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# Herbicide application on Genetically Modified Maize influence bee visitation

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## Abstract

Brazil is one of the world's largest producers of maize (*Zea mays* L.). Cry proteins derived from the bacterium *Bacillus thuringiensis* (Bt) have been widely used in transgenic maize due to their toxicity and specificity against insects that damage crops. In addition, these plants have been stacked with different herbicide tolerance genes. Non-target insects end up being exposed to Bt proteins and herbicide applications. There is little information on the effects of Bt transgenics and their cultural practices on the behavior of pollinators in genetically modified crops. The aim of this research was to verify the impact of genotypes of genetically modified maize, Herculex®, PowerCore®, and the conventional isohybrid, pulverized or not with herbicides (atrazine, glufosinate-ammonium and nicosulfuron) in bee populations. In order to evaluate the presence of insects, a zig-zag tour was carried out throughout the experimental field, ascertained from visual analysis and direct counting of six plants per plot (the dimensions of the plots were 2.5 x 10 m with five maize lines spaced 0.50 m between rows and 0.36 m between plants) randomly, 18 days after spraying herbicides in the area. *Apis mellifera* (L.) (Hymenoptera: Apidae), *Tetragonisca angustula* (L.) (Hymenoptera: Apidae) and *Trigona spinipes* (F.) (Hymenoptera: Apidae) were the pollinator species identified in the crop. It was observed that the incidence of pollinator insects varied according to cultivars and herbicides tested; however, the PowerCore® genotype experienced more visitation of pollinating bees independently of the herbicide treatments.

## Introduction

Brazil is one of the world's largest producers of maize (*Zea mays* L.) with a total production of 60 million tons and average yield in the first harvest of 5.4 t $ha^{-1}$ (Conab, 2016). However, the average national productivity is considered low, since the industries that operate on a high technological level obtained values three times higher than the average productivity presented for the same harvest. Several factors are responsible for the low productivity of the crops, though interference imposed by weeds and pests is highlighted (Constantin et al., 2007). Several technologies, including the use of herbicides and toxic proteins, help increase agricultural productivity. However, there are consequences linked to technologies use, such as the different effects on non-target organisms (Rosa et al., 2010).

Bees play an important role in plant production, which are secondary targets of the effects of chemicals. Thus, several researchers in the laboratory and in the field have attempted to evaluate and determine the effect of herbicides on bees (Chambó et al., 2010). Reductions and delays of plant flowering is caused by herbicide lesions that can disrupt pollinator communities (Bohnenblust et al., 2016). Herbicide lesions can also create a direct negative impact on pollinators. Bees exploit floral sources of pollen and nectar influenced by the selection pressure of the environment, noting that environmental contaminants and the time that food sources remain available for the visitor are fundamental variables (Brizola-Bonacina et al., 2012).

Crystal proteins derived from the bacterium *Bacillus thuringiensis* (Bt) have been widely used in transgenic crops due to their toxicity against insects that damage crops.

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However, the distribution and metabolism of these toxins in the tissues and organs of non-target insect has remained obscure (Zhao et al., 2016). The purpose of this innovative technology is to reduce insects that are considered crop pests. Thus, the advancement of biotechnology in the field of transgenic development is very critical. In addition to the positive impact to principal crops of the global economic market, the technology can also give rise to biological, environmental and social problems.

The effect of Bt maize cultivation on non-target insects seems to depend on the geographic location and crop management of the agricultural ecosystem. Other factors such as spraying chemicals in the maize field may thus exert a stronger influence in this process (Resende et al., 2016). The impact of Bt proteins on non-target arthropods is less understood than their effects on target organisms, where the mechanism of toxic action is known (Yuan et al., 2014). Some studies have been carried out investigating possible effects of transgenic plants on non-target organisms, but there is little information on the effects on the behavior of pollinator populations when visiting transgenic cultivars.

The aim of this research was to verify the impact of genotypes of genetically modified maize, Herculex®, PowerCore®, and the conventional isohybrid, pulverized or not with herbicides (atrazine, glufosinate-ammonium and nicosulfuron) in bee populations.

## Material and Methods

The experiment was set up at the Rio Manso experimental farm of The Federal University of the Jequitinhonha and Mucuri Valleys (18° 4' 25''S; 43° 28' 16''O), located in the municipality of Couto de Magalhães de Minas, Minas Gerais State, Brazil.

The experiment was performed in a field in DBC (randomized block design) in a 3 × 4 factorial scheme with 3 blocks where parcels were drawn within each block. As the A factor represented by the genotypes Herculex® - transgenic maize [Bt Cry1F protein (resistant to *Spodoptera frugiperda*, JE Smith, Lepidoptera: Noctuidae)], PowerCore® - stacked transgenic maize [Bt Cry1F, Cry1A.105, Cry2Ab2 proteins (resistant to *S. frugiperda*) and CP4 EPSPS protein (glyphosate tolerance) and the conventional isohybrid - maize sensitive to the *S. frugiperda* and herbicide. Factor B was represented by the herbicides: atrazine, glufosinate-ammonium and nicosulfuron applications, plus a control without application. Each plot was assembled in the dimensions 2.5 × 10 m, with five maize lines spaced 0.50 m between rows and 0.36 between plants, containing 3 blocks. The experiment was composed of a total of 36 plots with a total experimental area of 10 × 90 m.

The soil was fertilized and corrected according to a soil analysis conducted one month prior to planting, which also indicated a medium texture. With the chemical analysis, the following results were obtained: pH (water) of 5.2; organic matter content of 5.1 daq kg$^{-1}$; P and K of 3.3 and 65 mg dm$^{-3}$, respectively; Ca, Mg, Al, H + Al and effective CTC of 1.9; 0.8; 0.1; 7.1 and 2.9 cmolc dm$^{-3}$, respectively. 0.90 tonnes ha$^{-1}$ of dolomitic limestone was applied. Fertilizer with 500 Kg ha$^{-1}$ of the formulation 04:14:08 of N:P:K was applied and irrigation was monitored throughout the experiment.

Planting occurred on March 9, 2016. Three seeds per linear meter were sowed. The plants emerged on March 13, 2016 and the herbicides (atrazine 6.0 l/ha$^{-1}$, glufosinate-ammonium 2.0 l/ha$^{-1}$ and nicosulfuron 1.5 l/ha$^{-1}$) were applied on April 8, 2016; 26 days after the emergence of maize plants. Maize is tolerant to the atrazine herbicide, which is recommended for the control of dicot plants in grass crops and its site of action is the protein D1 in photosystem II. Nicosulfuron is recommended for the control of monocot and dicot plants in maize crops whose mechanism of action is the inhibition of the acetolactate synthase (ALS) enzyme. Glufosinate-ammonium is a herbicide that inhibits the glutamine synthetase (GS) enzyme in the nitrogen assimilation log and is classified as a non-selective herbicide (Silva & Silva, 2007). These herbicides are alternatives to glyphosate in Brazil, where some weeds have resistance.

To identify the natural presence of bees, six plants per plot were analyzed during the flowering period (18 days after spraying herbicides in the area) by zig-zag walking in the useful area of each plot, with direct counting of individuals in each plant. Bee visitation was observed on the maize plants tassel (the tops of maize plants) and male part of the maize plants, where the pollen grains are produced. The observations at the different treatments were performed at the same time for each herbicide/transgenic factor.

The data were submitted to analysis of variance and when significant, to the Scott-Knott grouping criterion at 5% probability of error. The analyses were developed using SISVAR computer program (Ferreira, 2014).

## Results

*Apis mellifera* (L.), *Tetragonisca angustula* (L.) and *Trigona spinipes* (F.) (Hymenoptera: Apidae) were the pollinator species identified in the crop field.

There was interaction among all the genotypes and herbicides in this research. *Apis mellifera* was the most frequent visitor in maize plants in relation to the other species, *T. angustula* and *T. spinipes* (Fig 1), in genetically modified and in the isohybrid genotypes, independent of herbicide treatments (Table 1).

The incidence of *A. mellifera* bees in the Herculex® genotype was lower in the plots treated with atrazine and nicosulfuron (Table 1). The plots treated with glufosinate-ammonium and the control were more visited by this species, showing difference in comparison to the other treatments. When evaluating the PowerCore® genotype, it was observed that the incidence of bees showed no difference for the herbicide and control treatments (Table 1). On the other hand, the isohybrid with glufosinate-ammonium showed less bee visitation than all the other genotypes, demonstrating that the isohybrid was severely affected by the application of this herbicide.

**Table 1**. Presence of *A. mellifera* on the genetically modified maize submitted to herbicide application.

| | Herculex® | PowerCore® | Isohybrid |
|---|---|---|---|
| Atrazine | 7.00 bB | 23.33 aA | 22.00 aA |
| Gluphosinato | 13.33 aB | 23.33 aA | 6.67 bC |
| Nicosulfuron | 8.00 bB | 22.00 aA | 20.00 aA |
| Control | 14.33 aB | 24.33 aA | 22.33 aA |
| Coefficient of Variation CV (%) | | 23.04 | |

*Average number of bees observed followed by the same lowercase letter in the column and the same uppercase letter in the row do not differ by Scott-Knott's Grouping Criterion.

When evaluating the herbicides within each genotype, the Herculex® genotype plots treated with atrazine, nicosulfuron and control had a lower incidence of *A. mellifera* bees. In the plots cultivated with the PowerCore® genotype, no difference was observed between the herbicide treatments and the control. In the isohybrid, a lower occurrence of bees was observed in plants treated with glufosinate-ammonium compared to the other treatments. The isohybrid was highly sensitive to the glufosinate-ammonium, with severe developmental delays, which may have reduced the demand for this cultivar by *A. mellifera* (Table 1).

The incidence of *T. angustula* in the Herculex® and PowerCore® genotypes showed no difference between the herbicide treatments and the control (Table 2). The isohybrid demonstrated higher visitation of *T. angustula* in the cultivated plants without the application of herbicides (control).

In the plots treated with atrazine, glufosinate-ammonium and nicosulfuron, no difference was observed in the visitation of bees among the maize genotypes evaluated (Table 2). Regarding the control, there was a lower incidence of *T. angustula* on the Herculex® and PowerCore® genotypes when compared to the isohybrid (Table 2).

**Table 2**. Presence of *T. angustula* on the genetically modified maize submitted to herbicide application.

| | Herculex® | PowerCore® | Isohybrid |
|---|---|---|---|
| Atrazine | 1.67 aA | 4.33 aA | 2.67 bA |
| Gluphosinato | 1.00 aA | 1.67 aA | 1.33 bA |
| Nicosulfuron | 3.00 aA | 4.00 aA | 2.33 bA |
| Control | 3.67 aB | 3.00 aB | 6.33 aA |
| Coefficient of Variation CV (%) | | 63.79 | |

*Average number of bees observed followed by the same lowercase letter in the column and the same uppercase letter in the row do not differ by Scott-Knott's Grouping Criterion.

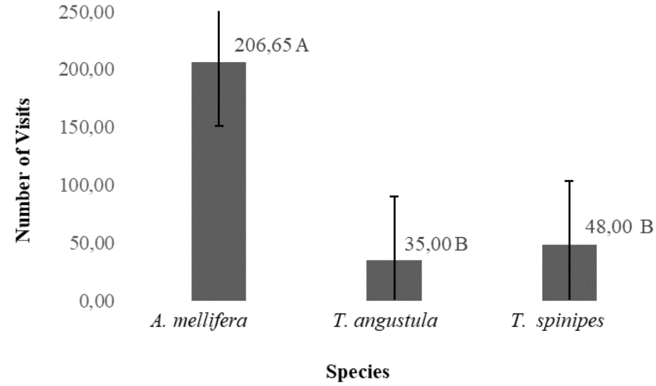


**Fig 1**. Verification of bees on the genetically modified maize submitted to herbicide application.

*Averages followed by the same letter do not differ by Scott-Knott's Grouping Criteria at 5% of probability.

The incidence of *T. spinipes* in the Herculex® genotype was lower when these plants were treated with the herbicides. With the PowerCore® and isohybrid genotypes, no difference of visitation between herbicide and control treatments was observed (Table 3). The Herculex® genotype submitted to nicosulfuron application showed a lower incidence of this species when compared to the other genotypes. In the control, the lowest visitation of *T. spinipes* was observed on the isohybrid (Table 3).

The PowerCore® genotype was the most visited by all species of bees regardless of herbicide application (Table 1, 2 and 3).

**Table 3**. Presence of *T. spinipes* on the genetically modified maize submitted to herbicide application.

| | Herculex® | PowerCore® | Isohybrid |
|---|---|---|---|
| Atrazine | 4.00 bA | 5.00 aA | 2.67 aA |
| Gluphosinato | 3.33 bA | 4.33 aA | 2.67 aA |
| Nicosulfuron | 1.33 bB | 4.00 aA | 3.67 aA |
| Control | 7.00 aA | 6.33 aA | 3.67 aB |
| Coefficient of Variation CV (%) | | 41.51 | |

*Average number of bees observed followed by the same lowercase letter in the column and the same uppercase letter in the row do not differ by Scott-Knott's Grouping Criterion.

## Discussion

The side effects of herbicides and toxic proteins on non-target insects such as pollinators need to be studied. Indirectly, this class of insects ends up being exposed to genetically modified organisms and their cultural tracts.

*Apis mellifera* being the most frequent visitor in maize plants can be explained by Pires et al. (2014) who studied genetically modified cotton and observed that the visitation of the species *A. mellifera* dominated in relation to other species of bees on the crop. This can be explained by the greater natural occurrence of this species. It is also a species of economic interest and can thus be multiplied and commercialized in large scale. Along with having an abundant presence in several environments *A. mellifera* is considered a dominant species.

Naturally, *T. spinipes* demonstrates highly aggressive behavior to other bee species (Brizola-Bonacina et al., 2012). Considering this aggressive behavior, *T. angustula* avoids areas where *T. spinipes* are present (Damascena et al., 2017). Therefore, *T. spinipes* can negatively influence pollination, inhibiting grain production and reducing the attractiveness of these plants for other pollinators. Thus, the species *T. spinipes* and *T. angustula* could be avoided themselves in the field and, consequently, controlling the population level of each other. Considering that *T. angustula* is small in size and less aggressive than the others studied, it could be occupying a habitat of lesser interest to the others.

In addition to the verification of more occurrence of *A. mellifera* in this experiment, it is also possible to conclude that the visitation of this species on the maize cultivars varied according to each genotype and the herbicide treatments. Thus, herbicides may have had a strong influence on the visitation of pollinating species on maize genotypes. The isohybrid was highly sensitive to herbicides tested and the Herculex® and PowerCore® genotypes demonstrated some resistance. Physiological and anatomical variations may exist due to this sensitivity and resistance. The toxic proteins seem not to have influenced the visitation of bees at this experiment, and this is corroborated by other authors. Grabowski and Dabrowski (2012) tested the effect of genetically modified maize on the behavior of *A. mellifera* and found that there was no impact on the choice of bees between the flowers of the genetically modified cultivars and the isogenic line (without the Cry1Ab protein).

It is possible that the exogenous protein made the transgenic cultivars less sensitive to the physiological and anatomical changes caused by herbicides application. The changes occurring in the isohybrid genotypes caused by the herbicides may have reduced bee visitation. Such changes are confirmed by field observations, with the genotype PowerCore® was more visually attractive and with outstanding anatomical conditions.

## Conclusions

Visitation of bees on the maize is affected by genotypes and herbicide application. The stacked PowerCore® was the only genotype that did not show alteration in bee visitations, regardless of the applied herbicide, with dominance of the species *A. mellifera*. This work constitutes an approach for evaluating the impact of genetically modified maize on pollinating insects. The method applied is sufficiently sensitive to detect differences between plants and herbicides applications; however, further work on the methodology, as well as the study of plants expressing other gene products and herbicides would be necessary before proposing a reliable test for verifying the safety of such plants.

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