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Gene Flow Between Conventional and Transgenic Soybean Pollinated by Honeybees

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1. Introduction

Among the main agricola commodities of Brazil, the soybean (*Glycine max* L. Merrill) keeps a highlight place. In last years, the cultivated area with this Leguminosae has been growing, as well as was testified the increase of productivity by area in Brazilian crops. The weeds are the principal problems that interferes in soybean production. After several decades of searching alternatives to control plagues of considerable periods of time, genotype of genetically modified (GM) soybean were developed to be resistant to herbicide glyphosate, an herbicide of chemical group of substituted glycine.

Wild soybean *G. max* is distributed in the Far East of Russia, eastern China including Taiwan, Korean peninsula and Japan (Lu, 2004). This annual plant grows in edges of crop fields, roadsides and riverbanks (Kuroda et al., 2010). The centre of domestication is controversial, may have occurred in China or independently in several regions of East Asia (Hymowitz, 1970, Xu et al., 2002). Only at the late l940's and early l950's, the U.S. exceeded China and eventually the entire Orient in soybean production (Hymowitz, 1970). The tolerance to herbicide was obtained by addiction in soybean genome of a gene named CP4, which came from common bacteria of soil of whole world *Agrobacterium* sp. The herbicide acts in aromatic chain amino acids synthesis inhibiting the enzyme EPSPs, in which the metabolic route synthesizing proteins, vitamins, hormones, and other essential products to growing and development of invading plants (Gazziero et al., 2009a). Therefore, the farmer can use the glyphosate to chemical weeding without risk to soybean plants (Paula-Júnior & Venzon, 2007). The herbicides are items of highest cost in the production system. The glyphosate in post emergence in soybean crops represents the possibility of using a new tool in management of plagues; easily handle, flexible and efficient. By presenting these features, the farmers of soybean were stimulated to adopt this technology (Gazziero et al., 2009b).

The cultivation of GM plants in 2010 overtaken, in whole world, the mark 148 million hectares, and the soybean plays 60% of total GM plants (James, 2010). The transgenic

soybean cultivation was regulated by the Brazilian Government – Bio security Law from March 2005, which authorizes the production and commercialization of GM products, and set security rules and mechanisms of controlling of any activities with organisms genetically modified. Its utilization by farmer is increasing year by year more than conventional varieties (Menegatti & Barros, 2007), and this Bio security Law also regulates the research about stem cells (Bruno, 2008). Besides, the government creates a commission named National Technical Commission of Bio security (CNTBio).

Brazil increased the planted area more than any other country, with an impressive rise of four million hectares in relation to 2009, and then, Brazil is now the second position of the commercial cultivation of GM plants, 25.4 million hectares, in whole word (James, 2010). The GM soybean, in 2010, occupied 50% of cultivated global area with OGMs, reaching 73.3 million ha, followed by corn – 46.8 million ha (31%), by cotton – 21 million ha (14%), and by canola – 7 million ha (5%). Since the beginning of commercialization in 1996 until 2010, the tolerance to herbicides is the dominant attribute. In USA, in 2010, the tolerance to herbicides used in soybean, corn, canola, cotton, beetroot, and alfalfa occupied 61% or 89.3 million ha of global area of 148 million ha cultivated with biotechnological planting (James, 2010).

The green revolution gets reduce the percentage of word-wide population that suffer hunger from 50% in sixties years to 20% nowadays. In plantation free of agrotoxic, the loss of production is 10% to 40%. If it not been used this technique, about two billion of six billion of inhabitants of the planet would starving. The use of transgenic crops may help to increase the productivity, avoiding, more deforesting, and more erosion of the soils (Souza, 2006).

Despite of evaluation of risk of the release of OGMs had been made cautiously by CTNBio, following procedures that avoid or minimize the adverse consequences of OGMs and their derivates to human being and environment (Mendonça-Hagler, 2001), one of the most worries of Brazilian farmers in relation to GM plants is the unexpected and cumulative effect of cross-contamination between GM plants and the conventional. The instability and risk of propagation of a gene to wild species are critical to maintenance of the environment (Chiari et al., 2008). Although the cross-pollination provides an increase in gene flow between plants there is a diversification with results remarkably favourable (McGregor, 1976; Free, 1993; Malerbo-Souza et al., 2004; Chiari et al., 2005a; Chambó et al., 2010). The genetic pollution is inevitable, whereas the transgenic pollen may contaminate conventional or biological fields located several kilometres from GM plantation (Scottish Crop Research Institute, 1999). Therefore, questions about bio security had been discussed, including gene flow via pollen (Ray et al., 2003; Schuster et al., 2007; Silva & Maciel, 2010). Besides, the impact of cross-pollination between transgenic cultivar and conventional in assays of improvement is not known.

In an insect-pollinated crop, gene flow occurs when pollinator moves between fields and cross-pollinate flowers with 'foreign' pollen (Cresswell, 2010). The gene flow via pollen by biological agents is a primordial factor in cross-pollination rates. Even though the soybean is considered auto pollinated, or not is beneficiate of insect pollination (Rubis, 1970, Ahrent & Caviness, 1994, Wolff, 2000), some authors considered the occurrence of cross-pollination in this species attributed to action of insect pollinators (Erickson, 1975; McGregor, 1976; Chiari et al., 2005a,b). However, the cv. BRS-133, not GM (Chiari et al., 2005a,b) was intensively visited by *A. mellifera* Africanized honeybees. The researchers reported an increase of 61.38% in number of pods, and 58.86% in seeds production, when it is compared with protected plants of the insect visitation.

The possibility of gene flow occurrence of the GM variety to conventional and the possibility of detect soybean seeds resistant to glyphosate mixed with conventional soybean are relevant subject with coming and spread of OGMs. In economical globalization and expansion of transgenic in agriculture, the diversity of market requires cultivar and these products must be precisely identified to commercialization. There are several commercial niches in agribusiness, and is imperative the need of obtain tools to detect the main characteristics of cultivar, as for seeds production with genetic purity guaranteed, as for the certification of the products (Pereira, 2007).

The transgenic flow becomes a problem especially because the farmer is interested in growing organic or conventional soybeans. Contamination with the transgenic will provide damage to the farmer.

Non-transgenic cultivars could be contaminated by transgene via wind-pollination. Yoshimura (2011) investigated this potential contamination by assessment of soybean pollen dispersion. The airborne soybean pollen was sampled using Durham pollen samplers located in the range of 20 m from the field edge. The dispersal distance was assessed in a wind tunnel under constant airflow and it was compared with the anticipated distances based on the pollen diameter. It was detected little airborne pollen in and around the field and the dispersal is restricted to a small area from the field edge even when soybean flowers were in full bloom. Considering soybean characteristics with a stigma invisible from the outside and a short pollen life, wind-mediated pollination in soybeans appears to be negligible.

Coexistence among genetically modified (GM) with non-GM cropping systems and identifying preservation at the field level are increasingly important issues in many countries (Beckie & Hall, 2008). In several types of cultivated plants of economical interest, the gene flow by cross-pollination is possible between separated spatially fields (Beckie & Hall, 2008). To preserve the purity of the varieties of a crop or to restrict the introduction of genes of GM soybean in conventional crops, a better understanding about gene flow is unexpected. Besides, there is a great need to generate knowledge about OGMs so that can perform an evaluation about benefits and what their implications in the ecosystem. One aspect to be studied refers to floral biology and pollination. In reproduction of several plants, the pollination represents an important mechanism to increase the gene flow in the species.

The insects play a fundamental role in transfer these genes by cross-pollination, this process that provides equilibrium in the ecosystem. Meanwhile the insects searching floral resources like pollen and nectar, they offer to plant a diversity of genetic material. Therefore, it is necessary to consider the possibility of these OGMs provoke alterations in this equilibrium (Chiari et al., 2005b).

The importance of bee pollination in particular can be understood (Pasquet et al., 2008) in Kenya using carpenter bees *Xylocopa flavorufa* main pollinator of *Vigna unguiculata* (cowpea). The experiment was carried out with the release of an insect-resistant, genetically engineered. The authors found out bees visited wild and domesticated populations, can mediate gene flow and, in some instances, allow transgenic escape over several kilometres.

In this research was evaluated the cultivar BRS-245 RR, developed by Empresa Brasileira de Pesquisa Agropecuária (Embrapa Soybean), and it has the characteristic Roundup Ready™ (Soja RR), developed by Monsanto, compared with conventional cultivar BRS 133, a isoline of BRS 245 RR, not transgenic. The choice of this OGM was made based on the importance of the soybean crop to the state of Paraná, and for Brazil, the greatest word-wide exporter, and for the availability of the cultivar by Embrapa Soja of Londrina city. The objective of this research was evaluating the gene flow provoked by cross rate between transgenic soybean and conventional and to verify the influence of Africanized honeybees *A. mellifera* in this process on cultivars BRS 245 RR and its isoline the BRS 133.

2. Material and methods

This research was carried out in experimental area of Empresa Brasileira de Pesquisa Agropecuária (Embrapa Soja), located in Londrina city (23° 08'47" S and 51º 19'11" W), which is situated in North region of state of Paraná, Brazil. The planting season, the cultivation, and management of the culture and crop occurred in appropriated time, and followed technical recommendations to soybean plant (Embrapa, 2003).

It was used the completely randomizely design with three treatments and six repetitions each. It was evaluated three treatments: covered area with cages, and inside there was a colony of honeybees during the flowering (Figure 1); a covered area without honeybee colony; and an uncovered area, free for insect visitation. In each area, of 24 m² each, the soybean planting was made in eight lines, of 6 m, interlaced two by two, with cultivars BR 245 RR and BRS 133. The stand used was 0.5 m between lines and 30 seeds by linear metre (Figure 2).

In covered areas, pollination cages were installed, made with nylon screen (two mm), supported by PVC tubes (¾ inch), and iron (3/8 inch), forming cages in a semi-arch with four metres wide, six metres length and two metres high, covering an area of 24 m² (Figure 3) and this avoids the passage of insects (Chiari et al., 2005a).

Immediately before the blooming phase, the cages were mounted and inside of each cage of covered treatment with honeybees, it was put an Africanized colony – *A. mellifera* in a Langstroth hive model with five frames, thus three frames with brood and two with pollen and honey (Figure 1). In covered treatment without honeybees, the cage avoids the insect visitation to soybean flowers, and third treatment was a delimited area with free visitation of insects and other pollinators and or visitors (Figure 3).

The soybean plantation was monitored during all the period with particular attention to the flowering phase, which started on December 31st 2003 to January 28th 2004, where the cages were dismounted. The insect visitation was evaluated in covered area with honeybee colony inside and in uncovered area, by two individuals that tracked two lines of transgenic soybean and two lines of conventional cultivar, during 10 minutes per hour, from 8:00h a.m. to 5:00h p.m., with three repetitions.

The harvesting of soybean grains was performed separately in each line, and the borders were discarded, computing 12m² of each cage. Cleaning, drying, classifying, and weighing of harvested grains obtained the production. Evaluating the gene flow, 1,000 seeds harvested of each line of conventional soybean were seeded in an experimental field. After the germination, the viable seedlings were counted, and where the fourth leaf arises from



Fig. 1. A honeybee colony inside the pollination cage.



Fig. 2. Planting of transgenic and conventional soybean in alternate lines.



Fig. 3. Pollination cage model used in the assays. Measures: 4m x 2m x 6m.

seedling, it was applied glyphosate (Figure 4). The evaluation and quantification of seedlings that survived after the pulverization with glyphosate was recorded after a week.

Data were analyzed with the software Statistical Analysis System (SAS, 2004), using the following model:

Yijklm = μ + Bi + Hj + Tk + Vl + (BH)ij + (BT)ik + (BV)jl + (HT)jk + (HV)jl + (TV)kl + e ijklm.

Wherein:

Yijklm = Observation as to variable of Block i, Herbicide j, Treatment k, Cultivar l

μ is the effect of general average;
Bi is the effect of Block (i = 1, 2 ... 6);
Hj is the effect of Herbicide (j = 1, 2);
Tk is the effect of Treatment (1, 2, 3);
Vl is the effect of cultivar;
(BH)ij is the interaction of Block i and Herbicide J;
(BT)ik is the interaction of Block i and Treatment k;
(BV)jl is the interaction of Block i and Cultivar l;
(HT)jk is the interaction of Herbicide j and Treatment k;
(HV)jl is the interaction of Herbicide i and Cultivar l and;
eijklm the error associated to observation ijklm.

Data were analyzed by variance analysis (Anova), and means of treatments compared using Tukey's test, at level of 5% of probability.



Fig. 4. The application of glyphosate in transgenic and conventional soybean plants.

3. Results and discussion

The percentage of germination of 1,000 seeds planted in experimental field did not differ ($p>0.05$) between covered area with pollination cages within Africanized honeybee colony, pollination cage without honeybees inside and uncovered area for free insect visitation. In Table 1 are shown data referred to summary of analysis of variance and means of percentage of seeds germination of conventional soybean resistant to herbicide glyphosate to all treatments.

The germination rate of seeds from conventional plants, in different treatments, was 79.43% ± 18.42. This value is considered satisfactory to cultivar BRS 133 (EMBRAPA, 2003). However, the average percentage of germination obtained in this research was lower than 91.02% reported by Chiari et al. (2005a) working with the same cultivar. A possible explanation for that difference in germination rate is, while in this research this rate was measured in a field, Chiari et al. (2005b) evaluating this rate in laboratorial conditions.

The similarity in germination rate between covered area with honeybee colony, covered area without honeybee colony, and uncovered area for free insect visitation and other visitors suggests that the presence of Africanized honeybees and other insects did not interfere in germination rate of the seeds. In other species such as canola (cv. OAC Triton) was found out the presence of pollinators, e.g. honeybees, increasing the germinability of resulting seeds from 83% to 96% (Kevan & Eisikovitch, 1990).

Variation source	Germination rate (%)
Treatment	0.51 P=0.6372
CV %	0.51
Means of treatments	79.43 ± 18.42
Covered area with honeybee colony	86.30 a (± 11.97)
Covered area without honeybee colony	72.83 a (± 26.15)
Uncovered area for free insect visitation	79.15 a (± 14.75)

Means followed by same small letters did not differ between them by Tukey's test (P>0.05)

Table 1. F values with their probability (P), coefficient of variation (CV%), means and their standard deviation of percentage of germination of 1,000 seeds obtained by resistant plants to glyphosate from seeds of soybean *Glycine max* L. Merrill, variety BRS 133 (conventional soybean) to treatments: covered area with honeybee colony, covered area without honeybee colony, and uncovered area for free insect visitation in experimental field of EMBRAPA Soja, in Londrina-PR city.

The environmental and physiological factors of the plant are considered the responsible directly of the better physiological quality of the seeds, including the vigour. Nevertheless, some researchers think about the insects can in several situations influence indirectly by better quality of the seeds, and then, provide seeds of the most high quality, for example, with more vigour and oil (Singh et al., 2001; Paiva et al., 2003; Camacho & Franke, 2008; Toledo et al., 2011).

The percentage of cross-pollination of plants from conventional seeds that show resistance to glyphosate in covered area with honeybee colony, covered area without honeybee colony, and uncovered area for free insect visitation are in Table 2. The percentage of transfer via pollen flow from transgenic soybean to conventional did not differ ($p \geq 0.05$) between pollination cages with honeybee colony and uncovered area for free insect visitation (1.57%). However, it was higher ($p=0.0224$) than percentage of pollen transfer of the covered area without honeybee colony, and the average was 0.20%. Therefore, the Africanized honeybees and other insects were responsible by cross-pollination, increasing the gene flow from transgenic to conventional soybean. Afterward, the gene flow from conventional to transgenic soybean may also have occurred, but was not evaluated in this assay.

It was possible to estimate an increase of 685% by cross-pollination in pollen transfer from transgenic to conventional soybean plants when Africanized honeybees visited the soybean flowers in relation to these areas in which the honeybees were avoided to visit. Therefore, the Africanized honeybees are able and efficient in transferring pollen from a plant to another.

Honeybees collecting mainly nectar, while collecting nectar and pollen was observed less frequently and foraging behavior of pollen was found out only rarely (observed data, not shown). These findings show that although literature data indicate that soybean flowers are not attractive to honeybees because there is not a large quantity of nectar (Erickson, 1975; Alves et al., 2010) they visit flowers and collect nectar and/or pollen, and an increase of yield like 37.84% when honeybee visits were allowed (Chiari et al., 2008).

Variation source	Cross-pollination (%)
Treatment	2.67 P=0.0224
CV %	77.73
Means of treatments	1.12 (± 0.20)
Covered area with honeybee colony	1.5726 a* (± 0.35)
Covered area without honeybee colony	0.2016 b (± 0.05)
Uncovered area for free insect visitation	1.5712 a (± 0.22)

Means followed by different small letters differ between them by Tukey's test (P<0.05)

Table 2. F values with their respective probability (P), coefficient of variation (CV%) of percentage of resistant plants to glyphosate from seeds of soybean *Glycine max* L. Merrill, variety BRS 133 (conventional soybean) to treatments covered area with honeybee colony, covered area without honeybee colony, and uncovered area for free insect visitation in an experimental field of EMBRAPA Soja, in Londrina-PR city.

Figures 5 and 6 are the flowers of conventional (BRS 133) and transgenic (BRS 245 RR) soybean plants.



Fig. 5. Flowers of conventional soybean plants – var. BRS 133.



Fig. 6. Flower of transgenic soyben plants var. BRS 245 RR.

The cross-pollination rate was 0.04% in Wisconsin with different varieties of soybean in adjacent rows, in different places (Woodworth, 1922), 0.70% and 0.18% in Virginia, in successive years (Garber & Odland, 1926), and less than 1% in Iowa and Maryland (Weber & Hanson, 1961). In honeybee colony near transgenic canola crops, it was detected that transgene was incorporated to intestinal bacteria chromosomes of the honeybee (Lean et al., 2000).

Furthermore, Ahrent & Caviness (1994) measured the cross-pollination rate in soybean when the plants grown in adjacent lines. The researchers used the colour of flowers, pubescence colour, and leaves fifth foliate as genetic markers. These authors worked with 12 cultivars, in two periods of planting in subsequent years. The cross-pollination rates in this research, probably, were near of maximum because honeybees and other pollinator insects were presented in experimental area during all flowering period. The means in this research varied from 0.09% to 1.63%, these values are averages from two years in different cultivar that differed significantly in relation to cross-pollination. Thus, there is some cultivars have a potential increase to cross-pollination towards to relate earlier.

The dispersion of pollen in soybean was reported by Abud et al. (2003), and they observed more dissemination of trangenic pollen in neighbour lines (0.44% to 0.45%), far way 0.5 m of central parcel, the frequency was reduced drastically in line two (0.04 to 0.14%) and reached zero in line 13, far way 6.5 m of central parcel. Africanized honeybees and other pollinator, not only disseminate the transgenic pollen, but pollinating and insects can carry some plant diseases and can themselves be infected while foraging at flowers (Kevan et al., 2007).

The observation of harvesting behaviour shows that Africanized honeybee has a tendency to collect pollen and nectar in flowers of neighbour plants, in the same line, suggesting that this behaviour can minimize the cross-pollination performed by them, once that cultivar were planted in intercalated lines. Increasing the pollination rate obtained can be explained by intense visitation of *A. mellifera,* means 97.25% of insect visitor in uncovered area. Other bees and Lepidoptera represented, only 1.65% and 1.33%, respectively of visitor to this area.

Evaluations of percentage of GM plants from seeds harvested of conventional plants, in eight directions, far way 1 to 5 m beginnings from pollen source make clear that cross-fecundation average rate observed until 5 m from pollen source was 0.29%, and that was calculated based on number of plants that survived to glyphosate. The cross-fecundation rate reduced from 0.61% far way 1 m of distance to 0.29% far way 2 m, and to 0.23% far way 3 to 5m. The cross-fecundation means, from two to 5 m distance from pollen source did not differ, but when the distance passed from 1 to 2 m, the reduction of cross-fecundation rate was significant. This rate diminished by exponential form, from pollen source, that indicates an allogamy as zero far way 7.76 m.

One of the concerning with advancement of transgenic soybean crops in several regions of Brazil, including the North region of state of Paraná is the occurrence of gene flow. However, Schuster et al. (2007) reported that for a soybean plantation free of grain GM, the isolation recommended is 8 m. Silva & Maciel (2010) reported 0.25% of gene flow between transgenic and conventional soybean plants in Minas Gerais, Brazil. Therefore, the authors reported a potential of contamination, in non-transgenic material, in planting close to RR transgenic soybean or other types of soybean GM that would be released in Brazil.

A mathematic model developed by Cresswell (2010) was used to evaluate the gene flow in experimental fields of safflower pollinated by honeybee *A. mellifera* and *Bombus* spp. This model estimated that the maximum feasible level of bee-mediated, field-to-field gene flow ranged between 0.05% and 0.005% of seed set (95% upper confidence intervals of 0.23% and 0.023%), depending on the composition of the bee fauna. The recommended strategy for minimizing GM gene flow is the utilization of a conventional safflower variety possessing a high capacity for automatic self-fertilization, allowing the conventional plants to grow in large stands to encourage long foraging bouts by bees.

Evaluations of methods for detection of soybean seeds resistant to glyphosate, and gene flow from transgenic to conventional soybean cultivar, performed in Florestal and Viçosa counties, Minas Gerais, Brazil evidenced that seedlings resistant to glyphosate presented cross-fecundation. The highest percentage of hybridization, 1.27% in Florestal and 0.25% in Viçosa counties occurred far way 0.5 m, between pollen source and pollen receptor (Pereira et al., 2007). These rates were about zero far way 2.26 and 1.16 m, to Florestal and Viçosa, respectively. However, the distance is an important factor and able to isolate transgenic soybean fields from conventional fields, preventing the gene escape.

Mathematical models (Baker & Preston, 2003; Cresswell, 2010), empirics (Weekes et al., 2005; Damgaard & Kjellsson, 2005; Gustafson et al., 2006) and deterministic (Colbach et al., 2001; 2004; 2005; Walklate et al., 2004) that simulate the gene flow in fields of trangenic and conventional plants are found in literature. However, each model has its advantages and disadvantages. Some are limited by scarcity of data of environmental variable or spatial scale, others, like empirics are attractive due to relative simplicity and easy utilization,

although the deterministic model offers potentially greater capacity predictive and inference. Recent advances in modelling pollen-mediated gene flow in commercial fields are encouraging, but simulating gene flow in heterogeneous landscapes remains an elusive goal. Moreover, practical, user-friendly decision-support tools are needed to inform and guide farmers in implementing coexistence measures (Beckie & Hall, 2008).

4. Conclusion

The Africanized honeybees provided a considerable increase of gene flow from transgenic to conventional cultivar (1.57%), besides these cultivar of soybean were attractive to the honeybees, and that they may perform the cross-pollination in these tested varieties of soybean.

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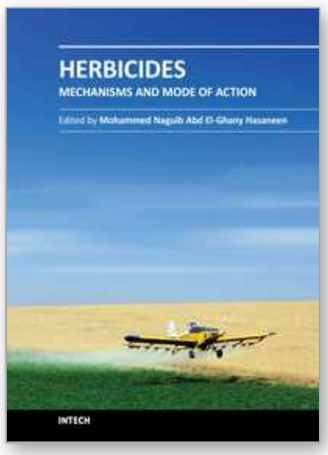
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Herbicides - Mechanisms and Mode of Action

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This volume contains two sections: Mechanisms of herbicidal action (chapters 1-4) and Mode of action of selected herbicides on controlling diseased, weed growth and productivity and/or growth and development of field crops (chapters 5-10). Topics by chapters are: molecular mechanism of action, immunosensors , laboratory studies, molecular modeling, weed resistance, community response, use of herbicides in biotech culture, gene flow, herbicides and risk, herbicides persistence. These recurring themes reinforce my view, held over a very long time, that experience with one crop or problem can sometimes be relevant, often to an unexpected extent, to an apparently dissimilar situation in a different crop. I hope that readers interested in herbicides and pesticides will be satisfied with all the chapters in the book as its content might be of interest and value to them in the future.

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