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Gene flow from transgenic to nontransgenic soybean plants in the Cerrado region of Brazil

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ABSTRACT. Evaluation of transgenic crops under field conditions is a fundamental step for the production of genetically engineered varieties. In order to determine if there is pollen dispersal from transgenic to non-transgenic soybean plants, a field release experiment was conducted in the Cerrado region of Brazil. Nontransgenic plants were cultivated in plots surrounding Roundup Ready transgenic plants carrying the cp4 *epsps* gene, which confers herbicide tolerance against glyphosate herbicide, and pollen dispersal was evaluated by checking for the dominant gene. The percentage of cross-pollination was calculated as a fraction of herbicide-tolerant and -nontolerant plants. The greatest amount of transgenic pollen dispersion was observed in the first row, located at one meter from the central (transgenic) plot, with a 0.52% average frequen-

cy. The frequency of pollen dispersion decreased to 0.12% in row 2, reaching 0% when the plants were up to 10 m distance from the central plot. Under these conditions pollen flow was higher for a short distance. This fact suggests that the management necessary to avoid cross-pollination from transgenic to nontransgenic plants in the seed production fields should be similar to the procedures currently utilized to produce commercial seeds.

Key words: Transgene flow, Transgene dispersal, Transgenic soybean, Isolation distance, Pollen dispersal, Outcross

INTRODUCTION

Recombinant DNA technology, associated with sexual breeding methods, can accelerate the development of plants with useful traits. Indeed, genetic engineering development of several crops carrying useful traits has become a reality (Aragão et al., 2000, 2002, 2005; Dias et al., 2006; Nunes et al., 2006; Faria et al., 2006). The USA, followed by Argentina, Brazil, Canada, and China, have lead the world in the cultivation of genetically modified crops; some products have been approved for commercialization, such as cotton, soybean, and maize (James, 2005). Brazil is the world's second largest soybean producer, with 53.4 million tons produced, cultivated on 22.2 million hectares, 46.4% of which is produced in the central region of Brazil (www.conab.gov.br).

Questions about biosafety of transgenic plants have been raised, such as gene flow via pollen. This matter has received considerable attention and effective evaluation in other countries (Rogers and Parkes, 1995; Llewellyn and Fitt, 1996). Brazilian soybean producers have expressed concern about the possibility of having their nontransgenic soybean areas pollinated by transgenic soybean varieties in nearby fields. In addition, there is interest in evaluating cross-pollination between transgenic and nontransgenic lines and its significance for plant breeding and for seed-production fields.

Soybean flowers are highly self-pollinating (Ahrent and Caviness, 1994); pollination occurs in the advanced bud stage when the stigma becomes receptive. The anthers dehisce, covering the stigma with pollen before the flowers open. Cross-pollination frequency in soybeans has generally been found to be less than 1% (Poehlman, 1987). However, Ahrent and Caviness (1994) found as high as 2.5% cross-pollination in some varieties. The effect of insects has been evaluated, especially those belonging to the order Hymenoptera, which can act as pollinators (Caviness, 1970; Beard and Knowees, 1971). Natural cross-pollination in soybean was found to be rare across distances greater than 4.6 m from the pollen source and did not vary greatly over a period of three years (Caviness, 1966; Ahrent and Caviness, 1994). Nelson and Bernard (1984) suggested that an appropriate distance from a pollen source would be at least 5 m; while Boerma and Moradshahi (1975) found that cross-pollination decreased to 0% at a distance of 7 m from the pollen source.

When field-testing transgenic crops, the regulatory authorities generally specify physical distance isolation to avoid cross-pollination. To maintain seed purity, it is important to de-

termine the distance over which effective gene flow can occur. Several experiments have been carried out to determine the rates of cross-pollination between transgenic and nontransgenic plants of some species, such as potatoes (Tynan et al., 1990; Conner and Dale, 1996), rapeseed (Scheffler et al., 1993), and cotton (Umbeck et al., 1991; Llewellyn and Fitt, 1996; Shen et al., 2001). Because of the limited available information regarding gene flow in soybean field tests under Brazilian conditions, we conducted studies to help develop safeguards for biological pollen containment, through an evaluation of pollen dispersal from transgenic to nontransgenic soybean plants cultivated in a Cerrado region of Brazil.

MATERIAL AND METHODS

Transgenic plants

Ttransgenic soybean seeds of the variety BR00-69515 Conquista derivated (BRS Valiosa, Roundup Ready, RR), genetically modified with the *epsps* gene (*Agrobacterium* sp strain CP4) (Padgette et al., 1995), which confers tolerance to glyphosate, a herbicide that is utilized in controlling annual and perennial mono- and dicot weeds were planted. All transgenic plants were homozygous for the *epsps* gene. Nontransgenic seeds of the variety Conquista were used as controls.

Field planting

The field test was located at Embrapa Cerrados, Planaltina, DF, Brazil (15°31'53" S, 47°42'30" W; at an altitude of 970 m). The experimental field conditions consisted of a central 64-m^2 plot planted with transgenic soybeans and four 80-m^2 plots with nontransgenic soybean plants surrounding this central plot. These were named North (N), West (W), East (E), and South (S), which were potential pollen receptors (Figure 1). Seeds were planted directly in the field in December 2001. Each plot consisted of 10 rows and had four experimental units, while each experimental unit had about 2-12 plants covering 1 m^2.

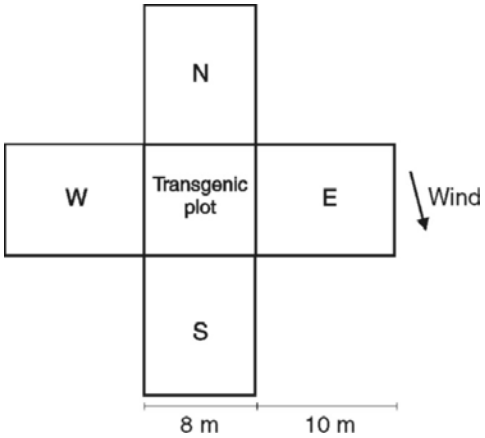

Figure 1. Field trial plot design. Plots North (N), West (W), East (E), and South (S) are indicated surrounding the central plot. The arrow indicates the predominate wind direction.

All seeds generated by the nontransgenic plants were harvested by hand, counted, and planted in another field at Embrapa Cerrados, in May 2002. Gene flow from transgenic to nontransgenic plants was evaluated by testing for the dominant *cp4 epsps* gene. All plants were sprayed at the third trifoliolate stage with the herbicide glyphosate (Transorb®, Monsanto) at 5 L/ha; the nontransgenic plants died within two weeks after the herbicide treatment. A leaf-disk was collected for PCR analyses to assay for the *cp4 epsps* gene in all tolerant plants. The frequency of cross-pollination around the central plot was determined by calculating the fraction of transgenic seeds in each row that developed in each nontransgenic plot.

Polymerase chain reaction

For PCR analyses, DNA was isolated from leaf-disks by the method of Edwards et al. (1991). Each reaction was carried out in a 25-µL mixture containing 10 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 160 µM of each dNTP, 200 nM of each primer, 2 U *Taq* polymerase (Gibco BRL), and about 20 ng genomic DNA. The mixture was overlaid with mineral oil, denatured for 5 min at 95°C in an MJ Research thermal cycler (USA), and amplified for 35 cycles (95°C for 1 min, 55°C for 1 min, 72°C for 1 min) with a final 7-min cycle at 72°C. The reaction mixture was then loaded directly onto a 1% agarose gel, stained with ethidium bromide, and visualized with UV light. The primers 5'-ATGTCG CACGGTGCAAGCAG-3' (EPSPS 1) and 5'-CGTCTCGACGGTAAGGTTGG-3' (EPSPS 654c) were used to amplify a 654-bp sequence to screen the transgenic plants for the presence of the *epsps* cassette.

Statistical analysis

Data were examined with regression and association analysis. The Student *t*-test was utilized to compare means. Statistical analyses were performed using SAS® (SAS Institute Inc., SAS Campus Drive, Cary, NC, USA), and the graphical analyses were conducted with GNUPLOT 4.0 (available at: http://www.gnuplot.info/download.html).

RESULTS AND DISCUSSION

As expected, all herbicide-tolerant plants contained the 654-bp sequence within the *epsps* cassette, showing that no nontransgenic plants escaped herbicide treatment. Another 30 nontransgenic plants utilized as a negative control revealed neither herbicide tolerance nor presence of the transgene, validating the screening method using herbicide to identify events of cross-pollination.

The highest percentage of pollen dissemination from transgenic to nontransgenic varieties occurred in the first row around the central plot, up to one meter from the transgenic plot (Table 1). The frequency of pollen dispersal already decreased in the second row at a 2-m distance, and continued decreasing gradually from 0 to 0.01% at 10 m from the central plot.

Table 1. Percentage of cross-pollination from transgenic to nontransgenic soybean plants in North (N), South (S), East (E), and West (W) plots.

Distance (m)	Direction				
	N (%)	S (%)	E (%)	W (%)	Average (%)
1	0.40	0.43	0.52	0.73	0.52
2	0.09	0.12	0.05	0.21	0.11
3	0.10	0.01	0.13	0.11	0.08
4	0.00	0.05	0.03	0.15	0.05
5	0.01	0.00	0.01	0.02	0.01
6	0.01	0.00	0.01	0.02	0.01
7	0.00	0.00	0.05	0.06	0.02
8	0.00	0.00	0.02	0.02	0.01
9	0.01	0.00	0.01	0.00	0.00
10	0.00	0.00	0.00	0.01	0.00
Number of seeds	92,792	81,325	88,559	82,588	86,316
Cross-pollination events[a]	51 (0.06%)	40 (0.05%)	67 (0.08%)	103 (0.13%)	65 (0.08%)

[a]Numbers in parentheses represent the percentage of transgenic cross-pollination events.

An association between distance and cross-pollination frequency was found (Figure 2). There was significantly more pollen dispersal at 1 m in comparison with all the other distances evaluated. However, there were no significant differences among the distances of 2, 3, and 4 m; 5, 6, and 7 m; as well as 8, 9, and 10 m (Figure 2). Considering only the 8, 9, and 10 rows, the frequency of transgenic soybean pollen transference was 0%, showing no significant difference according to the statistical analyses (Figure 3). However, in absolute terms, there was an allelic transference from transgenic to nontransgenic soybeans. At 10 m, the frequency of

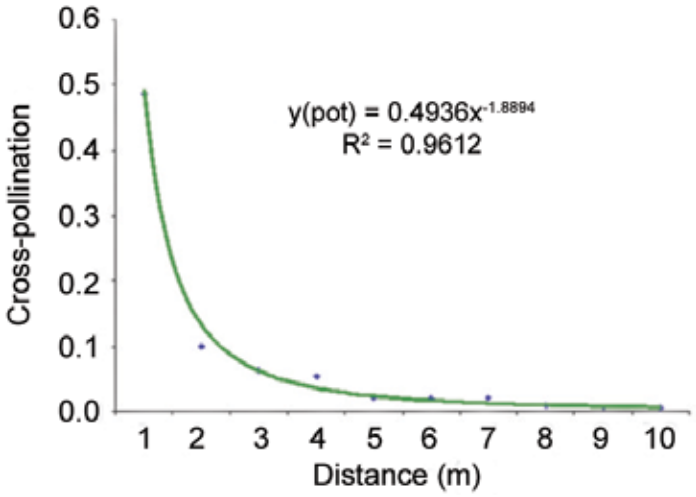

Figure 2. Regression curve for the cross-pollination from transgenic to nontransgenic soybean plants as a function of the distance (m).

cross-pollination was 0.0179% in the W plot, representing 18 in 100,000 plants. The average percentage of cross-pollination was 0.0044% at 10 m. The rate of cross-pollination in soybean is very low, tending to zero in distances greater than 8 m (Figure 2). Therefore, a distance of isolation greater than 10 m would ensure purity in a seed-production field.

Because the wind direction was preferentially N-S in the experimental area, a higher frequency of cross-pollination in the direction S would be expected. There was no significant effect of wind direction on RR allelic transmission (data not shown). However, cross-pollination in soybean is mainly due to pollinators, and it is evident that there was no wind effect on the insect pollination. Adequate control of insects during the period culture should greatly reduce gene flow levels. Although the insect populations were not monitored during the flowering period, potential pollinating insects, such as *Apis mellifera* L. and *Trigona spinipes* Fa-

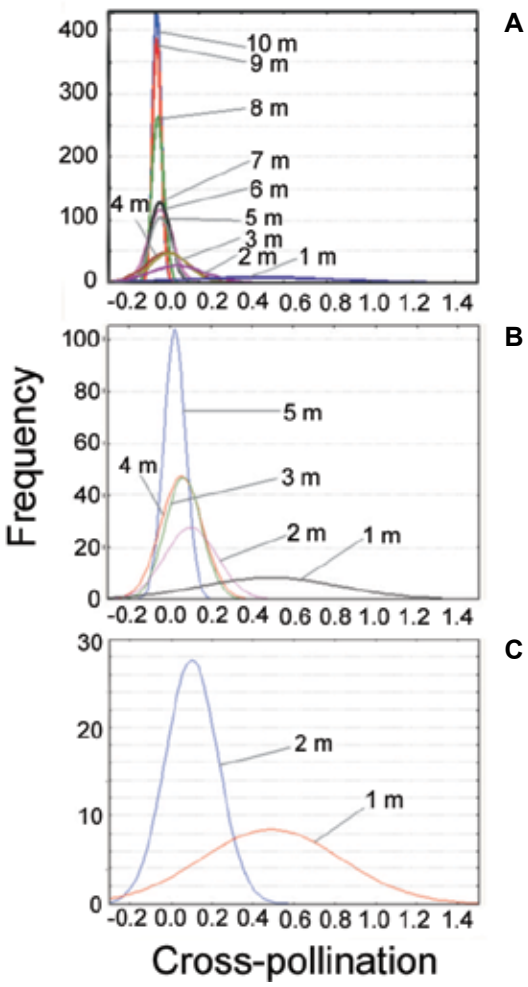

Figure 3. Frequency distribution of the distances of cross-pollination from transgenic to nontransgenic soybean plants. (A) Distance distribution; (B) details of (A); (C) cross-pollination rates and differences between 1-m and 2-m distances.

bricius (Hymenoptera: Apidae), were observed. In our study, the amount of insecticide applied during the flowering period was lower than usual under Brazilian conditions. This may have increased the population of pollinators. Thus, normal gene flow may be even lower than that observed in our study.

Cross-pollination in soybean has generally been found to be less than 1% (Poehlman, 1987); in some cases it can reach as high as 1.6 to 2.5% (Ahrent and Caviness, 1994). Although soybean is predominantly inbred, transfer of transgenes from transgenic to nontransgenic plants occurred at a frequency of 0.52% in the first row, apparently through the activity of pollinators. Though it is not possible to guarantee absolute containment of transgenes from transgenic to nontransgenic soybean plants, based on our results we suggest that, under the current soybean production systems in Brazil, a distance greater than 10 m, separated by nontransgenic rows surrounding the transgenic area, would be sufficient to provide an acceptable level of containment. This distance is greater than the current distance of 3 m, which is recommended by the regulatory framework for field testing of transgenic soybean lines in Brazil. For more effective control of cross-pollination within the breeding programs, the isolation distance should be greater than 10 m to avoid unintentional transference of transgenes into nontransgenic lines. In addition, the induction of a different flowering season might also be a practical isolation procedure in the plant-breeding experimental fields. Additional aspects, such as long-distance pollen dispersal and the effect of insect populations should be further evaluated. Cross-pollination may also vary with the variety of the pollen donor and the recipient.

Considering a distance of 10 m and based on a constant allelic transference rate $(1 - \mu)^n$, where n is the number of generations and μ is the allelic transference rate, the average frequency of the RR allelic was 0.00508%. In addition, it can be projected that 5% of the plant population would carry the RR alleles after 10 generations. Considering selection in favor of the RR alleles, the increment in the allelic frequencies would be even more accelerated and influenced by the imposed selection rate.

Under the conditions of this study, pollen dispersion from transgenic to nontransgenic plants was very low, even at short distances. Consequently, the possibility of gene flow will be similar to that usually assumed for nontransgenic seed production, improvement, or production fields.

At a distance of 8 m, the pollen dispersal showed no significant difference when compared with the pollen dispersal at 0 m, indicating that a distance greater than 8 m is necessary and sufficient to separate different seed production fields. Moreover, at 8 m the absolute value of pollen dispersal was not zero; more adequate insect control in this field would be advisable to reduce outcrossing among soybean plants.

These results contribute to our understanding of gene flow involving transgenic plants in the Cerrado region and to our knowledge of the actual impact of transgenic soybean plants on the environment.

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REFERENCES

Ahrent DK and Caviness CE (1994). Natural cross-pollination of twelve soybean cultivars in Arkansas. *Crop Sci.* 34: 376-378.

Aragão FJL, Sarokin L, Vianna GR and Rech EL (2000). Selection of transgenic meristematic cells utilizing a herbicidal molecule results in the recovery of fertile transgenic soybean [*Glycine max* (L.) Merril] plants at a high frequency. *Theor. Appl. Genet.* 101: 1-6.

Aragão FJL, Vianna GR, Albino MMC and Rech EL (2002). Transgenic dry bean tolerant to the herbicide glufosinate ammonium. *Crop Sci.* 42: 1298-1302.

Aragão FJL, Vianna GR, Carvalheira SBR and Rech EL (2005). Germ line genetic transformation in cotton (*Gossypium hirsutum* L.) by selection of transgenic meristematic cells with an herbicide molecule. *Plant Sci.* 168: 1227-1233.

Beard BH and Knowees PF (1971). Frequency of cross-pollination of soybeans after irradiation. *Crop Sci.* 11: 489-492.

Boerma HR and Moradshahi A (1975). Pollen movement within and between rows to male-sterile soybean. *Crop Sci.* 15: 858-861.

Caviness CE (1966). Estimates of natural cross-pollination in Jackson soybeans in Arkansas. *Crop Sci.* 6: 211-212.

Caviness CE (1970). The indispensable pollinators. University of Arkansas Extension Service Publication, Fayetteville.

Conner AJ and Dale PJ (1996). Reconsideration of pollen dispersal data from field trials of transgenic potatoes. *Theor. Appl. Genet.* 92: 505-508.

Dias BBA, Cunha WG, Morais LS, Vianna GR, et al. (2006). Expression of an oxalate decarboxylase gene from *Flammulina* sp. in transgenic lettuce (*Lactuca sativa* L.) plants confers resistance to *Sclerotinia sclerotiorum*. *Plant Pathol.* 55: 187-193.

Edwards K, Johnstone C and Thompson C (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Res.* 19: 1349.

Faria JC, Albino MMC, Dias BBA, Cançado LJ, et al. (2006). Partial resistance to bean golden mosaic virus in a transgenic common bean (*Phaseolus vulgaris* L.) line expressing a mutated *rep* gene. *Plant Sci.* 171: 565-571.

James CA (2005). Global review of commercialized transgenic crops: 2005. The International Service for the Acquisition of Agri-biotech Applications (ISAAA). http://www.isaaa.org/kc/bin/briefs34/pk/index.htm. Accessed August 21, 2006.

Llewellyn D and Fitt G (1996). Pollen dispersal from two field trials of transgenic cotton in the Namoi Valley, Australia. *Mol. Breed.* 2: 157-166.

Nelson RJ and Bernard RL (1984). Production and performance of hybrid soybeans. *Crop Sci.* 24: 549-553.

Nunes AC, Vianna GR, Cuneo F, Maya-Farfan J, et al. (2006). RNAi-mediated silencing of the myo-inositol-1-phosphate synthase gene (GmMIPS1) in transgenic soybean inhibited seed development and reduced phytate content. *Planta* 224: 125-132.

Padgette SR, Kolacz KH, Delannay X, Re DB, et al. (1995). Development, identification, and characterization of a glyphosate-tolerant soybean line. *Crop Sci.* 35: 1451-1461.

Poehlman JM (1987). Breeding field crops. 3rd edn. Van Nostrand Reinhold Publ., New York.

Rogers HJ and Parkes HC (1995). Transgenic plants and the environment. *J. Exp. Bot.* 46: 467-488.

Scheffler JA, Parkinson R and Dale PJ (1993). Frequency and distance of pollen dispersal from transgenic oilseed rape (*Brassica napus*). *Transgenic Res.* 2: 356-364.

Shen FF, Yu YJ, Zhang XK, Bi JJ, et al. (2001). Bt gene flow of transgenic cotton. *Yi. Chuan Xue. Bao.* (Acta Genet. Sin.) 28: 562-567.

Tynan JL, Williams MK and Conner AJ (1990). Low frequency of pollen dispersal from a field of transgenic potatoes. *J. Genet. Breed.* 44: 303-306.

Umbeck PF, Barton KA, Nordheim EV, McCarty JC, et al. (1991). Degree of pollen dispersal by insects from a field test of genetically engineered cotton. *J. Econ. Entomol.* 84: 1943-1950.