

Technical Note

Abstract

The objective of this study was to assess and quantify the gene flow in soybean. It were used two batches of soybean seeds, the cultivar CD 201 (non transgenic) and the CD 212 RR (transgenic, with the RR gene), both from the breeding program of COODETEC (Cooperativa Central de Pesquisa Agrícola), located in Cascavel (Paraná State-Brazil). They were sown in an experimental design of concentric rings, totaling an area of 100 m of diameter and 2.500 m². The sampling points were delimited in eight halfway rays, in the directions S, SW, SE, N, NW, NE, E and W, on which 480 collection points were defined. The 96 seeds collected from all these points were analyzed by the standard germination test, using paper towel soaked in 0.03% of glyphosate herbicide solution. The rate of cross pollination was calculated by the percentage of seedlings which showed to be resistant to the herbicide, in relation to the total of analyzed plants. The results showed a cross pollination of 0.027%. It is evidence that the gene flow is very low in soybean plants.

Key words: cross pollination; glyphosate; *Glycine max* (L.) Merrill; soybean RR.

Estimate of the gene flow between transgenic and non transgenic soybean cultivars

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Estimativa do fluxo gênico entre cultivares de soja transgênica e não transgênica

Resumo

O objetivo deste trabalho foi avaliar e quantificar o fluxo gênico em soja. Foram usados dois lotes de sementes de soja, a cultivar CD 201 (não transgênica) e a cultivar CD 212 RR (transgênica, com o gene RR), ambas do programa de produção da COODETEC, localizado em Cascavel (Paraná). O experimento foi semeado em sistema de anéis concêntricos, totalizando uma área de 100 m de diâmetro e 2,500 m². Os pontos foram delimitados em 8 raios medianos, nas direções S, SW, SE, N, NW, NE, E e W em nos quais foram definidos 480 pontos de avaliação. As 96 sementes coletadas nos 480 pontos foram analisadas pelo teste de germinação, usando o substrato papel-toalha embebido em solução saturada do herbicida glyphosato, na concentração de 0,03%. A taxa de polinização foi calculada pela porcentagem de plantas que mostraram resistentes ao herbicida, em relação ao total de plantas analisadas. Os resultados revelaram uma polinização cruzada de 0.027%, comprovando que o fluxo de genes é muito baixo em plantas de soja.

Palavras chave: polinização cruzada; glifosato; *Glycine max* (L.) Merrill; soja RR.

Estimación del flujo de genes entre cultivos de soja transgênica y no transgênica

Resumen

El objetivo de este estudio fue evaluar y cuantificar el flujo de genes en la soja. Fueran utilizados dos lotes de semillas de soja cultivar CD 201 (no transgênica) y el cultivar CD 212 RR (transgênica, con el gen RR), ambos del programa de producción de COODETEC, ubicada en Cascavel (Paraná). Se sembraron en un diseño experimental de anillos concêntricos, con una superficie total de 100 m de diâmetro y 2.500 m². Los puntos se delimitaron en 8 rayos medianos, en las direcciones S, SW, SE, N, NW, NE, E y W, donde se definirian 480 puntos de evaluación. Las 96 semillas recolectadas en los 480 puntos fueran analizadas mediante la prueba de la germinación, utilizando el sustrato papel-toalla embebido en solución saturada del herbicida glyphosate a una concentración de 0,03%. Se calculó la tasa de polinización por el porcentaje de plantas que

Received at: 06/09/2013

Accepted for publication at: 03/03/2014

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mostrarán ser resistente al herbicida, con relación al total de plantas analizadas. Los resultados revelaron una polinización cruzada de 0,027%, lo que confirma que el flujo de genes es muy bajo en las plantas de soja.

Palabras clave: polinización cruzada; glyphosate; *Glycine max* (L.) Merrill; soja RR.

Introduction

genetically modified species (GMOs) are a reality and the amount of available cultivars in the market increase every day. The production of transgenic plants is led by the United States, with 42% of the planted world surface, followed by Brazil, with approximately 23% of the world market (ISAAA, 2014). Soybean is a great expression of culture in Brazil, and for the 2013/2014 crop, the estimated production is about 85.4 million tons in an area of 29.7 million hectares (CONAB, 2014).

When the subject is transgenic, many questions come out. They refer to the safety of these materials, being the natural crossing, gene flow, with conventional cultivars, what draws more attention (RAY et al., 2003). In Brazil, specifically in the case of soybeans, this risk should not be considered negligible, given the absence of other sexually compatible species with *Glycine max* in the country (BORÉM and MIRANDA, 2009). Thus, measures that prevent gene flow between cultivars in seed production fields are required. Several papers mentioned in the literature had as objective estimating the frequencies crosses pollination among transgenic and nontransgenic plants in potato (CONNER and DALE, 1996), canola (SCHEFFLER et al., 1993), cotton (SHEN et al., 2001, FREIRE, 2002), species of the genus Brassica (SCHEFFLER et al., 1993) and soybean (BORÉM, 2000), among others.

The gene flow can be defined as a change in the frequency of particular alleles, that is, the transfer of alleles of a cultivar/specie to another. The rate of gene flow is superior in allogamy species, which it is usual cross pollination. In the case of the soybean, in which there is autogamy situation (AHRENT and CAVINESS, 1994), the consequences of the gene flow are less drastic. According to BORÉM (2000), there are no reasons for concern, because many factors have to occur in order to allow the gene flow or gene escape of a transgenic cultivar to a conventional one. The flowing factors should be considered: a) Individuals alleles should be sexually compatible; b) it should happen temporarily and it needs the same time of flowering and, c) the resulting hybrid should be viable (CHÉVRE et al., 1998). In function of these requirements, the rate of cross pollination,

in soybean, is around 1% (BORÉM, 2000), and can reach up to 2.5% (AHRENT and CAVINESS, 1994).

The gene flow can be measured direct or indirectly. The most common method to measure the gene flow is in a direct way. In plants, it is based on the observation of the dispersion of the pollen grains of seeds, which allows the estimate of gene flow potential or of the gene dispersion. Other direct methods use genetic markers and, in that case, it can be considered the real gene flow (MALONE et al., 2004).

Some manners of determining the frequency of cross pollination of an OGM cultivar at different distances are to surround it with other nontransgenic cultivars, similar in the flowering period. The experimental design should have a central circle in which the nontransgenic cultivar is sowed. At the next concentric ring, a transgenic variety is sowed. The remaining experimental area must be with nontransgenic cultivars (BORÉM, 2001). At this way, pollen from transgenic cultivar will be transferred by winds to nontransgenic cultivar inside or outside the circle.

This methodology was used in this study using two batches of soybean seeds, one of cultivar CD 201 (non-transgenic) and the other one of cultivar CD 212 RR (transgenic, carrying the gene RR), with the objective of estimating the rate of gene flow between these two cultivars.

Material and Methods

This study was developed at the Laboratory of Seed Test at the Federal University of Pelotas (UFPel), Pelotas (Rio Grande do Sul, Brazil) and COODETEC (Cooperativa Central Agropecuária de Desenvolvimento Tecnológico e Econômico Ltda.), located in Cascavel (Paraná State-Brazil).

Both cultivars of soybean, CD 201 and CD 212 RR, came from the COODETEC breeding soybean program. The cultivar CD 212 RR contains the resistance gene to the glyphosate and, in function of this, it was used as a pollen donor.

The experimental design was in concentric rings, proposed by BORÉM (2001), and cultivar CD 201 was sowed in the center of the design, totalizing 120 plants in an area of 4 m². Around that circle,

forming a ring of two meters of width, it was sowed the cultivar CD 212 RR, conforming an intermediate ring of 36 m². And in the periphery it was put, again, the cultivar CD 201, until a distance of 50 m, from the center of the experiment (Figure 1).

The delimitation of portions for sampling was determined by eight rays of 50 m of length, in all directions (S, SW, SE, N, NW, NE, E and W). They were distributed in equivalent distances and were defined 60 sampling points in each one of the eight geographical orientations, totalizing 480 sampling points. The sampling points were defined at each 0.80 meters, for each one of the eight rays, starting from the center of the experiment, not considering the intermediate transgenic cultivar ring of two meters. Only the seeds of plants of the cultivar CD 201 were collected for the laboratory evaluations. In function of that, if the pollination happens, the individual hybrid will be heterozygote (Aa) for the transgene. The seeds sampled were sowed again to allow the expression of the transgene and the resulting plants were analyzed for the resistance to glyphosate.

The area where the experiment was installed has as physical characteristics, a slightly wavy topography, with the area of the central and intermediate ring slightly higher, favoring, in that way, the exhibition of the flowers and, consequently, the action of the pollinator agents, mainly wind and insects.

A total of 96.000 seeds (4 repetitions of 50 seeds for each one of the 480 sampling points) were analyzed, using the standard germination test, in agreement with the rules of analysis of seeds - RAS (BRAZIL, 2009). It was used as substratum, paper-towel soaked in aqueous solution containing the glyphosate herbicide (4-hidroxi (metil) fosfinol-diholoalamina salt of ammonium) in the concentration of 0.03%, in equivalent amount to 2.5 times the weight of the dry paper. The germination test was accomplished in a germination chamber, regulated to the constant temperature of 25 °C. The counting of the seedlings was accomplished at 10 days after sowing. The rate of genetic contamination was estimated as the percentage of plants that showed to be resistant to the herbicide, in relation to the total of analyzed plants.

Results and Discussion

The seed germination was verified, and the resistant seeds to the herbicide presented a normal

initial development. Instead, the nonresistant seedlings, died after 5 days.

From 96.000 seeds analyzed, only four seeds were resistance to the herbicide. Considering that the genetic contamination happened until a maximum of 4 meters, from the donor pollen ring, the index of genetic flow was estimated excluding the sampling points outside of that radio of 4 meters. Then, 14.400 seeds from the 72 sampling points were used to estimate the rate of genetic flow. That rate was of 0.027%. The index of contamination obtained in the present work was superior to the obtained by MALONE et al. (2004), when analyzed the index of genetic contamination among commercial cultivars of soybean by molecular markers, they obtained contamination indexes (CI) of 0.07 and 0.006. According to BORÉM (2001), for the specific case of the soybean tolerant to herbicides, the risk of gene escape is minimized due to the low taxes of natural crossing and cleystogamous flowers. However, the tax of crossed fecundation among soybean cultivars with similar vegetative cycle and the same habitat, can overcome 1%.

In Alfenas-MG, SILVA and MACIEL (2010), also evaluating the flow of pollen from transgenic and conventional soybean plants obtained outcrossing rates of 0.025% for 1.0 plants m away and 0.01% for 2.0 m away. In the assessment of gene flow in maize in nine different locations in Brazil, NASCIMENTO et al. (2012) observed that in most cases, rates higher than 1% of gene flow occur only up to 20 m away, and in only one location was observed more than 10% of gene flow at 50 m, but in all locations gene flow rate was less than 1% at 100 m distance.

The reduced rate of crossed pollination observed in this work can be based upon some factors such as the application of insecticides during the experiment, which might have reduced the presence of pollinator insects. Although, the flowers of soybean plants are not attractive for the insects, according to ARIAS et al. (1994), especially the members of the order Hymenoptera, which can act as pollinators. The eolic distribution of the soybean pollen is extremely limited to some very close areas, due to the density of the pollen grain of soybean, it is difficult to be transported by wind. Because of this, the entomophylous distribution of pollen has been told as the main source of crossed pollination in soybean.

Another factor that could have influenced in the drop rate of identified crossed pollination, regards

incorporation and expression of the transgene (RR). Many times, depending on the degree of dominance of the transgene, it could be able or not to have the capacity to express with just one copy (an allele) present. As it was mentioned previously, the seeds, and consequently the seedlings were analyzed as F1 generation. The fact of they came from two pure lines homozygotes, the whole population F1 has composition genetic heterozygote for absolutely all genes, or at least for the gene selector, in this case the RR gene.

Thus, in the case of occurring gene flow, the resulting plants will be carrying of just one copy of the transgenic, being constituted in heterozygotes (Aa) or hemizygotes (A_) for the RR gene. If the RR gene is dominant, that situation is very probable; its expression is guaranteed in all plants F1 that suffered natural crossing with the transgenic cultivar. In the case that the RR gene presents partial dominance or incomplete dominance, only the homozygote

constitutions will express the character, and this situation could be masked in the heterozygotes constitutions. That sort of question can be solved accomplishing more deepened molecular analyses, seeking to identify the presence of the transgenic in the conventional cultivar genome. Technologies like hybridization of nucleic acids (Southern Blot) or amplification of the transgene (Polymerase Chain Reaction - PCR) have been used thoroughly with that purpose.

Equal importance should be addressed to the size of the donor pollen plot. The scarce distance that the pollen grain of soybean can be reached transported by wind, a larger population of transgenic plants, pollen donors, could have contributed for a higher rate of cross pollination. Probably, the fact that one of the four identified resistant seeds in the present analysis was collected in the central ring of the experiment, in the area totally surrounded by a ring of plants of transgenic cultivar, it gives support to

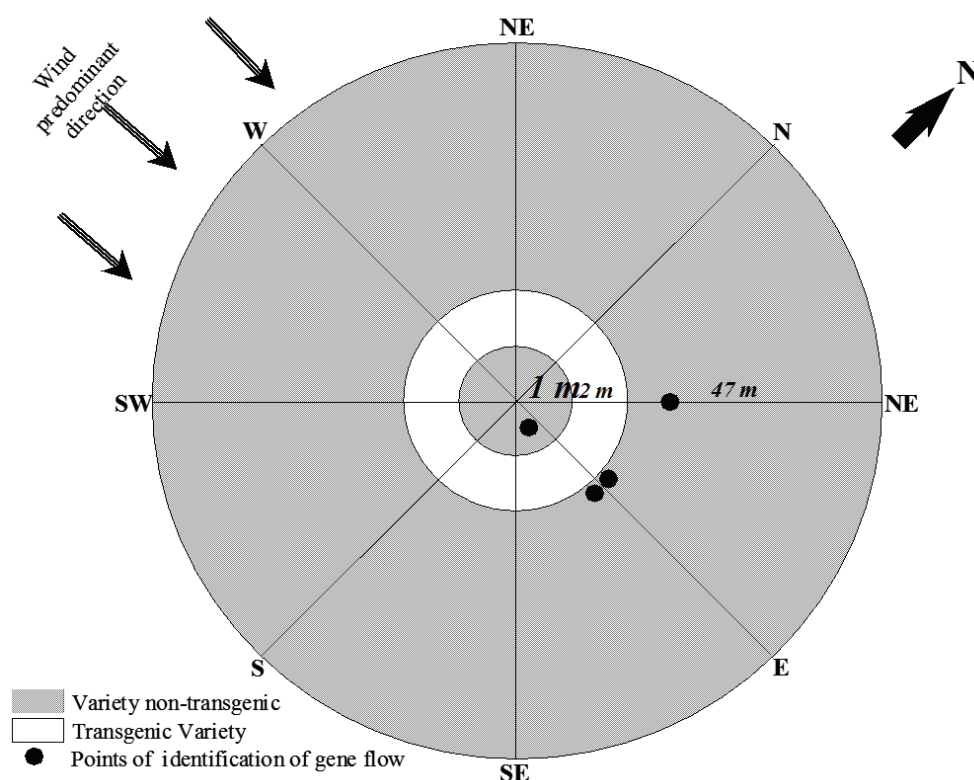


Figure 1. Schematic of the experimental design used, showing the disposition of transgenic and nontransgenic cultivars, the direction of the predominant winds and the points where it was identified the presence of gene flow. Pelotas-RS, 2012.

conjectures about that. From the remaining resistant seeds, two were collected in two spots of different sampling in the East ray, as consequence, located in the predominant direction of winds during the flowering stage. The remaining seed was collected in the NE ray. Two seeds were collected with 0.5 meters of distance of the pollen source in the East ray, and one collected 4.0 meters of distance from the pollen source in the NE ray (Figure 1), they evidence that, indeed, besides those distances, the rate of cross pollination seems to be extremely reduced.

The results of some studies accomplished during three years with nontransgenic soybean, indicated that the occurrence of crossed pollination among superior distances to 4.6 m, is rare (AHRENT and CAVINES, 1994). According to PEREIRA et al. (2012), starting from three meters between soybean cultivars it is rare the occurrence of gene flow. As stated by NODARI and DESTRO (2014), in most cases where there was contamination, it was found that the transgenic soybean cultivar resistant to the herbicide Roundup was being grown side by side or a few feet away from conventional varieties.

The results obtained in this study and in other scientific studies – that tried to identify and quantify rates of gene flow among transgenic and conventional cultivars of soybean - are good news for the Brazilian soybean growers, that are worried because their conventional cultivars could be contaminated with genes of transgenic cultivars and consequently, they should begin to pay for a technology that they did not ask to have.

However, the limits of geographical isolation, of 3 meters, established in the current Brazilian rules for production of Certified Seed (BRAZIL, 2006), do

not guarantee a safe isolation of production areas of soybean seed. Therefore, it is suggested the increase of geographical isolation limits from three, to five meters, for areas of soybean seed production.

Conclusions

The present study contributed for the search of strategies to reduce the occurrence of adventitious plants, seeking an appropriate handling on farming, implementing crop rotation and herbicides rotation with the objective of reducing the appearance of resistant harmful plants and, mainly, providing conscience about the application and execution of the rules of seeds production.

The results of this study emphasize that the rate of genetic contamination is about 0.027% as the obtained in this study, it does not appear to be worrying, and, the important knowledge observed is that the standards for certified seed production of soybean should be changed its geographical isolation, from three meters to five meters of distance, from one cultivar to another cultivar, even if it is not a transgenic cultivar.

Acknowledgements

We would like to thank COODETEC for the support that the Company gave us when this research was conducted, offering all the conditions for performing the study. We also are thankful to CNPq, the Brazilian Counseling Research Institution, which gave support to the Graduate Student O. Levien for accomplishing part of this work.

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