 1.46% and at 100 m it dropped to 0.19%. Only here (at 100 m) we can safely recommend placing a lot of hybridization. The highest percentage of contamination by MON 810 in the F1 generation, was recorded in the local population P 285, cultivated in the vicinity of MON 810 maize (38.31%).

Although no Xenia were identified at the phenotypic level (at the time we thought that it was a case of gametophyte incompatibility), exchange of genetic material held in full.

Much more interesting were the results from the F2 generation (variant 11 - 50% MON 810). If seeds of F1 generation (derived from inter-pollination of a hybrid of conventional maize with MON 810), arrive in culture in the following years, the F2 generation accumulates more genetically modified DNA (through pollination with other transgenic plants from crop but also through the mutational activity to which transgenic organisms are prone to).

Following the pace of decline in the level of contamination with increasing distance from MON 810, one can see that from 20 m to 30 m (10 m difference) 0.07 percent is lost and from 30 m to 50 m (20 m difference) 0.14 percent is lost.

By a simple calculation we find that with every row cultivated (0.7 m) 0.0049% of the level of contamination is lost. By dividing the actual number of rows existing in the field, at the distance of 10 m we have 14 rows, which means 0.005% and at the distance of 20 m we have 29 rows, which means 0.0048% per row, very close to theoretical calculation.

Going backwards from 20 m to 0 m, the 0.33 percent allocated to each row (0.7 m) means 0.01%. In fact, there are 29 rows, each having 0.01% - which is the maximum level of contamination, because these are the nearest rows to MON 810 plot.

Theoretically, up to the permissible level of contamination (0.9%) we can approach more to the MON 810 plots even up to 1-2 rows. However, from the analysis of DNA percentage to MON 810 (Variant 12 - 38.31%), one can see that in the F1 generation, the degree of contamination is also determined by the amount of MON 810 pollen, respectively by the size of the plot and the ratio between the donor plot (MON 810) and the pollen receiver plot (conventional maize). So, for greater certainty, we can cultivate genetically modified maize (MON 810) in the vicinity of conventional corn with the recommendation to eliminate a band of 20 m all around it (considering it to be genetically modified maize).

Phenotypic aspects of transgenic genotypes obtained from Xenia

Experimental variants obtained from Xenia in 2012 and 2013 were a mixture of genotypes (inbred line A 188 – with white grain; sweet hybrid ”Deliciul verii”; hybrid DKC 5784 YG - MON 810), each plant coming out of a grain with another genetic constitution.

We planted these grains in 2014 in a Xenia micro-culture with free pollination and the resulting cobs (F2 generation) were analysed in terms of ear appearance, colour and type of grain. Test plots being planted next to each other and pollination being free, the probability of combining genes was much higher and the number of Xenia was very high (Photos 1 A and 1 B).

Photo 1 A. Xenia – F2 generation obtained through free pollination

Photo 1 B. Intermediate phenotype MON 810 + A 188 (cob with white grains)

There was a great phenotypic variability in terms of colour and grain type and general appearance of the ear type of grain. Dent grain type (specific for MON 810), wrinkled (shrunken) - specific for sweet hybrid, but also new or intermediate types appeared.

Type of grain and grain colour is actually the phenotypic expression of the F2 generation, and as a result of free pollination between different transgenic genotypes, mutational activity appears to be very intense. At the level of the same variant we obtained cobs of several shapes, types and sizes and of different colours (Photo 2).

Photo 2. Ears of different shapes, types, sizes and colours within the same variant

The colour of the pericarp of the grain varied greatly from white to dark red (scarlet). This proves once again that genetically modified plants F1 are more prone to epigenetic mutational events. Methylation or hypo-methylation at the level of the gene *p1* (pericarp colour) as mechanisms of epigenetic changes, affect the level of biosynthesis and expression of flavonoid type flobafen scarlet.

P1 gene has more than 100 alleles and epi-alleles which are identified in the coloration of cob and pericarp. While the genetic pattern *P1-rr–* presents a red pericarp and a red cob, *P1-wr–* combination presents white pericarp and red cob.

According to Chopra et al., (1998), the two alleles have more than 99% of the similar DNA segments but the *P1-rr–* is composed of a single-unit gene while *P1-wr–* has 6 or more copies related head to toe. Furthermore, *P1-wr–* is heavily methylated in the promoter region. There is another transcriptional factor acting dominantly over gene *P1-wr*, *Unstable factor for orange one (Ufo1)*, which modifies the pattern of phenotypic expression of flobafen from pericarp. *Ufo1* associated with *P1-wr* can induce phenotypes which express pericarp pigmentation from red to black (in our case to dark-red, scarlet). There is a direct correlation between the level (amount) of pigment in the pericarp and the hypo-methylation of alleles *P1-wr*. Furthermore, there is a proper pattern of methylation for each cell, expressing that somatic mosaic of methylation.

CONCLUSION

The rate of cross-pollination of studied conventional genotypes with genetically modified maize (MON 810) was dependent on: the coincidence of flowering of the pollen donor (MON 810) with the apparition of silk at the pollen receptor; the amount of pollen emitted by donor (plot size); isolation distance between conventional and genetically modified maize and the presence or absence of protection curtains.

By analysing gene flow through phenotypic changes due to the action of dominant genes (*Su1 Su1*) on recessive genes (*su1 su1*) of sweet corn, it can be said that the greater the distance from the source donor of pollen (MON 810) the more the percentage of Xenia (*Su1 su1*) decreases.

DNA % Quantification - MON 810 showed that:

- There is a possibility of cultivation of genetically modified maize MON 810 without risk, in the same area with conventional consumption maize, provided that the protective strip (maize or other plants even taller) between the two cultures has 20 m. The level of impurities at 20 m was 0.33%.
- At 100 m distance from MON 810 we can safely recommend placing a lot of hybridisation. The level of contamination was 0.19%.
- In the F1 generation (variant 12 - P 285-38.31%) the degree of contamination is also determined by the amount of MON 810 pollen, respectively by the size of the plot and the ratio between the donor plot (MON 810) and the pollen receiver plot (conventional maize).

If F1 generation seeds (obtained from inter-pollination of conventional corn hybrid

with MON 810), arrive in culture in the following years, the F2 generation accumulates more and more genetically modified DNA (through free pollination with other transgenic plants from crop but also through mutational activity that these transgenic organisms are prone to).

In the transgenic plants obtained from Xenia there is great phenotypic variability in terms of colour and grain type and also general appearance of the ear. This proves once again that transgenic plants F1 are more prone to epigenetic mutational events.

Based on these results we can recommend several options of conventional maize cultivation within the same agro-ecosystem with genetically modified maize MON 810, which remain at the discretion of the farmer:

- Careful selection of genotypes to be grown in the same area, according to the precocity group (preferably different groups in order not to have coincidence in blooming).

If selected genotypes belong to the same earliness group:

- Staggered sowing (depending on the time of genotypes vegetation) so that there is no coincidence in blooming.

- Minimum isolation distance, at which the minimum allowable threshold of contamination is not exceeded, is 20 m for consumption maize and 100 m for lots of hybridization.

- Use of protective curtains (preferably plants taller than maize or maize).

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