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Acute contact toxicity test of insecticides (Cipermetrina 25, Lorsban 48E, Thionex 35) on honeybees in the southwestern zone of Uruguay

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ABSTRACT

Glyphosate-resistant soybean cultivation is expanding rapidly in Uruguay, with its land area having increased by 95 times during the past 10 years. Because of the region's Neotropical conditions, insecticide use is required to ensure adequate soybean productivity. However, in areas shared by soybean crops and beekeepers – such as the southwestern zone of Uruguay (SWZU) – the use of insecticides can increase the risks of honeybee death and honey contamination. Uruguayan commercial and legal guidelines set out practices and field doses designed to prevent acute intoxication with insecticides. However, honeybees in the SWZU are predominantly a polyhybrid subspecies different from that used to set international reference values, and hence they may have a different acute toxicity response, thus rendering such precautions ineffective. The aim of this work was to assess the acute toxicity response of polyhybrid honeybees in the SWZU to cypermethrin (commercial formulation: Cipermetrina 25 Agrin®), chlorpyrifos (commercial formulation: Lorsban 48E®), and endosulfan (commercial formulation: Thionex 35®). Acute toxicity bioassays were conducted to determine the median lethal dose (LD_{50}) of each insecticide for the honeybees. The results indicate that, compared with EU reference values, SWZU honeybees have a higher toxicological sensitivity to chlorpyrifos and endosulfan, and a lower toxicological sensitivity to cypermethrin, based on the commercial formulations tested. However, when these results were adjusted according to their field dose equivalents, only chlorpyrifos emerged as a potential problem for beekeeping, as the maximum recommended field dose of Lorsban 48E® for soybean crops in Uruguay is 23 times the corresponding LD_{50} for honeybees in the SWZU.

1. Introduction

Uruguay has a total agricultural land area of 16.4 million hectares, of which 13.2 million hectares is used for cattle production and 1.16 million hectares for crops. Currently, soybean is cultivated across 849,000 hectares – an area that has increased by 95 times during the past 10 years – with soybean production reaching 1793,000 tons in 2008/9 (DIEA, 2010). This agricultural intensification is raising many questions about the potential environmental impacts (Céspedes-Payret et al., 2009), and trends in Europe indicate that such intensification may have negative collateral effects for beekeeping (Le Féon et al., 2010).

Beekeeping is an economically important agricultural activity for Uruguay, as indicated by the value of annual exports of honey, which account for at least 0.5% of gross domestic product, according to the Uruguay's Department of Agricultural Statistics (DIEA, 2005, 2006, 2007, 2008, 2009). In 2009, Uruguay exported 6484 tons of honey, produced by a total of 504,514 hives and 3180 hive owners, to the total value of US$17.6 million (DIEA, 2010; DIGEGRA, 2010). The southwestern zone of Uruguay (SWZU; Fig. 1) is historically one of the country's most important areas for honey production, hosting 38% of its hives and 29% of the hive owners (DIGEGRA, 2010). However, the recent expansion of soybean cultivation has occurred in this same zone, increasing the risk of honeybee exposure to insecticides. The expansion of soybean cultivation also explains the increase in the volume of insecticide imports, which grew from 895 tons in 1999 to 2000 tons in 2009 (DGSSAA, 2011). This situation has raised concerns among honey producers in the SWZU concerns supported by the recent detection of honey contaminated with insecticides used in soybean cultivation (Rios et al., 2010). Of additional concern are the findings of a study by Suchail et al. (2000) on the potential toxicity of insecticides to honeybees. Their test results indicate that the same chemical compounds can have

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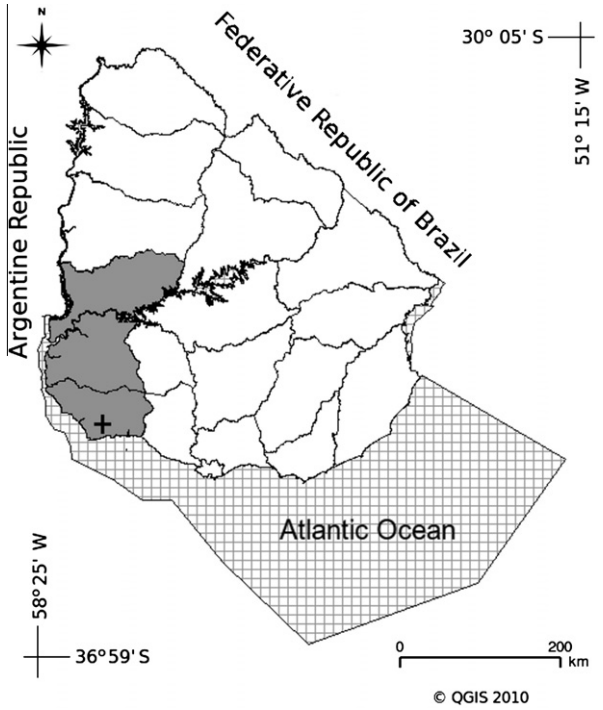

Fig. 1. Geographic position of the southwestern zone of Uruguay (dark gray), within the Oriental Republic of Uruguay. The black cross indicates Experimental Station Alberto Boerger INIA La Estanzuela.

different median lethal doses (LDs_{50}) for different subspecies of honeybee. These findings are particularly relevant for the SWZU, where a polyhybrid subspecies of honeybee is predominant (Diniz et al., 2003). This suggests that the acute LD_{50} values that apply in this region may differ from the reference values used in international guidelines, which are based on *Apis mellifera mellifera* (PPDB, 2010), and which constitute the toxicity reference values normally considered in defining insecticide field doses (Atkins and Kellum, 1981; Mayer et al., 1999; Sanford, 2003). Moreover, commercial formulations of insecticides include excipients, which are an unknown group of chemical compounds with the capacity to modify the ultimate toxic effect of active compounds, through undefined antagonistic and synergistic effects (Pilling et al., 1995; Rozman et al., 2010).

Several genetic approaches had been employed for the characterization of the honey bee colonies (Daly et al., 1982; Rinderer et al., 1987; Del Lama et al., 1988; Estoup et al., 1995; Franck et al., 2001). Different authors had employed the analysis of the mitochondrial DNA to define the haplotype of the honeybee (Hall and Smith, 1991; Garnery et al., 1998; Franck et al., 2001). Since it is inherit by maternal via it represent half of the story of the bee, so complementary analysis should be included in order to study the drone contribution. The morphometric approach is an alternative (Daly et al., 1982; Rinderer et al., 1987). There are different methodologies in order to characterize the honey bees, but most of them are time consuming, and needs expensive equipment and qualified personal. Rinderer et al. (1987) proposed a morphometric simple methodology which allows the statistical discrimination between Africanized and European honey bees.

The aim of this study was to assess the acute toxicity response of SWZU honeybees to some of the commercial formulations of insecticides normally used on soybean crops in the SWZU. To achieve this, the LD_{50} of the following insecticides were determined: Cipermetrina 25 Agrin®, the commercial formulation of cypermethrin (a synthetic pyrethroid insecticide); Lorsban 48E®, the commercial formulation of chlorpyrifos (an organophosphate insecticide); and Thionex 35®, the commercial formulation of endosulfan (an organochlorine insecticide).

2. Materials and methods

Honeybees used in this study were a polyhybrid subspecies of *A. mellifera* from SWZU. Bees were obtained from experimental apiaries kept by the Beekeeping Unit of Experimental Station Alberto Boerger INIA La Estanzuela (34° 20′ 22.20″ S, 57° 41′ 14.93″ W, Colonia, Uruguay). Colonies denominates as INIA-LE's colonies for this study.

In order to demonstrate the polyhybrid origin of the colony a genetic and morphometric analysis was employed. The mitochondrial DNA was assessed, which allow the differentiation of haplotypes A (African origin), M (West European origin), C (North Mediterranean origin), and O (Near and middle eastern) (Franck et al., 2001). The DNA extraction was carried out as described by Aguirre C., INIA La Platina, Chile (personal communication) using a modification of the protocol described by Walsh et al. (1991). The posterior leg was incubated at 37 °C for 30 min. After that Chelex 5% (Sigma) and proteinase K (Promega, 5 mg ml^{-1}) were added and incubated during 1 h at 55 °C, 15 min at 99 °C, 1 min at 37 °C and 15 min at 99 °C (T1 Biometra Thermocycler). A 2 μl of DNA were used for the amplification of the intergenic region COI–COII using the primers E2: 5'GGCAGAATAAGTGCATTG3' and H2: 5'CAATATCATTGATGACC3' (Garnery et al., 1998). The 20 μl reaction mixture contained 1 X buffer, 1 mM $MgCl_2$, 0.15 μM of each primer, 0.5 mM of each dNTP and 5 U/μl of Taq polymerase (Invitrogen). The PCR cycling program consist on 5 min at 94 °C, 35 cycles of 45 s at 92 °C, 45 s at 48 °C, 2 min at 62 °C, and a final extension of 20 min at 65 °C (T1 Biometra Thermocycler) (Aguirre C., INIA La Platina, Santiago de Chile, personal communication). The size of the amplified product was determined by electrophoresis on a 1% w/v agarose gel, stained with GelRed (Biotum, USA) and visualized by UV (Biometra T13). The amplified product was digested with FastDigest DraI (Fermentas), according to manufacturer's recommendations, and analyzed under electrophoresis on polyacrilamide gel under native conditions during 15–16 h at 80 V, and staining with GelRed (Biotium, USA).

The African origin probability was calculated by a morphometric approach as described by Rinderer et al. (1987).

The honeybees used in the bioassays were newborn bees (age 1–7 d), obtained from hive frames isolated with bags of plastic mesh (square cells, 1 × 1 mm) in hives without any treatment against varroosis. The honeybees used were closely monitored after treatment, and then observed for mortality and signs of intoxication at 48 h.

The acute toxicity bioassay used in this study was developed according to the European and Mediterranean Plant Protection Organization (EPPO, 1992) and the United States Environmental Protection Agency (US-EPA, 1996) with the following criteria: 48 h in the dark, with humidity (60%) and temperature-controlled (25 °C) conditions. Five doses of each insecticide were tested, each one in triplicate. Each replicate was conducted on a group of 10 honeybees; to make each group, two honeybees were taken from each of five hives, randomly selected from an apiary of 50 hives. All insecticide's dilutions was done in acetone. The dose of insecticide was applied to the thorax of the honeybees using a micropipette, and all the dilutions was prepared to avoid the use of volumes higher than 5 μl per bee. The honeybees were anesthetized with CO_2 (g) (US-EPA, 1996) for the grouping and dose administration. Each group of 10 honeybees was kept in a glass Petri dish (i.d. 10 cm), the bottom of which was lined with clean filter paper, containing a feeder with 1 ml of sucrose 50% w/v for *ad libitum* consumption. For the control treatment, a similar