UNIVERSIDADE DE SÃO PAULO
FFCLRP - DEPARTAMENTO DE BIOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ENTOMOLOGIA

Efeito da soja geneticamente modificada DAS-81419-2, que expressa as proteínas Cry1Ac e Cry1F, sobre os organismos-alvo e na comunidade de artrópodes não-alvo

Luiz Henrique da Silva Fagundes Marques

Tese apresentada à Faculdade de Filosofia, Ciências e
Letras de Ribeirão Preto da USP, como parte das
exigências para a obtenção do título de Doutor em
Ciências, Área: ENTOMOLOGIA

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RESUMO

Efeito da soja geneticamente modificada DAS-81419-2, que expressa as proteínas Cry1Ac e Cry1F, sobre os organismos-alvo e na comunidade de artrópodes não-alvo

A soja (*Glycine max* (L.) Merrill) com o evento DAS-81419-2 (Conkesta™ technology) que expressa as proteínas de *Bacillus thuringiensis* (Bt) Cry1F e Cry1Ac protege a cultura de danos de alimentação de lepidópteros-praga primários e secundários. Experimentos de campo foram conduzidos de 2011 à 2016 na região sul e central do Brasil para caracterizar a eficácia da soja DAS-81419-2 infestada com *Anticarsia gemmatalis* (Lepidoptera: Erebididae), *Chrysodeixis includens* (Lepidoptera: Noctuidae), *Chloridea virescens* (Lepidoptera: Noctuidae), *Spodoptera cosmioides* (Lepidoptera: Noctuidae), *Elasmopalpus lignosellus* (Lepidoptera: Pyralidae), *Agrotis ipsilon* (Lepidoptera: Noctuidae) e *Helicoverpa armigera* (Lepidoptera: Noctuidae) durante os estágios vegetativo (V4) e reprodutivo (R2 e/ou R4) da cultura. A eficácia da soja DAS-81419-2 foi comparada com a variedade isogênica não-Bt manejada com ou sem inseticidas foliares comerciais para controle de lepidópteros. DAS-81419-2 reduziu significativamente o dano de alimentação causado por *A. gemmatalis*, *C. includens*, *C. virescens*, *S. cosmioides* e *H. armigera* durante os estágios vegetativo e reprodutivo, bem como, a mortalidade de plântulas causada por *E. lignosellus*. Entretanto, DAS-81419-2 apresentou moderada atividade em *A. ipsilon*. Além disto, um estudo de campo de três anos avaliou os potenciais efeitos adversos de DAS-81419-2 por meio da inspeção da diversidade e abundância de artrópodes não-alvos comparado com a isolinha não-Bt com ou sem aplicações convencionais de inseticidas. Estes estudos foram conduzidos em três regiões produtoras de soja onde os artrópodes não-alvos da parte aérea e da superfície do solo foram monitorados. Em geral, a abundância e diversidade total não foram afetadas significativamente pela soja Bt. Desta maneira, DAS-81419-2 mostrou eficácia consistente ao longo dos anos, localidades e diferente estágios da cultura contra lepidópteros-pragas primários e secundários de importância econômica da cultura da soja e não afetou adversamente os artrópodes não-alvos que ocorrem nos campos de soja.

Palavras-chave: Soja; *Bacillus thuringiensis*; Conkesta™; Eficácia; Efeito; Organismos-alvos; Lepidoptera; Artrópodes não alvos

ABSTRACT

Effect of genetically modified soybean DAS-81419-2 expressing the proteins Cry1Ac and Cry1F on the target-organisms and non-target arthropod community

Soybean (*Glycine max* (L.) Merrill) event DAS-81419-2 (Conkesta™ technology) expresses *Bacillus thuringiensis* (*Bt*) Cry1F and Cry1Ac proteins to provide protection against primary and secondary lepidopteran pest feeding damage. Field experiments were conducted from 2011 to 2016 in southern and central Brazil to characterize the efficacy of DAS-81419-2 soybean infested with *Anticarsia gemmatilis* (Lepidoptera: Erebididae), *Chrysodeixis includens* (Lepidoptera: Noctuidae), *Chloridea virescens* (Lepidoptera: Noctuidae), *Spodoptera cosmioides* (Lepidoptera: Noctuidae), *Elasmopalpus lignosellus* (Lepidoptera: Pyralidae), *Agrotis ipsilon* (Lepidoptera: Noctuidae), and *Helicoverpa armigera* (Lepidoptera: Noctuidae) during vegetative (V4) and reproductive (R2 and/or R4) crop developmental stages. The efficacy of DAS-81419-2 was compared to a non-*Bt* isogenic variety managed with or without applications of commercial foliar insecticides for lepidopteran control. DAS-81419-2 significantly reduced the feeding damage caused by *A. gemmatilis*, *C. includens*, *C. virescens*, *S. cosmioides*, and *H. armigera* during both vegetative and reproductive crop growth stages as well as the seedling stage plant mortality caused by *E. lignosellus*. However, DAS-81419-2 showed moderate activity on *A. ipsilon*. In addition, a 3-yr in-field study evaluated the potential adverse effects of DAS-81419-2 by examining the diversity and abundance of non-target arthropods (NTAs) compared to the non-*Bt* near isoline with and without conventional insecticide applications. These studies were conducted in three Brazilian soybean producing regions where both soil surface and aerial NTA were monitored. Overall, the total abundance and diversity were not significantly affected by *Bt* soybean. Therefore, DAS-81419-2 showed consistent efficacy across years, locations, and crop stages against the most economically important primary and secondary lepidopteran pests of soybean and did not adversely affect non-target arthropods occurring in soybean field.

Keywords: Soybean; *Bacillus thuringiensis*; Conkesta™; Efficacy; Effect; Target organisms; Lepidoptera; Non-target arthropods

1. GENERAL INTRODUCTION

One of the most critical challenges facing the world population in the coming decades is the need to increase food production. Recent estimates indicate that the current world population of 7.3 billion will reach 9.6 billion by the year 2050 and further to 11.2 billion by 2100 (United Nations, 2015). Current levels of food production must increase despite limited availability of water, arable land, and fossil fuels, conditions that are exacerbated by climate change. Current yield growth trends are simply insufficient to keep up with a growing demand (FAO, 2009; Alexandratos & Bruinsma, 2012; Ray et al., 2013). The demand for safety food, together with demand from other competing uses (e.g.; animal feed and fuel), put growing pressure on already scarce agricultural resources and production systems across the world (FAO, 2009). Agricultural productivity must increase while the deleterious impact on biodiversity and the services provided by complex ecosystems (Cardinale et al., 2012). To meet the increasing demand for quality and quantity of food in the face of anticipated climate changes, farmers need increased access to genetic resources (Juma, 2011) and new technologies to meet the need for increased productivity on available land (FAO, 2009).

1.1. Soybean Plant

Soybean, *Glycine max* (L.) Merrill, is globally important annual agricultural plants and valuable in the nutrition of humans and animals and for processing in various industries (Vlahović et al., 2013). The first recorded introduction of this crop in Brazil occurred around the year 1882. It was followed by introductions in Argentina, Colombia, and Paraguay during the first third of the 20th century. These initial introductions marked the first phase of soybean production in the region, which mostly aimed at experimentation and adaptation (Bonato & Bonato, 1987) and for human consumption by Asian immigrants (Pérez, 2007). Brazil was the largest soybean producer globally in the 2015/2016 season with an estimated production of 96.50 million metric tons of soybean (USDA, 2016).

1.2. Soybean Pests

The agricultural sector faces systematic annual losses due to pests. The damage caused by insect pests is one of the primary factors leading to the reduced production of major crops

(Metcalf, 1996; Oliveira et al., 2014). There are a great number of species that may reach pest status and affect soybean production in Brazil and in other regions around the world. The most important phytophagous arthropods include insect defoliators mainly of the family Noctuidae (Lepidoptera), and stink bugs within the Pentatomidae family (Hemiptera) (Bortolotto et al., 2015).

The velvetbean caterpillar, *Anticarsia gemmatilis*, Hübner, 1818 (Lepidoptera: Erebiidae), is an important defoliator that occurs in soybeans from Brazil to the Southeastern United States (King & Saunders, 1985; Panizzi, 1990; Gazzoni et al., 1994; Panizzi & Corrêa-Ferreira, 1997). Each larva consumes from 85 to 150 cm² of leaf area throughout larval stage. The majority of the larval consumption occurs from 4th to 6th instar (Moscardi & Carvalho, 1993; Cardoso et al., 1996; Bueno et al., 2011, Moscardi et al., 2012). In some cases, the infestation can lead to a complete defoliation of soybean plants.

The soybean looper, *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae), is also another important defoliator commonly associated to soybean. Its occurrence has increased in soybean fields due to the indiscriminate use of non-selective chemicals, mainly fungicides and insecticides that reduce natural biological control used to prevent *C. includens* outbreaks (Sosa-Gómez et al., 2003). Until the 1990s, the soybean looper was considered a secondary pest with a limited distribution. Its occurrence was observed only in isolated situations, and represented no more than 10% of the velvetbean caterpillar incidence (Corrêa et al., 1977; Moraes et al., 1991; Moscardi, 1993). Its incidence has increased recently, causing significant damage to soybean fields, especially in commercial producing regions of Brazil (Marsaro, 2010; Guedes, 2010) occurring alone or simultaneously with *A. gemmatilis* (Bernardi, 2012).

Similarly, species of the genus *Spodoptera*, such as the Southern Armyworm, *Spodoptera eridania* (Cramer) and *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidae), are now considered key pests by some Brazilian soybean growers (Bueno et al., 2007). They have affected soybean fields in the Cerrado region, Central and Southern Brazil (Hoffmann-Campo et al., 2000; Santos et al., 2005) causing damaging defoliation to soybean plants (Bueno et al., 2011) and destroying flowers and pods (Hoffmann-Campo et al., 2000; Moscardi et al., 2012).

Moreover, Brazilian soybean growers around the country face several other caterpillar outbreaks, mainly from species of the subfamily Heliothinae: *Chloridea* (= *Heliothis*) *virescens* (Fabricius) (Tomquelski & Maruyama, 2009) and *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Ávila et al., 2013). *Chloridea virescens* is present in Brazil, Bolivia, Peru, Mexico, and throughout the United States and southern Canada (Poole et al.,

1993; Molina-Ochoa et al., 2010). This species occurs in all major cotton-producing areas of Brazil at different infestation levels (Degrande, 1998). It has recently become an important pest of the soybean crop (Tomquelski & Maruyama, 2009). Thus, this insect can be a more serious pest of soybean, especially in areas where cotton is abundant and soybean is present at the vegetative stage (Moscardi et al., 2012). *Helicoverpa armigera* is considered to be one of the most important agricultural pests in the world (Tay et al., 2013). This polyphagous species is widespread throughout Europe, Africa, Asia, and Australia, where it causes extensive damage to a wide range of crops (Zalucki et al., 1986; Pogue, 2004; Cunningham et al., 2014). *Helicoverpa armigera* represents a significant insect management challenge to cotton and soybean growers in different parts of the world (Downes, 2012a, 2012b; Yang, 2013). This species was recently reported in Brazil (Czepak et al., 2013) and presented a high potential to cause severe yield losses (Ávila et al., 2013).

Soybean production in Brazil has experienced an increasing number of soil pest outbreaks caused by *Elasmopalpus lignosellus* (Zeller, 1848) (Lepidoptera, Pyralidae) (Viana, 2007, Hoffmann-Campo et al., 2012; Afonso-Rosa, 2013) and *Agrotis ipsilon* (Hufnagel) (Lepidoptera: Noctuidae) (Hoffmann-Campo et al., 2012; Ferreira et al., 2015). These pests have significant potential to cause yield losses in localized areas. *Agrotis ipsilon* is a polyphagous pest distributed across the world (Rings & Johnson, 1975; El-Salamouny et al., 2003). Small larvae feed mainly on leaf tissue and larger larvae cut cotyledons and stems. Larger larvae feed on plant parts just above the soil surface, and occasionally below the surface if the soil is loose and dry near the base of the plant. Larvae can cut off the plant stem, causing the death of the young plant. If the stem is not completely cut, the inflicted damage may lead to plant wilting or lodging (Tonet et al., 2000; Moscardi et al., 2012). The larva of *E. lignosellus* prefers sandy soils and requires a prolonged drought period during the early stages of the crop. This species initiates its attack soon after soybean emergence. The young larva feeds on the stem being able to damage or destroy multiple plants and can remain actively feeding for 30-40 days. The damage often occurs in spots within a field and can affect up to 50% of the soybean stand causing significant yield loss (Tonet et al., 2000; Moscardi et al., 2012).

1.3. Transgenic *Bt* Plants

Foreign genes were successfully introduced into plants for the first time around 30 years ago (Herrera-Estrella, 1983). Genetically modified (GM) crops are a promising

technology and their use has been documented to significantly boost agricultural productivity while decreasing maintenance costs, such as pesticides costs (Scandizzo & Savastano, 2010). Since first commercialized in 1996, GM crops, that express δ -endotoxins (Cry proteins) from the soil bacterium *Bacillus thuringiensis* Berliner (*Bt*), have been adopted rapidly worldwide (James, 2015).

Bt Cry genes were first engineered into tobacco plants for insect pest control in 1987 (Vaecck et al., 1987). Since then, *Bt* genes have been transferred into many plant species including two major economic crops, maize, *Zea mays* L. (Koziel et al., 1993) and cotton, *Gossypium* spp. (Flint et al., 1995). The development of *Bt* technologies is an additional tool in Integrated Pest Management (IPM) to control insects pests and to complement chemical insecticides (Bernardi et al., 2012). Transgenic *Bt* technologies can offer control of insect pests generally difficult to manage with synthetic insecticides (Siebert et al., 2008).

The availability of *Bt* plants engineered to produce insecticidal proteins from *Bt* has provided valuable options for managing certain insect pests with considerable environmental and economic benefits (Brookes & Barfoot, 2014). *Bt* crops provide an effective alternative tool for controlling target insects, bringing additional social, environmental, and economic benefits (Tabashnik, 2010). These include reduced insecticide use (Cattaneo et al., 2006; Lu et al., 2012), increased or less variable yields (Cattaneo et al., 2006; Carpenter, 2010; Edgerton et al., 2012; Shi et al., 2013), regional pest suppression (Carrière et al., 2003; Wu et al., 2008; Hutchison et al., 2010; Tabashnik et al., 2010), and better economic performance (National Research Council, 2010; Carpenter, 2010).

The first transgenic *Bt* maize and *Bt* cotton were first approved for commercial release in the USA in 1995, and were first planted in 1996 (Hellmich et al., 2008; Naranjo et al., 2008; James, 2015). In Brazil, *Bt* maize and *Bt* soybean plants that express the *Bt* proteins have been commercialized since 2008 (Mendes et al., 2009; Omoto et al., 2016) and 2013 (Brookes & Barfoot, 2015; Yano et al., 2015), respectively. The first generation of transgenic *Bt* crops expressed only one single Cry protein, and thus, had a relatively narrow target spectrum. Lepidopteran species are the primary insects targeted by the first generation *Bt* plants (Perlak et al., 1991; Koziel et al., 1993; Sivasupramaniam et al., 2008; Burkness et al., 2010; Siebert et al., 2012). Pyramided or stacked *Bt* crop technologies (second generation) that express two or more *Bt* proteins for a target pest have become commercially available during recent years. Compared to single-toxin *Bt* crops, multi-toxin *Bt* crops may control a broader range of pests, improve control of individual pest species and reduce overall pest damage to crops (Adamczyk & Gore, 2004; Burkness et al., 2010; Siebert et al., 2012).

Pyramided *Bt* crops have been adopted rapidly due to their improved pest control and insect resistance management attributes. They are expected to become widely adopted in the future (Carrière et al., 2015).

1.4. Insect-Resistant Soybeans Plants

Parrott et al. (1994) reported that the transgenic soybean plants carrying a native cryIAb gene from *B. thuringiensis* var. kurstaki HD-1 prevented *A. gemmatilis* larval feeding and growth. This was the first report of the successful introgression and expression of *B. thuringiensis* Berliner (*Bt*) gene in soybeans. Stewart Jr. et al. (1996) demonstrated that transgenic soybeans expressing a synthetic cryIAc gene protected the plants from damage caused by *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), *C. includens*, *C. virescens*, and *A. gemmatilis* in laboratory bioassays. Similarly, Walker et al. (2000) evaluated a transgenic soybean line for resistance to four lepidopteran pests in the field. Compared with untransformed lines, the *Bt* line showed three to five times less defoliation from corn earworm and eight to nine times less damage from velvetbean caterpillar, but lower resistance to soybean looper. In addition, the *Bt* soybeans showed four times greater resistance than non-*Bt* soybean to natural infestations of lesser cornstalk borer, *E. lignosellus*. Transgenic lines of soybean, expressing a synthetic cry1A gene (tic107) from *B. thuringiensis* were evaluated in screen house and in conventional field trials for efficacy against lepidopteran pests. They exhibited excellent efficacy against several lepidopteran pests (MacRae et al., 2005; McPherson & MacRae, 2009). Miklos et al. (2007) reported on three transgenic soybean lines expressing a cry1A synthetic gene, exhibiting high levels of resistance (100% of mortality) against *C. includes*, *H. zea* and *A. gemmatilis* in leaf disc and whole-leaf bioassays. Similarly, insect bioassays with a transgenic soybean cultivar IAS5 expressing a modified Cry1Ac endotoxin demonstrated high toxicity to *A. gemmatilis* in both lab and greenhouse studies; thus, offering a potential for effective insect resistance in soybean (Homrich et al., 2008a, 2008b).

Genetically modified MON 87701 × MON 89788 soybean, which expresses the Cry1Ac and EPSP-synthase proteins, has been registered for commercial use in Brazil, demonstrating good efficacy on *A. gemmatilis*, *C. includens*, *C. virescens* (Bernardi et al., 2012, 2014a), and *H. armigera* (Azambuja et al., 2015) but with low control efficacy against the *Spodoptera* complex (Yu et al., 2013; Bernardi et al., 2014b). In recent years, Dow AgroSciences developed soybean transgenic event DAS-81419-2 (trademarked as Conkesta™

technology) via *Agrobacterium*-mediated transformation to express Cry1Ac, Cry1F, and phosphinothricin acetyltransferase (PAT) proteins derived from *Bacillus thuringiensis* subspecies *kurstaki*, *Bacillus thuringiensis* subspecies *aizawai*, and *Streptomyces viridochromogenes*, respectively, where Cry1Ac and Cry1F provide protection against lepidopteran insect species, and PAT confers tolerance to the herbicide glufosinate as a selectable marker (Fast et al., 2015). Cry1Ac and Cry1F proteins provide protection against several lepidopteran pests affecting the soybean crop and the PAT protein provides tolerance to the herbicide glufosinate which is used as a selectable marker during the development of DAS-81419-2 soybean.

1.5. Effects of *Bt* Crops on Non-Target Arthropods

The potential for adverse effects of *Bt* crops on the non-target arthropods (NTAs) have been extensively studied by researchers and regulatory agencies worldwide (Wilkinson et al., 2003; Romeis et al., 2006; Romeis et al., 2009) and also has been evaluated as part of the environmental risk assessment (ERA) process that takes place before the decision to cultivate these crops commercially (Nap et al., 2003; Conner et al., 2003). One of the risks associated with the growing of insect resistant populations to genetically modified crops is their potential to adversely affect non-target arthropods (NTAs) which may include a range of arthropod species that fulfill important ecological functions such as biological control, pollination, and decomposition (Romeis et al., 2008). The most appropriate and currently recognized as rigorous approach to assess for potential effects on NTAs in many regulatory frameworks is a tiered testing system using surrogate species within the framework of problem formulation for *Bt* crops (Rose, 2006; Romeis et al., 2008; Wolt et al., 2010). In this tiered approach, risk (a function of hazard and exposure) is evaluated within different tiers. They progress from worst-case exposure scenarios to real-world field scenarios if the earlier tiered tests fail to indicate adequate certainty of acceptable risk (Romeis et al., 2008; Duan et al., 2010). In general, it is necessary to conduct field studies when the main potential effects of *Bt* crops are caused by complex interactions that cannot be evaluated in simple laboratory conditions (Comas et al., 2014). In the few past decades, field studies have been conducted in many countries, where researchers assessed the impact of *Bt* crops on NTAs (Priestley & Brownbridge, 2009; Comas et al., 2013).

1.6. Objectives and Dissertation Organization

This dissertation consists of three main chapters, preceded by this general introduction. Chapter I presents the field performance of the soybean transgenic event DAS-81419-2 expressing Cry1F and Cry1Ac in comparison to a non-*Bt* isogenic variety with and without the use of insecticidal sprays against *A. gemmatilis*, *C. includens*, *C. virescens*, and *S. cosmioides* in Brazil. In chapter II, the objective was to assess the efficacy of *Bt* soybean with transgenic event DAS-81419-2 to control the secondary pests *E. lignosellus*, *A. ipsilon*, and *H. armigera*. Chapter III presents the results from field studies assessing the potential for adverse effects of soybean event DAS-81419-2 on NTAs using a multi-year survey of the diversity and abundance of NTAs associated with the crop relative to those of its non-*Bt* near isoline and a non-*Bt* soybean near isoline managed with conventional insecticide applications under field conditions.

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2. CHAPTER I¹ - Efficacy of Soybean's Event DAS-81419-2 Expressing Cry1F and Cry1Ac to Manage Key Tropical Lepidopteran Pests under Field Conditions in Brazil

2.1 Abstract

Bacillus thuringiensis (*Bt*) event DAS-81419-2 (Conkesta™ technology) in soybean, *Glycine max* (L.) Merrill, expresses Cry1F and Cry1Ac proteins to provide protection from feeding by several lepidopteran pests. A total of 27 field experiments across nine locations were conducted from 2011 to 2015 in southern and central Brazil to characterize the efficacy of DAS-81419-2 soybean infested with *Anticarsia gemmatilis* Hübner (Lepidoptera: Erebidæ), *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidæ), *Heliothis virescens* (F.) (Lepidoptera: Noctuidæ), and *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidæ) during vegetative (V4) and reproductive (R2 and R4) crop developmental stages. The efficacy of DAS-81419-2 was compared to that of a non-*Bt* isogenic variety managed with or without applications of commercial foliar insecticides for lepidopteran control. DAS-81419-2 soybean consistently experienced defoliation levels of 0.5% or less (compared with 20.05–56.74% in the non-*Bt*, nonsprayed treatment) and larval survival of <0.1% in all four species across the vegetative and reproductive plant stages evaluated. The efficacy of DAS-81419-2 was significantly higher than commercial foliar insecticides applied to the non-*Bt* variety. DAS-81419-2 soybeans containing two highly effective *Bt* proteins are expected to be a more robust IRM tool compared to single-trait *Bt* technologies. The consistent efficacy of DAS 81419-2 soybeans across years, locations, and crop stages suggests that it will be a valuable product for management of hard-to-control key lepidopteran pests in South American soybean production.

Keywords: Conkesta; *Anticarsia gemmatilis*; *Chrysodeixis includes*; *Heliothis virescens*; *Spodoptera cosmioides*

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2.2. Introduction

Soybean, *Glycine max* (L.) Merrill (Fabaceae: Phaseoleae), is the world's most important legume crop, with 90% of its global production grown in the United States, Brazil, Argentina, China, and India (Chang et al., 2015). Brazil is the second largest soybean producer with an estimated production of 100 million metric tons during the 2015–2016 growing season, following the United States with an estimated 107 million metric tons (USDA, 2016). Economic losses to soybean production caused by a wide number of arthropod pests are experienced every year (Higley & Boethel, 1994; Funderburk et al., 1999). Almost all major soybean-producing areas suffer significant crop losses from a complex of lepidopteran pests. Brazil is no exception and important pests include the velvetbean caterpillar, *Anticarsia gemmatilis* Hübner (Lepidoptera: Erebidae), and the soybean looper, *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae) (Panizzi, 1990; Funderburk, 1994; Sullivan & Boethel, 1994; Walker et al., 2000; Moscardi et al., 2012; Sosa-Gómez et al., 2014). Additional lepidopteran species such as *Spodoptera cosmioides* (Walker, 1858), *Spodoptera eridania* (Stoll, 1782), and *Heliothis virescens* (F., 1781) (Lepidoptera: Noctuidae) are becoming notorious in Brazil for their ability to inflict significant crop damage (Tomquelski & Maruyama, 2009; Bueno et al., 2011; Moscardi et al., 2012; Bortolotto et al., 2014).

Synthetic insecticides are commonly used for controlling lepidopteran infestations in soybeans, often with limited success on target pests due to differences in intrinsic activity of products, developing insecticide tolerance, and sheltered feeding habits within the plant canopy (Thomas & Boethel, 1994; Aragon et al., 1997; di Oliveira et al., 2010; Martins & Tomquelski, 2015). Alternate control strategies such as biological insecticides and the use of natural enemies are available (Luttrell et al., 1998; Moscardi, 1999; Bueno et al., 2012). However, they are difficult to successfully and consistently implement on commercial large-scale production systems. Technological advances and commercial implementation of crop biotechnology for lepidopteran control offer a promising alternative and an additional tool to complement chemical insecticides in soybean (Bernardi et al., 2012, 2014a). Since commercially introduced in 1996, transgenic crops expressing δ -endotoxins (Cry proteins) from the soil bacterium *Bacillus thuringiensis* Berliner (*Bt*) have become important tools for effective pest control (Kumar et al., 2008). The successful adoption of *Bt* biotechnology in cotton, *Gossypium* spp., has enabled more effective management of lepidopteran pests and reduced synthetic chemical insecticide use (Perlak et al., 2001; Qaim & de Janvry, 2005; Wu

et al., 2008, Krishna & Qaim, 2012). Similarly, the adoption of *Bt* biotechnology in corn, *Zea mays* L., has reduced the economic impact of lepidopteran pests while protecting yield potential (Pilcher et al., 2002; Fernandes et al., 2003; Buntin, 2008; Siebert et al., 2008; Burkness et al., 2010; Siebert et al., 2012). Single-trait *Bt* soybeans were first commercialized in Brazil, Paraguay, Argentina and Uruguay in 2013 (Brookes & Barfoot, 2015; Yano et al., 2015), demonstrating good efficacy on *A. gemmatilis*, *C. includens*, and *H. virescens* (Bernardi et al., 2012, 2014a). However, this single-trait *Bt* soybean has showed poor control against the *Spodoptera* complex (Yu et al., 2013; Bernardi et al., 2014b). To date, there are no commercially available multi *Bt*-trait soybeans in South America.

Dow AgroSciences has developed soybean event DAS-81419-2 (trademark Conkesta™ technology) via *Agrobacterium*-mediated transformation to express Cry1Ac, Cry1F, and phosphinothricin acetyltransferase (PAT) proteins derived from *Bacillus thuringiensis* subspecies *kurstaki*, *Bacillus thuringiensis* subspecies *aizawai*, and *Streptomyces viridochromogenes*, respectively, where Cry1Ac and Cry1F provides protection against lepidopteran insect species, and PAT confers tolerance to the herbicide glufosinate as a selectable marker (Fast et al., 2015). Event DAS-81419-2 is a dual *Bt* technology for soybeans developed to provide South American agricultural producers with wide-spectrum control of key lepidopteran pests.

The objective of this study was to demonstrate the field performance of DAS-81419-2 (Conkesta™) and to assess its comparative efficacy against a non-*Bt* isogenic line with and without the use of insecticidal sprays to manage a complex of lepidopteran pests including *A. gemmatilis*, *C. includens*, *H. virescens*, and *S. cosmioides* in Brazil.

2.3. Materials and Methods

2.3.1. Field Sites and Growing Seasons

Field experiments were conducted from 2011 to 2015. All field experiments were conducted during the summer rainy season (October to March) in Brazil for commercial soybean production. Field sites were distributed across southern and central Brazil (Fig. 1). These areas were selected based on the commercial importance of soybean production and reflected a range of different environmental and agronomic conditions normally observed in soybean-producing areas of Brazil. All trials followed strict adherence to Brazilian regulatory requirements and were conducted at accredited certified field research sites which included

Dow AgroSciences, Coodetec - Desenvolvimento, Produção e Comercialização Agrícola LTDA, and SGS Gravena field stations (Table 1).

Fig. 1. Locations of field experiments in Brazil from 2011 to 2015. Location markers are slightly staggered for a better perception of the number of trials per region; see Table 1 for GPS coordinates.

Table 1. Trials locations and pests artificially infested in each year, from 2011 to 2015 in Brazil.

Trial Location				
City, State	Latitude	Longitude	Year	Pest Infested
Cravinhos , SP	21°18'05.53" S	47°44'27.66" W	2011	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i> , <i>H. virescens</i>
Indianópolis , MG	18°57'39.19" S	47°51'12.95" W	2011	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>H. virescens</i>
Uberlândia , MG	19°02'28.16" S	48°11'52.01" W	2011	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>H. virescens</i>
Cascavel, PR	24°53'20.84" S	53°32'37.36" W	2012	<i>A. gemmatalis</i>
Castro, PR	24°47'32.33" S	49°53'56.97" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i>
Cravinhos, SP	21°18'02.98" S	47°44'26.47" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i>
Cravinhos, SP	21°18'02.60" S	47°44'25.40" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>H. virescens</i>
Indianópolis, MG	18°57'55.00" S	47°51'28.00" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>H. virescens</i>
Indianópolis, MG	18°57'54.00" S	47°51'32.00" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>H. virescens</i>
Indianópolis, MG	18°57'23.54" S	47°51'22.81" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i> , <i>H. virescens</i>
Montividiu, GO	17°22'33.15" S	51°23'46.36" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i> , <i>H. virescens</i>
Montividiu, GO	17°22'37.46" S	51°23'31.08" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i> , <i>H. virescens</i>
Uberlândia, MG	19°02'30.40" S	48°11'49.90" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>H. virescens</i>
Uberlândia, MG	19°02'33.40" S	48°11'50.00" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>H. virescens</i>
Uberlândia, MG	19°02'30.31" S	48°11'44.27" W	2012	<i>A. gemmatalis</i> , <i>C. includens</i>
Cravinhos, SP	21°18'02.11" S	47°44'16.15" W	2013	<i>S. cosmioides</i> , <i>H. virescens</i>
Indianópolis, MG	18°57'28.08" S	47°51'22.09" W	2013	<i>S. cosmioides</i> , <i>H. virescens</i>
Cascavel, PR	24°53'13.30" S	53°32'24.05" W	2014	<i>S. cosmioides</i>
Montividiu, GO	17°22'38.08" S	51°23'44.62" W	2014	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i> , <i>H. virescens</i>
Montividiu, GO	17°22'37.57" S	51°23'44.02" W	2014	<i>S. cosmioides</i> , <i>H. virescens</i>
Castro, PR	24°47'34.42" S	49°53'57.18" W	2015	<i>S. cosmioides</i>
Conchal, SP	22°24'11.69" S	47°06'52.03" W	2015	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i> , <i>H. virescens</i>
Mogi Mirim, SP	22°26'49.81" S	47°04'14.79" W	2015	<i>H. virescens</i>
Palotina, PR	24°21'19.42" S	53°45'15.69" W	2015	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i> , <i>H. virescens</i>
Palotina, PR	24°21'18.49" S	53°45'15.56" W	2015	<i>C. includens</i> , <i>S. cosmioides</i> , <i>H. virescens</i>
Indianópolis, MG	18°57'27.85" S	47°51'22.35" W	2015	<i>S. cosmioides</i>
Indianópolis, MG	18°57'28.00" S	47°51'20.97" W	2015	<i>A. gemmatalis</i> , <i>C. includens</i> , <i>S. cosmioides</i>

2.3.2. Experimental Design

All field experiments were arranged in a randomized complete block design (RCBD) with four replications, except in four trials which had three replications. Plots sizes across locations ranged from four to 20 rows wide (45.0- to 50.0-cm row centers) by 5.0 to 20.0 m in length. The germplasm used for these experiments consisted of Maverick (University of Missouri, Columbia, MO) or Maverick crossed with DM16 (Don Mario Seeds, Buenos Aires, Argentina). All fertilization and weed control programs followed locally recommended practices to grow the crop. No foliar applied insecticides were used on the test plots, except in the plots for the non-*Bt* isogenic variety managed with insecticides. Most experimental sites relied on natural rainfall. However, artificial irrigation was available and occasionally used to avoid water stress during times of drought.

2.3.3. Treatments

The efficacy of a soybean variety expressing the *Bt* proteins Cry1F and Cry1Ac (DAS-81419-2 event, Conkesta™ technology, Dow AgroSciences LLC, Indianapolis, IN) was compared to a non-*Bt* isogenic soybean variety sprayed with insecticides (Brazil commercial insecticide program). The third treatment consisted of the non-*Bt* isogenic, and received no pest control treatments.

2.3.4. Insecticide Applications

The specific products used for the commercial insecticide treatment were selected based on standard soybean IPM recommendations in Brazil in the year in which the trials were conducted. The commercial insecticide-treated plots always used three sequential insecticide applications. To avoid drift, all adjacent plots were covered with tarps during the insecticide application. The insecticide program during the 2011 season consisted of chlorpyrifos at 480 g a.i./ha (Lorsban® 480BR EC insecticide, Dow AgroSciences LLC, Indianapolis, IN), methomyl at 215 g a.i./ha (Lannate BR SL, DuPont, Wilmington, US) and imidacloprid+beta-cyfluthrin at 112.5 g a.i./ha (Connect SC, Syngenta, Basel, Switzerland). These products were sprayed during the vegetative (V4) and reproductive (R2 and R4) soybean crop stages (Ritchie *et al.* 1985), respectively. The insecticide program used during the 2012 to 2015 growing seasons consisted of chlorantraniliprole at 10 g a.i./ha (Premio SC, DuPont,

Wilmington, US), thiamethoxam + lambda-cyhalothrin at 49.4 g a.i./ha (Engeo Pleno SC, Bayer CropScience, Monheim, Germany) and imidacloprid+beta-cyfluthrin at 112.5 g a.i./ha were sprayed at V4, R2, and R4 crop stages, respectively.

2.3.5. Artificial Infestations

All treatments were evaluated against *A. gemmatalis*, *C. includens*, *H. virescens*, and *S. cosmioides*. Field trials were subjected to artificial pest infestations to ensure uniform pest pressure across plots in all locations. All larvae were obtained from insect colonies maintained by SGS-Gravena at its location near the city of Jaboticabal in the state of São Paulo in Brazil. Colonies were maintained either in a room with controlled temperature or in an incubator at $25\pm3^{\circ}\text{C}$, $60\pm5\%$ RH, at a photoperiod of 14:10 (L:D) h and reared on artificial diet. Vigor in all insect colonies was boosted by introducing new field-collected larvae every year. Larvae of *A. gemmatalis*, *C. includens*, *H. virescens*, and *S. cosmioides* were infested at V4, R2, and R4 stages of soybean growth. A total of 10–20 plants per plot at each growth stage, randomly selected from one of the mid four rows of each plot, were marked and infested with 10–20 first-instar larvae per plant. Infestations were performed manually using a camel's hair brush. Upon infestation, plants were covered with a fine-mesh cage to prevent larval escape. Field evaluations were conducted ~10 d after each artificial infestation at the three selected plant growth stages. Variables evaluated included the percentage of live larvae per plot and a visual estimation of feeding defoliation expressed in percentage of leaf surface. The number of surviving larvae was recorded from each of the artificially infested plants per plot. Prior to removing each cage, the infested plant was gently shaken inside the cage to recover all surviving larvae. The fine-mesh cages were removed from the plants and all larvae inside the cage were counted. Any remaining live larva from the plant was also counted. To estimate the percent defoliation, the plant foliage that was enclosed within the cage was observed for lepidopteran feeding damage. The amount of tissue consumed by the larvae was visually estimated and expressed in percentage. Defoliation values were estimated for each artificially infested plant per plot.

2.3.6. Statistical Analyses

Mean defoliation (%) values, for each insect species and growth stage, were analyzed with the linear mixed model:

$$\eta_{ijk} = \eta + \text{Treatment}_i + \text{Trial}_j + \text{Treatment} \times \text{Trial}_{ij} + \text{Block}_{k(j)} + \text{nlarvae}X_{ijk}$$

with observations normally distributed, $y_{ijk} \sim N(\mu_{ijk}, \sigma^2)$, and identity link function $\eta_{ijk} = \mu_{ijk}$; $\text{nlarvae}X_{ijk}$ is a covariate to account for the number of larvae used in the artificial infestation on each plant. In order to improve the normality and homogeneity of variance of the dataset, both requirements for linear model application, values were transformed using $\sqrt{x + 0.5}$. Least square means presented in tables are back-transformed values and their standard errors were estimated with the delta method (Stroup, 2012).

Percentage of live larvae, binomial response, was analyzed with the generalized linear mixed model:

$$\eta_{ijk} = \eta + \text{Treatment}_i + \text{Trial}_j + \text{Treatment} \times \text{Trial}_{ij} + \text{Block}_{k(j)}$$

with observations binomially distributed, $y_{ijk} \sim \text{Binomial}(N_{ijk}, \pi_{ijk})$, where N_{ijk} and π_{ijk} are the number of larvae and proportion of live larvae in each experimental unit respectively. The link function for the binomial distribution is the logit function $\eta_{ijk} = \log \left[\frac{\pi_{ijk}}{1 - \pi_{ijk}} \right]$.

In both models, treatment is modeled as a fixed factor and trial, block (trial) and the interaction treatment $\times$ trial are modeled as random factors. Significance of treatment effect was evaluated with F-approximate test ($\alpha=0.05$) and least square means from different treatments were compared with Tukey's test. In the case of linear mixed model, the estimation method was restricted maximum likelihood and Kenward Rodgers for degrees of freedom; for the generalized linear mixed model the estimation method was maximum likelihood with Laplace approximation. The proportions of variance explained by random factors were calculated; corresponding significance levels were determined with the likelihood ratio test at $\alpha=0.05$ (Stroup, 2012). Linear mixed models were estimated with Proc MIXED and generalized linear mixed models were estimated with Proc GLIMMIX (SAS Institute, 2011).

2.4. Results

Bt soybean event DAS-81419-2 significantly reduced the level of defoliation caused by *A. gemmatilis* (Fig. 2A) compared to the non-*Bt* isogenic treatment with and without insecticide sprays across all growth stages evaluated (V4, $F_{2,34.0} = 115.36$, $P < 0.0001$; R2, $F_{2,23.8} = 35.13$, $P < 0.0001$; R4, $F_{2,19.2} = 40.50$, $P < 0.0001$). The percent defoliation ranged from 30.8 to 56.7% in the non-*Bt*, nonsprayed treatment, which is above the economic injury threshold level adopted in Brazil of 30% defoliation during the vegetative stage and 15%

during the reproductive stage of soybeans. The use of insecticides reduced defoliation to ~8.2 to 19.3%. However, event DAS-81419-2 consistently reduced the percentage of defoliation to 0.1% or less (Table 2). DAS-81419-2 provided almost 100% mortality to *A. gemmatilis* larvae and was significantly better than all other treatments across different plant phenological stages (V4, $F_{2,34.0} = 47.91$, $P < 0.0001$; R2, $F_{2,24} = 34.65$, $P < 0.0001$; R4, $F_{2,20} = 30.20$, $P < 0.0001$). The percent of surviving larvae was 0.01% or less on DAS-81419-2, compared to 14.6 to 25.7% survival on the non-*Bt*, nonsprayed soybeans and around 1% when the non-*Bt* soybeans were managed with a commercial spray program (Table 3).

Chrysodeixis includes was also highly susceptible to the DAS-81419-2 event throughout the vegetative and reproductive soybean crop stages evaluated (Fig. 2B). Defoliation levels observed in DAS-81419-2 soybeans were significantly lower compared to all other treatments (V4, $F_{2,33.9} = 99.45$, $P < 0.0001$; R2, $F_{2,21.9} = 30.88$, $P < 0.0001$; R4, $F_{2,17.8} = 56.55$, $P < 0.0001$). Defoliation in the non-*Bt*, nonsprayed treatment was 49.0% during the vegetative stage and ranged from 26.2 to 27.8% in the reproductive stages, both of which are above the economic threshold levels (Table 2). The insecticide program brought defoliation levels down to a 13.5 to 21.5% range. Significant differences were also observed on the percentage of surviving larvae on DAS-81419-2 soybean compared to the sprayed and nonsprayed non-*Bt* isogenic treatments at V4, R2 and R4 stages (V4, $F_{2,33} = 47.42$, $P < 0.0001$; R2, $F_{2,22} = 24.30$, $P < 0.0001$; R4, $F_{2,18} = 21.74$, $P < 0.0001$; Table 3). Larval survival on event DAS-81419-2 was 0.01% or less across all trials and throughout the different crop stages evaluated. Surviving larvae on the non-*Bt*, nonsprayed treatment ranged from 10.6 to 20.2% and was reduced to 2.6 to 4.5% when the non-*Bt* crop was maintained with a commercial insecticide program.

DAS-81419-2 soybean significantly reduced (R2, $F_{2,19.9} = 27.24$, $P < 0.0001$; R4, $F_{2,25.9} = 92.10$, $P < 0.0001$) the defoliation injury caused during reproductive crop stages by another important pest, *H. virescens*, compared to both sprayed and nonsprayed non-*Bt* isogenic soybeans at the R2 and R4 stages evaluated (Fig. 2C) The levels of defoliation measured on event DAS-81419-2 ranged from 0.01 to 0.68% compared to 23.4 to 27.8% in the non-*Bt*, nonsprayed soybean, and compared to 10.0 to 15.3% in non-*Bt* soybeans managed with insecticides (Table 2). DAS-81419-2 caused significant levels of mortality to *H. virescens* compared to those observed on the non-*Bt* soybeans with and without insecticides (R2, $F_{2,14} = 24.64$, $P < 0.0001$; R4, $F_{2,26} = 22.57$, $P < 0.0001$). The percent of surviving *H. virescens* on DAS-81419-2 was near zero (0.01% or less) compared to 6.2 to 19.9% on the

non-*Bt*, nonsprayed treatment, and to 2.0 to 4.9% on the non-*Bt* soybeans managed with insecticides (Table 3).

Defoliation injury caused by *S. cosmioides* was significantly reduced by event DAS-81419-2 (Fig. 2D) and was successful in keeping defoliation damage to an average of less than 0.5% (Table 2.2) which was significantly lower (V4, $F_{2,15.7} = 22.94$, $P < 0.0001$; R2, $F_{2,7.2} = 37.49$, $P = 0.0002$; R4, $F_{2,25.8} = 65.48$, $P < 0.0001$) compared to non-*Bt* isogenic soybeans with and without insecticide sprays in which defoliation levels ranged from 7.3 to 11.0% and from 20.0 to 31.0%, respectively. Similarly, DAS-81419-2 inflicted significant mortality to *S. cosmioides* larvae compared to all other treatments across the different crop vegetative and reproductive stages evaluated (V4, $F_{2,15} = 16.45$, $P = 0.0002$; R2, $F_{2,8} = 22.9$, $P = 0.0005$; R4, $F_{2,26} = 25.24$, $P < 0.0001$). Survival on DAS-81419-2 was less than 0.1% (Table 3) and was significantly lower than non-*Bt* isogenic soybeans with insecticides which was significantly less damaged than the non-*Bt* soybeans without insecticides.

For all pest species, random variation was mainly explained by treatment $\times$ trial and trial effects, both for percentage of defoliation and percentage of live larvae. The proportion of variance explained by treatment $\times$ trial effect ranged from 31 to 75% for percentage of defoliation and from 21% to 75% for percentage of live larvae (all χ^2_{1df} likelihood ratio tests were significant, $P < 0.05$). The proportion of variance explained by trial effect ranged from 3 to 37% for percentage of defoliation (only 2 out of 11 χ^2_{1df} likelihood ratio tests were significant, $P < 0.05$) and from 18 to 68% for percentage of live larvae, (only 3 out of 11 χ^2_{1df} likelihood ratio tests were significant, $P < 0.05$). The proportion of variance explained by block (trial) was not relevant, with values ranging from 0 to 12%. The significance of the interaction treatment $\times$ trial and trial effects were due to the variation in performance of the insecticide applications across trials and also due to the variation in the response of the non-*Bt*, nonsprayed treatment. Performance of the event DAS-81419-2 was consistent across trials, with very low levels of defoliation and surviving larvae.

Fig. 2. Mean defoliation (%) caused by *A. gemmatalis*, *C. includens*, *H. virescens*, and *S. cosmioides* at V4, R2 and R4 soybean growth stages.

Table 2. Percentage of defoliation by *A. gemmatalis*, *C. includens*, *H. virescens*, and *S. cosmioides* at V4, R2, and R4 soybean growth stage.

Pest Species	Treatment	% Defoliation					
		Growth Stage V4		Growth Stage R2		Growth Stage R4	
		No. of trials	Lsq mean +SE	No. of trials	Lsq mean +SE	No. of trials	Lsq mean +SE
<i>A. gemmatalis</i>	DAS-81419-2 (Cry1F+Cry1Ac)		0.13 ± 0.58c		0.06 ± 0.73c		0.04 ± 0.71c
	non- <i>Bt</i> isoline sprayed	18	19.30 ± 3.24b	13	9.63 ± 3.12b	11	8.26 ± 2.86b
	non- <i>Bt</i> isoline nonsprayed		56.74 ± 5.52a		30.81 ± 5.48a		33.64 ± 5.64a
<i>C. includens</i>	DAS-81419-2 (Cry1F+Cry1Ac)		0.21 ± 0.61c		0.07 ± 0.65b		0.09 ± 0.61c
	non- <i>Bt</i> isoline sprayed	18	21.54 ± 3.45b	12	13.53 ± 3.22a	10	13.54 ± 2.98b
	non- <i>Bt</i> isoline nonsprayed		49.07 ± 5.04a		26.21 ± 4.44a		27.81 ± 4.24a
<i>H. virescens</i>	DAS-81419-2 (Cry1F+Cry1Ac)		—		0.01 ± 0.61c		0.68 ± 0.65c
	non- <i>Bt</i> isoline sprayed		—	8	10.01 ± 2.92b	14	15.35 ± 2.39b
	non- <i>Bt</i> isoline nonsprayed		—		27.88 ± 4.80a		23.45 ± 2.93a
<i>S. cosmioides</i>	DAS-81419-2 (Cry1F+Cry1Ac)		0.42 ± 0.95c		0.14 ± 0.67c		0.48 ± 0.63c
	non- <i>Bt</i> isoline sprayed	9	10.74 ± 3.57b	5	7.35 ± 2.38b	14	11.03 ± 2.15b
	non- <i>Bt</i> isoline nonsprayed		31.02 ± 5.59a		20.05 ± 3.85a		21.57 ± 2.98a

Values with the same letter in each column, within species, are not significantly different (Tukey's test, $P > 0.05$).

Table 3. Percentage of live larvae of *A. gemmatalis*, *C. includens*, *H. virescens*, and *S. cosmioides* at V4, R2, and R4 soybean growth stages.

Pest Species	Treatment	% of Live Larvae					
		Growth Stage V4		Growth Stage R2		Growth Stage R4	
		No. of trials	Lsq mean +SE	No. of trials	Lsq mean +SE	No. of trials	Lsq mean +SE
<i>A. gemmatalis</i>	DAS-81419-2 (Cry1F+Cry1Ac)		0.002 ± 0.003c		0.014 ± 0.012c		0.004 ± 0.005c
	non-Bt isoline sprayed	18	1.01 ± 0.46b	13	0.78 ± 0.38b	11	1.16 ± 0.52b
	non-Bt isoline nonsprayed		25.74 ± 8.25a		18.97 ± 6.99a		14.63 ± 5.33a
<i>C. includens</i>	DAS-81419-2 (Cry1F+Cry1Ac)		0.017 ± 0.012c		0.008 ± 0.008c		0.007 ± 0.009c
	non-Bt isoline sprayed	18	4.54 ± 1.64b	12	2.69 ± 1.20b	10	2.80 ± 1.31b
	non-Bt isoline nonsprayed		20.20 ± 5.79a		12.57 ± 4.83a		10.64 ± 4.37a
<i>H. virescens</i>	DAS-81419-2 (Cry1F+Cry1Ac)		—		0.018 ± 0.021c		0.005 ± 0.006c
	non-Bt isoline sprayed		—	8	4.91 ± 2.03b	14	2.09 ± 1.07b
	non-Bt isoline nonsprayed		—		19.90 ± 6.77a		6.27 ± 2.98a
<i>S. cosmioides</i>	DAS-81419-2 (Cry1F+Cry1Ac)		0.047 ± 0.042c		0.029 ± 0.036c		0.077 ± 0.047c
	non-Bt isoline sprayed	9	1.81 ± 1.21b	5	1.82 ± 1.27b	14	2.67 ± 1.18b
	non-Bt isoline nonsprayed		16.35 ± 8.28a		19.64 ± 10.59a		8.58 ± 3.52a

Values with the same letter in each column, within species, are not significantly different (Tukey's test, $P > 0.05$).

2.5. Discussion

Results from our multiyear and cross-geographic studies show that *Bt* soybean event DAS-81419-2 provides high efficacy against *A. gemmatalis*, *C. includens*, *H. virescens*, and *S. cosmioides* during vegetative and reproductive stages of crop development. These extremely low levels of defoliation were consistently and significantly observed in DAS-81419-2 soybeans compared to a non-*Bt* soybean managed with commercial insecticides. The percent of defoliation injury levels averaged around 0.5% or less in soybeans with DAS-81419-2 event, which will represent an important field attribute under South American conditions. Our results also indicate that DAS-81419-2 soybeans cause high levels of mortality to all four lepidopteran species evaluated, suggesting that survival to adult in the field will be extremely low. High levels of mortality are indicative of effective IRM when the product is planted with a suitable non-*Bt* refuge (Gould, 1998). The very limited survival of key target pest larvae tested in these studies supports the durability of DAS-81419-2 soybean when used in this manner.

Bernardi et al. (2012, 2014a) discussed results from recent studies evaluating the efficacy of events MON 87701 × MON 89788 soybean against key lepidopteran pests of soybeans in Brazil. MON 87701 × MON 89788 soybeans express a single *Bt* insecticidal protein, Cry1Ac, and was first commercially available in South America in 2013. The authors concluded that MON 87701 × MON 89788 soybeans provides a high-level efficacy against *A. gemmatalis*, *C. includens* (Bernardi et al., 2012), and *H. virescens* (Bernardi et al., 2014a). However, lower mortality was reported against species of the *Spodoptera* complex in laboratory and greenhouse trials, revealing low mortality on *S. cosmioides* and *S. eridania* of <13%, and ~50% mortality against *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) (Bernardi et al., 2014b). While *Bt* soybean events DAS-81419-2 and MON 87701 × MON 89788 provided excellent levels of control on three important lepidopteran pests (*A. gemmatalis*, *C. includens*, and *H. virescens*), the additional and consistent high level control of *S. cosmioides* demonstrated with DAS-81419-2 brings a much needed solution to South American soybean production, as this insect is gaining notoriety for becoming more damaging to soybean production in key commercial soybean-producing areas of Brazil.

The expression of two *Bt* insecticidal proteins in DAS- 81419-2 soybeans combines the efficacy of Cry1Ac and Cry1F. It provides dual control potential and is a significant advance over single-trait products. This is important because the ability to control damaging

pests is enhanced within the plant resulting in broad and high level efficacy, and also because the dual protein expression is an effective strategy for delivering increased product durability.

Our results also demonstrate that DAS-81419-2 soybean is significantly more efficient in controlling key lepidopteran pests compared to the non-*Bt* isogenic variety managed with a standard foliar insecticide program representative of the region in which these tests were conducted. The difficulties in achieving high levels of efficacy with foliar spray programs in soybeans is explained in part by the different degrees of susceptibility of key pests to commercial foliar sprays and also by the well-known behavior of some of these pests to feed deep within the plant canopy, which makes it difficult to be reached by lethal doses of foliar sprays. When managed properly, DAS-81419-2 soybeans is expected to reduce the number of foliar insecticides sprays to manage lepidopteran larvae. When commercially available, DAS-81419-2 soybeans will be a valuable and highly effective new tool to manage hard-to-control lepidopteran infestations in soybeans. As such, the durability of DAS-81419-2 soybean can only be maximized when used as part of both IPM and IRM programs and with the implementation of best management practices for *Bt* crop production that are relevant to South America conditions including planting of refuge areas consistent with manufacturer guidelines. Pest monitoring is still important to deploy on-time additional control tactics and help manage heavier than expected lepidopteran pest infestations or to control other insects not targeted by this technology.

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3. CHAPTER II² - Field Evaluation of Soybean Transgenic Event DAS-81419-2 Expressing Cry1F and Cry1Ac Proteins for the Control of Secondary Lepidopteran Pests in Brazil

3.1 Abstract

Genetically modified soybean with transgenic event DAS-81419-2 (Conkesta™ technology), expresses two *Bacillus thuringiensis* (*Bt*) derived proteins, Cry1Ac and Cry1F. Event DAS-81419-2 is a proposed new Integrated Pest Management (IPM) tool with demonstrated high efficacy for controlling the primary lepidopteran pests affecting soybean production in South America. Studies were conducted in Brazil from 2011 to 2016 to assess the efficacy of DAS-81419-2 against secondary lepidopteran pests including *Elasmopalpus lignosellus*, *Agrotis ipsilon* and *Helicoverpa armigera*, using artificial infestations to ensure uniform pest pressure. Results from research trials across nine localities showed that compared to a non-*Bt* isoline, DAS-81419-2 significantly reduced the seedling stage plant mortality caused by *E. lignosellus* and the feeding damage caused by *H. armigera* during both vegetative and reproductive crop growth stages. Event DAS-81419-2 showed moderate activity on *A. ipsilon*, and additional control tactics such as a chemical insecticide applied as a seed treatment or spray during early crop stages may be needed against this pest to provide consistently higher levels of protection in fields that are at risk of infestation. Collectively, the efficacy of soybean transgenic event DAS-81419-2 for the control of secondary lepidopteran pests, added to its high efficacy on primary lepidopteran pests suggest this new, dual protein technology will be an important tool where primary and secondary pests affect soybean production.

Keywords: Conkesta™; *Bacillus thuringiensis*; *Agrotis ipsilon*; *Elasmopalpus lignosellus*; *Helicoverpa armigera*

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3.2. Introduction

Soybean, *Glycine max* (L.) Merrill (Fabaceae: Phaseoleae) is one of the most important legume crops worldwide. It has experienced the highest percentage increase in planted area since the 1970s compared to any other major crop and is now grown in approximately 6% of the world's arable land (Hartman et al., 2011). The planted area in North and South America represents about 80% of total global soybean production (Chang et al., 2015). Brazil is the second largest producer, following the United States (US), with 96.5 million tons and an area of approximately 33 million hectares during the 2015-2016 growing season (USDA, 2016). The potential productivity of the soybean crop is influenced and often limited by various abiotic and biotic factors, including edapho-climatic conditions and the occurrence of diseases and pests throughout the crop cycle.

Hemiptera and Lepidoptera are the main orders of insect pests that attack the soybean crop and cause significant yield losses by reducing stand density (from plant death) and causing defoliation and feeding damage to pods (Sosa-Gómez et al., 2014). The most important and widely distributed defoliating lepidopteran pests of the soybean crop in Brazil include the velvetbean caterpillar, *Anticarsia gemmatilis* Hübner (Lepidoptera: Erebidiae) (Bernardi et al., 2012) and the soybean looper, *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae) (Bueno et al., 2011). However, an increasing number of reported outbreaks of secondary lepidopteran pests including *Elasmopalpus lignosellus* (Zeller, 1848) (Viana, 2007, Hoffmann-Campo et al., 2012; Afonso-Rosa, 2013), *Agrotis ipsilon* (Hufnagel) (Hoffmann-Campo et al., 2012; Ferreira et al., 2015) and *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Specht et al., 2013; Pomari-Fernandes et al., 2015) have been recently observed in Brazil. While not occurring regularly or extensively at economically-damaging levels, these secondary pests have significant potential to cause yield losses in localized areas. In the case of *H. armigera*, a recently introduced pest (Czepak et al., 2013), it is considered an emerging key pest in some Brazilian crop producing areas and requires close attention in terms of monitoring and control tactics.

The lesser cornstalk borer, *E. lignosellus*, is a polyphagous and cosmopolitan soil insect pest. It is more abundant in sandy soils and more damaging during hot and dry climatic conditions (Smith & Barfield, 1982; Funderburk & Mack, 1994). The larval stage is semi-subterranean and causes damage on seedlings and mature soybean plants (Leuck, 1966). The attack occurs in localized spots within a field, and can compromise up to 50% of the crop's production (Hoffmann-Campo et al., 2012). Another soil pest, *A. ipsilon*, is a polyphagous

insect with nocturnal habits. The larvae cause damage to soybean plants, cutting off seedlings and causing plant death, which leads to economic losses (Hoffmann-Campo et al., 2012; Sharaby & El-Nojiban, 2015; Xu et al., 2016). *Helicoverpa armigera* feeds on the aerial parts of the plant. It was recently reported in the main soybean producing regions of Brazil, causing losses by severely damaging leaves and pods (Czepak et al., 2013; Specht et al., 2013).

Until recently, lepidopteran pest management in soybeans relies primarily on the use of synthetic insecticides. However, the effectiveness of these products can be limited by an increase in insecticide resistance and the difficulty in reaching target insects at their resting and feeding sites, as they remain protected in the soil or within the plant canopy (Bernardi et al., 2012). An additional tool within an Integrated Pest Management (IPM) program is the use of genetically modified plants that express crystal (Cry) proteins derived from the soil bacterium *Bacillus thuringiensis* (*Bt*). The mode of action of Cry proteins has been documented in several studies (Bravo et al., 2007; Soberón et al., 2009). These proteins bind to specific receptors on the midgut epithelial cells of sensitive lepidopteran larvae, leading to pore formation, osmotic imbalance, and septicemia. The affected cells rupture and cause death of the insect (Federici & Siegel, 2008; Soberón et al., 2010; Bravo et al., 2013).

The use of *Bt* in genetically modified plants has been widely adopted in many crops such as corn and cotton across multiple countries, including Brazil (Romeis et al., 2008; Naranjo, 2014). In Brazil, genetically modified maize plants that express the *Bt* protein (*Bt* corn) have been commercialized since 2008 (Mendes et al., 2009; Omoto et al., 2016). *Bt* soybean plants expressing the Cry1Ac protein were recently approved for cultivation in Brazil and launched during the crop season of 2013 to 2014 (Bernardi et al., 2014; Yano et al., 2015). Since then, *Bt* soybeans have been considered as an important tool in lepidopteran insect-management programs (Bernardi et al., 2012; Yu et al., 2013; Bernardi et al., 2014; Bortolotto et al., 2014; Azambuja et al., 2015; Yano et al., 2015). Soybean transgenic event DAS-81419-2, known as ConkestaTM technology (Dow AgroSciences, LLC, Indianapolis, IN), was developed using an *Agrobacterium*-mediated transformation process to express Cry1Ac, Cry1F, and phosphinothricin acetyltransferase (PAT) proteins (Fast et al., 2015). Event DAS-81419-2 combines two *Bt* proteins in soybeans, Cry1Ac and Cry1F, derived from *Bacillus thuringiensis* subspecies *kurstaki* and *Bacillus thuringiensis* subspecies *aizawai*, respectively. They are highly similar to those expressed in cotton transformation events DAS-21023-5 and DAS-24236-5 respectively, combined through breeding in WideStrikeTM cotton (Dow AgroSciences, LLC, Indianapolis, IN), previously approved for cultivation in the United States (US EPA, 2005) and Brazil (CTNBio, 2009). Marques et al. (2016) reported

higher efficacy of DAS-81419-2 in soybeans against *A. gemmatilis*, *C. includens*, *Heliothis virescens* (Fabricius) and *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidae) than commercial foliar insecticides sprayed in soybean fields. There are limited reports of field studies evaluating the efficacy of *Bt* soybean to control other lepidopteran pests that are considered of secondary importance. The objective of the study was to assess the efficacy of the soybean transgenic event DAS-81419-2 expressing Cry1F and Cry1Ac to control *E. lignosellus*, *A. ipsilon*, and *H. armigera*.

3.3. Materials and Methods

Field and laboratory trials were designed to evaluate insect damage by *E. lignosellus*, *A. ipsilon*, and *H. armigera* to DAS-81419-2 (Conkesta™ technology) soybeans (Dow AgroSciences LLC, Indianapolis, IN, USA) and an isoline non-*Bt* soybean. Trials were conducted between 2011 and 2016 in southern and central Brazil (Table 1). Feeding damage to the soybean line expressing Cry1F and Cry1Ac was compared to that of a non-*Bt* isogenic soybean line. Each field experiment consisted on a randomized complete block design with four replications, except in 2011 trials which had three replications. Plots sizes across locations ranged from four to 20 rows wide (45.0 to 50.0-cm row centers) by 5.0 to 20.0 m in length. The germplasm used for these experiments was Maverick (Sleper et al., 1998) or Maverick crossed with DM16 (Don Mario Seeds, Buenos Aires, Argentina). All trials followed strict adherence to Brazilian regulatory requirements for confined field trials and were conducted at accredited certified field research sites. Field plots received no soil or foliar insecticide applications. Commercial herbicides and fungicides were applied in accordance with local weed and disease control management practices.

3.3.1. Insects

Natural insect infestations of secondary pest in Brazil vary across localities and growing seasons. Infestation pressure at specific research field sites is unpredictable and may be non-uniform. Therefore, artificial infestations of *A. ipsilon*, *E. lignosellus*, and *H. armigera* were performed on field trials to ensure pest pressure across plots in all locations. All of the insects used in these studies were obtained from a laboratory-reared colony (SGS, Jaboticabal – São Paulo State, Brazil). The insect colonies were reared on artificial diet and maintained at room

temperature of $25\pm 3^{\circ}\text{C}$, $60\pm 10\%$ RH, and a photoperiod of 14:10 h (L:D). Colonies were invigorated by introducing new field collected larvae every year from non-*Bt* fields in Brazil.

3.3.2. Field Trials

Elasmopalpus lignosellus

A total of 10 to 20 plants per plot, randomly selected from one of the center rows of each plot, was infested with two third-instar larvae per plant at the VC (cotyledon) soybean growth stage (Ritchie et al., 1982). Larvae were confined to an individual plant using a cut polyvinyl chloride (PVC) pipe (15 cm in diameter and 12 cm in height) placed at soil level. Prior to infestation, larvae were transferred from diet cups to Eppendorf tubes (one larva per tube). Two open Eppendorf tubes were positioned near the base of the plant inside the PVC pipe arena, allowing the larvae to exit the tubes and infest the plant. Plant injury was assessed 28 days after infestation (DAI) by counting dead plants that resulted from insect feeding and tunneling plants at the soil level. Percent plant mortality for each plot was calculated based on the number of dead plants versus the total number of infested plants.

Agrotis ipsilon

Between 10 and 20 plants were artificially infested at the VC soybean growth stage by placing one or two third-instar larvae at the base of each plant using a pair of soft forceps. Larvae were confined to the area of the seedling plants by placing barriers made of polyethylene plastic (20 cm in height, 1 m length and 25cm width) around each plot. Plant injury was assessed 15 DAI by counting the plants that presented insect damage, which is usually at soil level. Percent plant mortality in each plot was calculated based on the number of dead (cut) plants versus the total number of infested plants.

Helicoverpa armigera

Larvae were artificially infested at V4 (four node) and R4 (full pod) stages of soybean growth (Ritchie et al., 1982). A total of 8 to 15 plants per plot was randomly selected from one of the central four rows of each plot during each growth stage. The number of plants selected depended on the number of larvae available for infestation. Plants were marked and infested with 10 first-instar larvae or 5 third-instar larvae per plant. Infestations were performed manually using a camel's hair brush and then each individual plant was covered with a fine-mesh cage to prevent larvae from escaping. Field evaluations were conducted 10

DAI at the two selected plant growth stages. The percentage of live larvae per plot and a visual estimation of defoliation percentage were assessed.

3.3.3. Laboratory Trial

A replicated laboratory trial was conducted to assess the efficacy of the DAS-81419-2 soybean on *E. lignosellus*. Laboratory conditions were $27 \pm 1^\circ\text{C}$, relative humidity $60 \pm 10\%$ and a 14:10 h light:dark photoperiod. A completely randomized design was used, with six replicates per treatment. Each replicate consisted of six pots, with four seeds of either DAS-81419-2 or non-*Bt* isoline sown per pot. The number of emerged plants varied between 20 to 24 per replicate. One larva at the third instar was infested per plant three days after seedling emergence. Each pot was placed on top of a tray filled with water to provide needed moisture without disrupting the larva. In addition, pots were individually covered with a fine mesh bag to prevent larval escape. Plant mortality was assessed 10 and 35 DAI by counting the percentage of dead plants that resulted from insect feeding and tunneling plants at the soil level.

3.3.4. Statistical Analyses

The percentage plant defoliation caused by *H. armigera* was analyzed with a linear mixed model:

$$\eta_{ijk} = \eta + \text{Treatment}_i + \text{Trial}_j + \text{Treatment} \times \text{Trial}_{ij} + \text{Block}_{k(j)} + \text{nlarvae}X_{ijk}$$

with observations normally distributed, $y_{ijk} \sim N(\mu_{ijk}, \sigma^2)$, and identity link function $\eta_{ijk} = \mu_{ijk}$; $\text{nlarvae}X_{ijk}$ is a covariate to account for the number of larvae used in the artificial infestation on each plant.

The percentage plant mortality caused by *E. lignosellus* and *A. ipsilon*, and the percentage of live *H. armigera* larvae were analyzed with the generalized linear mixed model:

$$\eta_{ijk} = \eta + \text{Treatment}_i + \text{Trial}_j + \text{Treatment} \times \text{Trial}_{ij} + \text{Block}_{k(j)} + \text{nlarvae}X_{ijk}$$

with observations binomially distributed, $y_{ijk} \sim \text{Binomial}(N_{ijk}, \pi_{ijk})$. The link function for the binomial distribution is the logit function $\eta_{ijk} = \log \left[\frac{\pi_{ijk}}{1 - \pi_{ijk}} \right]$.

In both mixed models, treatment is considered a fixed effect while trial, block (trial) and the interaction treatment $\times$ trial are considered random effects. Linear mixed model was estimated by restricted maximum likelihood (REML) and Kenward Rodgers method for

degrees of freedom calculation. For the generalized linear mixed model the estimation method was maximum likelihood with Laplace approximation. The significance of treatment effect was evaluated with F-approximate test ($\alpha=0.05$) and least square means from different treatments were compared with Tukey's test. The proportions of variance explained by random effects were calculated and their significance levels were determined with a likelihood ratio test at $\alpha=0.05$ (Stroup, 2012). Linear mixed models and generalized linear mixed models were estimated with SAS PROC MIXED and SAS PROC GLIMMIX respectively (SAS Institute, 2011).

3.4. Results

DAS-81419-2 soybean significantly ($F_{1,21}=73.68$, $P < 0.0001$) reduced plant mortality caused by *A. ipsilon* (Fig. 1A). DAS-81419-2 plant mortality was 7.22% compared to 63.16% non-*Bt* soybean mortality (Table 2).

Plant mortality during the seedling stage of soybean caused by *E. lignosellus* was significantly lower on *Bt* soybeans containing the DAS-81419-2 event, compared with the non-*Bt* isogenic line 28 DAI ($F_{1,28}=127.29$, $P < 0.0001$). DAS-81419-2 soybean was highly effective at protecting plants and averaged only 1.16% plant mortality, even under a high infestation level and larval confinement on the plant (Fig. 1B and Table 2). In the laboratory trial, DAS-81419-2 soybean also provided consistent protection at 10 and 35 DAI and significantly reduced the percentage plant mortality, exhibiting 2.04 and 7.14% plant mortality compared to 55.43 and 57.61% observed for the non-*Bt* treatment at 10 DAI and 35 DAI, respectively (10 DAI: $F_{1,68}=30.16$, $P < 0.0001$; 35 DAI: $F_{1,68}=41.57$, $P < 0.0001$) (Fig. 2).

The effect of *H. armigera* larval instar (L1 and L3) on treatment comparisons was not statistically significant both at vegetative and reproductive stage for any of the evaluations (% Defoliation V4, $F_{1,3}=1.97$, $P=0.25$; % Defoliation R4, $F_{1,3}=0.21$, $P=0.67$; % Live larvae V4, $F_{1,18}=0.99$, $P=0.33$; % Live larvae R4, $F_{1,18}=1.45$, $P=0.24$), therefore the data were combined for subsequent analysis and graphical representation. Levels of defoliation caused by *H. armigera* and observed in DAS-81419-2 soybeans were significantly lower compared to those in the non-*Bt* isogenic treatment both at vegetative and reproductive stages (V4, $F_{1,6.8}=45.07$, $P=0.0003$; R4, $F_{1,4}=51.43$, $P=0.002$) (Fig 1C). Defoliation in the non-*Bt* treatment reached 62.60% during the vegetative stage and 36.56% in the reproductive stages, both of which are above the economic threshold levels (Table 3). *H. armigera* was highly susceptible to DAS-

81419-2 soybeans at the vegetative and reproductive soybean crop stages evaluated (Fig. 1D). *H. armigera* survival on DAS-81419-2 ranged from 0.64% to 0.05% compared to 37.7% to 20.06% on the non-*Bt* treatment in the vegetative and reproductive stages, respectively, with significant differences between both treatments (V4, $F_{1,8} = 32.13$, $P = 0.0005$; R4, $F_{1,19} = 37.31$, $P < 0.0001$) (Table 3).

Random variation was mainly explained by trial and trial $\times$ treatment effects. For those cases where variance components were significant (likelihood ratio tests $\chi^2_{1\,df}$, $P < 0.05$), percentages of variance explained by trial ranged from 39 to 61% and percentage of variance explained by trial $\times$ treatment interaction ranged from 26 to 70%. The relevance of both variance components was mainly due to the variation in the response of the non-*Bt* isoline treatment across trials, as can be observed in Fig. 1. In general, the performance of event DAS-81419-2 was consistent across trials for plant mortality, defoliation and surviving larvae. Damage caused by *A. ipsilon* and *E. lignosellus* was observed in certain trials to cause up to about 40% and 20% plant mortality, respectively, to DAS-81419-2 plots but was consistently lower than in the non-*Bt* treatment.

Table 1. List of pests, trial locations and soybean growth stage that at which pests were artificially infested, from 2011 to 2016 in several Brazilian regions.

Pest	Location (city, state)	Year (# of trials) ^a	Infestation method/soybean growth stage
<i>Elasmopalpus lignosellus</i>	Cascavel, PR	2012, 2014	Artificial infestations in VC stage soybean
	Castro, PR	2013, 2014, 2015	
	Cravinhos, SP	2011, 2012, 2013	
	Jaboticabal, SP	2016 (laboratory trial)	
	Indianópolis, MG	2011, 2012 (3), 2013 (2), 2015 (2)	
	Mogi Mirim, SP	2015 (2)	
	Montividiu, GO	2012 (2), 2013 (2), 2014 (2)	
	Palotina, PR	2014, 2015	
	Uberlândia, MG	2012 (3)	
<i>Agrotis ipsilon</i>	Cascavel, PR	2012, 2014	Artificial infestations in VC stage soybean
	Castro, PR	2013, 2015	
	Cravinhos, SP	2012 (2), 2013	
	Indianópolis, MG	2012, 2013 (2), 2015	
	Mogi Mirim, SP	2015 (2)	
	Montividiu, GO	2012, 2013 (2), 2014 (3)	
	Palotina, PR	2014, 2015	
	Uberlândia, MG	2012	
<i>Helicoverpa armigera</i>	Castro, PR	2015 (1, V4 / 1, R4) ^b	Artificial infestation in V4 and/or R4 stage soybean
	Indianópolis, MG	2015 (2, R4)	
	Mogi Mirim, SP	2015 (2, V4 / 1, R4)	
	Montividiu, GO	2014 (1, V4)	
	Palotina, PR	2014 (1, V4), 2015 (1, R4)	

^a If not specified then only one trial was conducted in the year listed. ^b Number of trials per location and soybean growth stages.

Table 2. Percentage plant mortality in *Bt* (event DAS-81419-2) and non-*Bt* soybean caused by *E. lignosellus* and *A. ipsilon* in field trials.

Pest species	Treatment	No. of trials	% Plant Mortality	
			Lsq mean	SE
<i>E. lignosellus</i>	DAS-81419-2 (Cry1F+Cry1Ac)	29	1.16	0.43 b
	non- <i>Bt</i> isoline		49.09	7.35 a
<i>A. ipsilon</i>	DAS-81419-2 (Cry1F+Cry1Ac)	22	7.22	2.75 b
	non- <i>Bt</i> isoline		63.16	9.06 a

Values with the same letter in each column, within species, are not significantly different (Tukey's test, $P>0.05$).

Table 3. Percentage live larvae and defoliation by *H. armigera* at V4 and R4 soybean growth stages.

Pest species	Treatment	Crop Stage / No. of trials	% Live larvae		% Defoliation	
			Lsq mean	SE	Lsq mean	SE
<i>H. armigera</i>	DAS-81419-2 (Cry1F+Cry1Ac)	V4 / 5	0.64	0.45 b	2.42	6.38 b
	non- <i>Bt</i> isoline		37.71	9.98 a	62.60	6.38 a
	DAS-81419-2 (Cry1F+Cry1Ac)	R4 / 5	0.05	0.06 b	0.08	3.80 b
	non- <i>Bt</i> isoline		20.06	8.16 a	36.56	3.80 a

Values with the same letter in each column, within species, are not significantly different (Tukey's test, $P>0.05$).

Fig. 1. Percentage plant mortality caused by *A. ipsilon* (A) and *E. lignosellus* (B) in field trials. Plant defoliation (%) by *H. armigera* (C) and percentage of live larvae of *H. armigera* (D) in field trials at V4 and R4 soybean growth stages (Dot markers represent individual trial mean values).

Fig. 2. Percentage plant mortality in *Bt* and non-*Bt* soybean caused by *E. lignosellus* under laboratory conditions (lsq mean $\pm$ SE; statistical comparison performed by evaluation time, Tukey's test, $P < 0.05$).

3.5. Discussion

Our studies conducted from 2011 to 2016 in Brazil present the first field data of a biotech soybean crop expressing two *Bt* proteins against pests that currently are considered secondary in importance. Recent studies from South America indicate that *Bt* transgenic technology in soybeans are highly effective against the main lepidopteran pests (Bernardi et al., 2012, 2014; Marques et al., 2016). The efficacy of the combined Cry1Ac and Cry1F insecticidal proteins expressed by soybean transgenic event DAS-81419-2 was characterized under a range of field environment conditions in several Brazilian regions. DAS-81419-2 reduced significantly the percent plant mortality caused by *E. lignosellus* and *A. ipsilon* as well as feeding damage caused by *H. armigera* during both vegetative and reproductive crop growth stages. The consistent results observed with DAS-81419-2 across the most important soybean producing geographies in Brazil are of extreme importance. Albeit these pests are not widely distributed at economically important densities, they have demonstrated a high

potential to cause locally severe yield losses (Viana, 2007; Hoffmann-Campo et al., 2012; Ávila et al., 2013).

Our results using a soybean transgenic event with dual *Bt* protein expression, Cry1Ac and Cry1F, agree with those of previous authors using single protein. Walker et al. (2000) reported a non-*Bt* soybean variety exhibited four-times higher infestation of *E. lignosellus* under natural conditions compared to a *Bt* soybean isogenic variety expressing the single Cry1Ac protein. In addition, Siebert et al. (2012) and Okumura et al. (2013) reported field maize expressing single protein Cry1F to provide high levels of control against *E. lignosellus* both in the U.S. and Brazil, respectively.

Soybean transgenic event DAS-81419-2 demonstrated moderate reductions in plant mortality caused by the black cutworm, *A. ipsilon*, compared to a non-*Bt* treatment. This result agrees with previous work showing that *A. ipsilon* is relatively insensitive or less susceptible to many current *Bt* Cry toxins such as Cry1Ac, Cry1Fa, and Cry1Fb (de Maagd et al., 2003). Cry1F in maize inflicted only 40% mortality but caused significant weight reduction to *A. ipsilon* larvae compared to a non-*Bt* maize (Binning et al., 2014). Seedling feeding laboratory bioassays using Cry1F maize revealed partial efficacy against black cutworm. However, better results with Cry1F maize were observed in field-strip trials. The authors indicated the addition of a seed treatment to the *Bt* maize contributed to achieve a higher larval mortality and reduced larval mortality of survivors and concluded that Cry1F maize alone or in combination with a seed treatment may be suitable for suppressing *A. ipsilon* in no-tillage corn fields (Kullik et al., 2011). In addition, previous work by Yu et al. (2013) evaluating the susceptibility of two *Bt* soybean lines expressing a single Cry1Ac protein provided almost no resistance to *A. ipsilon*. Rule et al. (2014) reported that Cry1F in maize protected plants and maintained mean plant stand densities of > 85%, even despite high infestation levels of *A. ipsilon* under confinement conditions with the plants. These results may suggest better activity on *A. ipsilon* observed with Cry1F than with Cry1Ac proteins. Willrich et al. (2005) reported a good efficacy against low-level infestations of black cutworm using WideStrikeTM cotton that expresses Cry1Ac and Cry1F protein and similar to our study on soybeans suggest compatibility of both proteins to manage *A. ipsilon* at moderate levels. However, Binning et al. (2014) demonstrated an initial, post-ingestive aversive response of *A. ipsilon* to Cry1F maize. This behavior may offer a possible explanation of the moderate levels of protection observed with the DAS-81419-2 soybean line. The two proteins expressed in the DAS-81419-2 event may elicit a feeding deterrent behavior with black cutworm larvae. Similar results were reported by Harris et al. (1997) in light brown apple moth, *Epiphyas*

postvittana (Walker), where neonate larvae abandoned diet containing *Bt* endotoxins Cry1Ac and Cry1Ba in choice feeding bioassays after initial ingestion. Despite the moderate levels of control of *A. ipsilon* observed, our studies and existing literature indicate additional control methods such as seed treatments or foliar insecticides, following close monitoring, may be needed when DAS-81419-2 soybeans are planted in areas prone to black cutworm infestations.

Results presented herein document the first detailed report for the susceptibility of *H. armigera* larvae to *Bt* soybean expressing two proteins with the DAS-81419-2 transgenic event. Our studies showed that DAS-81419-2 provides high efficacy against *H. armigera*, consistent low levels of defoliation damage, and high levels of mortality of *H. armigera* larva. Our results suggest that the probability of survival of this pest to adult stages in the field will be extremely low. The high efficacy exhibited by soybean transgenic event DAS-81419-2 event against *H. armigera* is in accordance with previous studies testing *Bt* soybean expressing a single Cry1Ac protein (Yu et al., 2013; Azambuja et al., 2015; Dourado et al., 2016). Laboratory studies testing purified protein showed high toxicity of Cry1Ac (Babu et al., 2002) and significant larval growth inhibition with Cry1Fa1 on *H. armigera* (Avilla et al., 2005). Additional studies by Ibargutxi et al. (2008) on *Bt* cotton concluded that Cry1Ac was significantly more toxic than Cry1Fa against *H. armigera*. However, when both proteins were expressed in the same plant, they showed an additive effect against this pest.

The concurrent expression of several toxins in the same plant has been recommended as one of the most effective strategies to delay insect resistance development to *Bt* plants (Ibargutxi et al., 2008). However, binding studies conducted by Hernández & Ferré (2005) revealed the occurrence of a common receptor for Cry1Ac and Cry1Fa in *H. armigera* larvae. Therefore, as with any *Bt* transgenic technology, a sound Insect Resistance Management (IRM) strategy, adapted to local conditions, must be implemented and adopted to promote a long duration of *Bt* technologies involving these two proteins. IRM strategies based on planting areas of non-*Bt* refuge crops alongside the *Bt* crop are designed specifically to delay the development of resistance in the primary target pests. Refuge strategies are targeted at delaying resistance in primary pests, and will need to be augmented with best management practices that reduce the level of infestation and include scouting for unexpected damage by primary or secondary pests followed by supplemental insecticide treatments if necessary.

Secondary pest outbreaks are a well-known phenomenon, resulting in unexpected yield losses to agricultural production in several localized areas. However, because they are not widely distributed, and often are referred to as occasional pests, they are generally not the

focus of transgenic crop research. Abundant research documents *Bt* crops as highly effective at controlling target key pests even though they may not be as effective at controlling other pests that historically pose less or even no threat across large areas, but are important locally. Over several years, suppression of primary pests using *Bt* technology and associated changes in crop production practices may allow secondary pests to increase in importance (Ho et al., 2009). For example, the problem of secondary pests may be exacerbated because they no longer will be controlled in the absence of broad-spectrum pesticide applications that previously were aimed at controlling key pests (Sharma & Ortiz, 2000).

Our results on the efficacy of soybean transgenic event, DAS-81419-2, to manage secondary lepidopteran pests, combined with the high efficacy on key lepidopteran pests (Marques et al., 2016) suggest this new technology will be an important tool that has a fit across soybean areas where either or both key and secondary pests affect a soybean crop. To extend the benefit offered by DAS-81419-2 event as with any other *Bt* technology, the product should be used as the basis of IPM programs, and strong IRM plans should be implemented that are designed to maintain the benefits and extend the lifetime of *Bt* transgenic crops against key and secondary pests.

3.6 References

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4. CHAPTER III³ - Impact of Transgenic Soybean Expressing Cry1Ac and Cry1F Proteins on the Non-Target Arthropod Community Associated with Soybean in Brazil

4.1 Abstract

Field-scale studies that examine the potential for adverse effects of *Bt* crop technology on non-target arthropods may supplement data from laboratory studies to support an environmental risk assessment. A three year field study was conducted in Brazil to evaluate potential for adverse effects of cultivating soybean event DAS-81419-2 that produces the Cry1Ac and Cry1F proteins. To do so, we examined the diversity and abundance of non-target arthropods (NTAs) in *Bt* soybean in comparison with its non-*Bt* near isoline, with and without conventional insecticide applications, in three Brazilian soybean producing regions. Non-target arthropod abundance was surveyed using Moericke traps (yellow pan) and pitfall trapping. Total abundance (N), richness (S), Shannon-Wiener (H'), Simpson's (D) and Pielou's evenness (J) values for arthropod samples were calculated for each treatment and sampling period (soybean growth stages). A faunistic analysis was used to select the most representative NTAs which were used to describe the NTA community structure associated with soybean, and to test for effects due to the treatments effects via application of the Principal Response Curve (PRC) method. Across all years and sites, a total of 254,054 individuals from 190 taxa were collected by Moericke traps, while 29,813 individuals from 100 taxa were collected using pitfall traps. Across sites and sampling dates, the abundance and diversity measurements of representative NTAs were not significantly affected by *Bt* soybean as compared with non-sprayed non-*Bt* soybean. Similarly, community analyses and repeated measures ANOVA, when applicable, indicated that neither *Bt* soybean nor insecticide sprays altered the structure of the NTA communities under study. These results support the conclusion that transgenic soybean event DAS-81419-2 producing Cry1Ac and Cry1F toxins does not adversely affect the NTA community associated with soybean

Keywords: Genetically modified crop; *Bacillus thuringiensis*; *Glycine max*; ConkestaTM; Non-target arthropods; Environmental risk assessment

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4.2. Introduction

Genetically modified (GM) crops that express Cry proteins derived from the soil bacterium, *Bacillus thuringiensis* (*Bt*), have been grown in many countries on increasing acreage since their commercial introduction in 1996 (James, 2015). Commercially available GM crops that express *Bt* proteins are widely used to control major lepidopteran and coleopteran insect pests and are an important tool within an integrated pest management (IPM) system (Romeis et al., 2006; Burkness et al., 2010; Siebert et al., 2012; Bernardi et al., 2012, 2014). Prior to commercialization of a GM crop, a science-based environmental risk assessment (ERA) is conducted to evaluate the potential for unreasonable adverse effects on beneficial non-target organisms that occur in agricultural ecosystems (Romeis et al., 2008; Wolt et al., 2010; Romeis et al., 2011). This process has been described in detail by a number of regulatory agencies worldwide (Rose, 2007).

Beneficial arthropods can play an important role in regulating populations of insect pests. Thus, understanding the potential impact of pest management tools on non-target arthropod (NTA) populations in the field is an important element of ERA for transgenic crops (Romeis et al., 2011). Problem formulation is conducted in the first stage of the assessment to identify the applicable risk hypotheses and an approach to testing them, which includes estimates of hazard (effects) and exposure (Raybould, 2006). Knowledge about the NTA species most likely to be exposed to the proposed insecticidal protein(s) enables researchers to determine which species should be the focus of the risk assessment (Romeis et al., 2013, 2014; Carstens et al., 2014). Within the scope of an ERA's hazard assessment, a tiered testing approach is used to characterize effects of GM crops on non-target organisms at progressively higher levels of biological organization. Lower-tier tests are conducted under controlled laboratory conditions representing worst-case or artificially high exposure scenarios, while higher-tier studies are conducted under field conditions representing realistic exposure pathways and levels and natural environments (Romeis et al., 2008). For practical reasons, only a small fraction of all possible organisms can be considered for laboratory testing. To examine the potential effects of GM plants on NTAs, representative species should be selected (Dutton et al., 2003; Garcia-Alonso et al., 2006; Romeis et al., 2008). The selected species should represent different ecological functions, such as herbivory, pollination, predation and parasitism of pest organisms, and decomposers (Romeis et al., 2008). The extrapolation of results from lower-tiered studies to inform risk assessments that consider the potential for field effects is well supported in the *Bt* literature, where meta-analyses

examining correlation of laboratory and field data concluded that laboratory results generally predict (Duan et al., 2009) or over-estimate field effects (Naranjo, 2009). Building on the lessons from empirical testing and the evolution of risk assessment for GM crops (Dutton et al., 2003; Garcia-Alonso et al., 2006; Raybould, 2006; Rose, 2007; Romeis et al., 2008; Wolt et al., 2010; Romeis et al., 2011, 2013, 2014; Carstens et al., 2014), the risk assessment community recognized that surrogate organism testing (Carstens et al., 2014) and data transportability (Nakai et al., 2015), when appropriately applied (Paula et al., 2016), provide high value through informing and simplifying risk assessments performed across geographies in which the same crop is cultivated (Wach et al., 2016). While lower tier hazard assessments have generally predicted low risk for *Bt* crops to NTA's under field conditions, field studies are sometimes required by government regulatory bodies allowing this prediction to be tested empirically (Garcia-Alonso et al., 2006).

Transgenic soybean event DAS-81419-2 (Conkesta™ technology, Dow AgroSciences LLC, Indianapolis, IN) was developed via *Agrobacterium*-mediated transformation to express the Cry1Ac, Cry1F, and phosphinothricin acetyltransferase (PAT) proteins derived from *Bacillus thuringiensis* subspecies *kurstaki*, *B. thuringiensis* subspecies *aizawai*, and *Streptomyces viridochromogenes*, respectively. Cry1Ac and Cry1F provide protection against certain lepidopteran pests, and PAT confers tolerance to the herbicide glufosinate ammonium as a selectable marker (Fast et al., 2015). Event DAS-81419-2 combines two *Bt* proteins in soybeans to provide South American agricultural producers with wide-spectrum protection from feeding damage by certain key lepidopteran pests (Marques et al., 2016, 2017). The Cry1Ac and Cry1F proteins expressed in event DAS-81419-2 soybean are highly similar to those expressed in cotton transformation events DAS-21023-5 and DAS-24236-5 respectively (combined through breeding in WideStrike™ cotton)⁴, which was previously assessed and approved for cultivation in the United States since 2004 (US EPA, 2005) and in Brazil since 2009 (CTNBio, 2009). Event DAS-81419-2 soybean has similarly been assessed and approved for cultivation in Argentina (2016) (Ministerio de Agroindustria Presidencia de la Nación, 2016), Brazil (2016) (CTNBio, 2016), Canada (2014), and the United States (2014) (ISAAA, 2016).

Problem formulation conducted as part of the ERA for DAS-81419-2 soybean considered the familiarity of the mode of action for Cry proteins (Aronson & Shai, 2001;

⁴™WideStrike is trademark of The Dow Chemical Company ("DOW") or an affiliated company of Dow

Rang et al., 2004; Lee et al., 2006; Gouffon et al., 2011), the narrow spectrum of activity for Cry proteins (McClintock et al., 1995; Romeis et al., 2006), the demonstrated history of safe use for *Bt* crops (Naranjo, 2005; Sanvido et al., 2007) and the agronomic equivalence of DAS-81419-2 soybean to conventional soybean (USDA, 2012). Previous laboratory studies using purified protein, lyophilized plant tissue, and/or leaf feeding bioassay in direct or indirect exposure test systems demonstrated no adverse effects of Cry1Ac or Cry1F on NTA (Rodrigo-Simón et al., 2006; González-Zamora et al., 2007; Li et al., 2011; Tian et al., 2012, 2014; Su et al., 2015; Ali et al., 2016; Wang et al., 2016). Comprehensive reviews (Romeis et al., 2006; Han et al., 2016) and meta-analyses (Marvier et al., 2007; Wolfenbarger et al., 2008; Naranjo, 2009) of NTA laboratory and field data have concluded the safety of transgenic *Bt* proteins across the spectrum of commercialized events and cropping systems, including cotton and maize, in which they have been deployed. The only differences in NTA populations between *Bt* crops and their conventional counterparts (in the absence of insecticide applications) have been attributed to reduction in target pest abundance or quality as prey or hosts for predators or parasitoids. Based on the existing data supporting the safety of *Bt* proteins, and Cry1Ac and Cry1F specifically, the problem formulation step for DAS-81419-2 concluded that additional testing was not required to refine the risk assessment.

Despite familiarity with *Bt* proteins, some regulatory agencies require in-country field studies to confirm the findings of lower tier studies and studies conducted in other countries. This study evaluated the potential for unreasonable adverse effects of cultivating soybean event DAS-81419-2 through a multi-year survey examining the abundance and diversity of NTAs associated with this dual *Bt* protein-expressing crop in comparison with its non-*Bt* near isoline with and without target pest management through conventional insecticide applications under field conditions. Subsequent community-level analyses were conducted with the data generated in key soybean-production regions of Brazil.

4.3. Materials and Methods

Field trials were conducted at three sites in Brazil during the 2011-2012, 2012-2013 and 2013-2014 soybean growing seasons. The selected field sites represented regions of distinct agronomic practices and environmental conditions for commercial soybean production (Table 1). Treatments included soybean event DAS-81419-2 (Conkesta™ technology, Dow AgroSciences LLC, Indianapolis, IN) expressing Cry1Ac and Cry1F, a non-

Bt near isoline without insecticides, and the same non-*Bt* variety managed under an insecticide program consistent with local commercial practices. The variety Maverick was used in all treatments (Sleper et al., 1998). Each site consisted of three replications of each treatment in a randomized complete block (RCB) design.

Table 1. Description of field sites used to assess the effect of *Bt* soybean Event DAS-81419-2 on non-target arthropods.

Location	Latitude	Longitude	Year	Plot Size (m x m)	Planting rate (seeds/m)	Soil type
Castro, PR	24°47'34.04"S	49°53'54.94"W	2012	10 × 20	15.0	Loam
	24°47'34.42"S	49°54'07.23"W	2013	10 × 40	18.0	
Montividiu, GO	17°22'37.46"S	51°23'31.08"W	2011	9 × 20	16.0	Loam
	17°22'36.44"S	51°23'49.44"W	2012	10 × 20	14.0	
	17°22'33.72"S	51°23'47.06"W	2013	10 × 40	18.0	
Uberlândia, MG	19°02'28.16"S	48°11'52.01"W	2011	9 × 20	15.0	Loam
	19°02'29.09"S	48°11'45.98"W	2012	10 × 20	10.0	

4.3.1. Agronomic Practices

All field experiments were conducted during the summer rainy season (November to January) in Brazil which paralleled commercial soybean production. All trials followed strict adherence to Brazilian regulatory requirements and were therefore conducted at accredited and certified field research stations operated by Dow AgroSciences or SGS Company. All field trials were conducted as regulated permits approved by the Comissão Técnica Nacional de Biossegurança (CTNBio). Standard agronomic practices were used for fertilization, irrigation, disease, and weed management. The tillage practices applied at each field site varied from minimum tillage (Uberlândia, MG and Montividiu, GO) to conventional tillage (Castro, PR). Thus, experiments covered the breadth of tillage practices normally observed in Brazil. Crop management practices during the study excluded the use of sprayed insecticides or miticides, except in the non-*Bt* plots managed with insecticides to simulate commercial practices. In field studies of risk assessment of *Bt* crops, the use of insecticide sprayed controls is important to reflect the replacement of existing agricultural practices (Wolfenbarger et al., 2008).

4.3.2. Insecticide Applications

Plots managed with insecticide sprays were inspected weekly following plant emergence. At all sites, insecticide applications were performed when any of the target pest population densities exceeded economic thresholds according to local integrated pest management (IPM) programs (Hoffmann-Campos et al.; 2012), but limited to three applications, to allow comparisons across sites. The insecticide sequence used was methomyl at 107.5 g a.i./ha (Lannate® BR SL, DuPont, Wilmington, DE), followed by chlorpyrifos at 480 g a.i./ha (Lorsban® 480BR EC insecticide, Dow AgroSciences LLC, Indianapolis, IN), followed by imidacloprid + beta-cyfluthrin at 84.38 g a.i./ha (Connect® SC, Syngenta, Basel, Switzerland). This sequence was applied in all cases when the treatment threshold was met. To limit the potential for spray drift to affect untreated plots, all adjacent plots were covered with tarps during insecticide application.

4.3.3. Arthropod Surveys

Aerial Arthropod Sampling. Monitoring was conducted at each site using Moericke traps (yellow pan traps). These traps consisted of rectangular plastic trays (30 x 25 x 10 cm) filled with a mixture of water, formaldehyde (10%), and a few drops of liquid detergent, and were deployed during soybean growth stages V4/V5, R2, R4/R5 and R7/8 (Ritchie et al., 1982). An additional early season trapping during VC/V1 was conducted for the 2012-2013 trials. At the initiation of each sampling period, two traps were placed randomly near the center of each plot and levelled at the upper third of the plant canopy. Traps were maintained for three days during each sampling period. The contents were sieved using a fine (0.5 mm) mesh sieve (Lara et al., 2009; González et al., 2015), labeled and preserved with 70% ethanol, and identified in the laboratory. The most representative arthropods were identified at least to the family level; assigned to ecological function on the basis of family habits or subfamily, for families with multiple feeding habits (Triplehorn & Johnson, 2005).

Surface-Dwelling Arthropod Sampling. Pitfall traps were used to monitor surface-dwelling arthropods during the soybean growth stages V4/V5, R2, R4/R5 and R7/8. For the 2012-2013 trials, an additional early season trapping during VC/V1 was conducted. During each sampling period, two pitfall traps were established near the center of each plot to reduce edge effects; and traps were spaced 2 m apart within a single row interspace. Each trap consisted of a plastic outer cup (12 cm in diameter x 15 cm depth) buried in the soil with the

upper rim set at ground level. A galvanized tripod shield was placed over each cup with a gap of 2-3 cm between the rim of the cup and shield cover to protect against rain water and to reduce debris contamination. The traps were filled with a mixture of water, formaldehyde (10%) and a few drops of detergent, and left in the field for three days during each sampling period. Trap contents were processed as described for Aerial Arthropod Sampling.

4.3.4. Statistical Analyses

Abundance and Diversity of Non-Target Arthropods

The abundance (N), richness (S) (Slade & Blair, 2000), Shannon-Wiener (H') (Shannon & Weaver, 1949), Simpson's (D) and Pielou's evenness (J) (Pielou, 1966) measures for arthropod samples were calculated for each treatment and sampling time (soybean growth stage) combination, using PAST 3.12 software (Hammer et al., 2001). Diversity measures were subjected to a two-way repeated-measures analysis of variance (RM-ANOVA; $\alpha = 0.05$) to test the interaction of the effect of treatments and sampling time (PROC MIXED; SAS Institute, 2008). When the RM-ANOVA was significant for the interaction of soybean treatment with sampling time, treatment means were compared using Tukey's test ($\alpha = 0.05$). Prior to analysis, data were subjected to Shapiro-Wilk and Bartlett's tests ($\alpha = 0.05$) to check the assumptions of normal distribution and homogenous variance, respectively (PROC UNIVARIATE; SAS Institute, 2008). Data that violated the ANOVA assumptions of normality and homogeneity of variance were $\log(x+1)$ transformed. Non-transformed means are presented.

Selection of Representative Taxa and Effects on Non-Target Arthropod Communities

A faunistic analysis was used to select the most representative NTA. Faunistic analyses were performed according to Silveira Neto et al. (1976) with indices calculated using ANAFAU software (Moraes et al., 2003). The most representative NTA, defined as those with the highest faunistic indices, were selected based on a combination of their dominance, abundance, frequency and consistency in the population samples. Calculations were performed for treatments within each field trial. The classification of indices was performed based on the 95% confidence interval (CI) for the mean of the total individuals collected. Before analyses, the target lepidopteran pests of *Bt* soybean were excluded. When the abundance or frequency of a taxon was higher than the upper CI limit, the taxon was classified as super-dominant (SD), super-abundant (SA), super-frequent (SF) and constant

(W). If these values were within the CI limits, the taxon was classified as dominant (D), very abundant (VA) and very frequent (VF). The taxa with values below the lower CI limit were not selected by faunistic analyses and were classified as others. The proportion of total abundance for each taxon (number of individuals within the taxon divided by the total number of all arthropods enumerated) was also calculated.

A step-wise approach to data analysis was applied. First, the organisms selected in the faunistic analyses were used to describe components of the NTA community structure associated with soybean, and to test for effects due to the treatments by examining the Principal Response Curves (PRC). The PRC method incorporates redundancy analysis (RDA) of repeated observations via application of a direct gradient based on a linear distribution model (Van den Brink & Ter Braak, 1999). PRC diagrams represent the part of the variation of the community structure that is explained by the first canonical axis constructed in RDA. The weight (b_k) associated with each taxon can be interpreted as the affinity of the taxon with the Principal Response Curve (C_{dt}). Only organisms with b_k values greater than 0.5 or lower than -0.5 are shown in the diagrams because organisms with weights between these value are likely to show either a weak response or a response that is unrelated to that depicted in the constructed curves (Van den Brink & Ter Braak, 1999). The significance of the canonical axes produced via RDA was examined by Monte-Carlo permutation test (999 permutations; $\alpha = 0.05$). Identification of a significant first axis indicates that some variation captured in the population survey is likely partly explained by the treatments, where the control treatment (non-*Bt* soybean) partly accounts for the background effect of predominant factors that influence the agroecosystem. The RDA produces estimates of the proportion of total variance explained by the constrained factors, which for this study included time (soybean growth stage) and field treatments. Prior to RDA, the abundance of organisms was $\log(x+1)$ transformed and subjected to Detrended Correspondence Analysis (DCA) to test the fit of the distribution model. The distribution model is determined by gradient length that measures the diversity in the community composition along the individual independent gradients. According to Lepš & Šmilauer (2003), if the longest gradient value is larger than 4.0 the linear method would not be appropriate, while if the gradient length is less than 3.0 the linear method is a better choice. Resulting gradient lengths for the present data set were less than 3.0, indicating that application of the linear method was appropriate (S1 Table). Monte Carlo simulations, RDA and DCA were conducted using CANOCO 4.5 software (Ter Braak & Šmilauer, 2002).

Table S1. Gradient lengths via Detrended Correspondence Analysis of the most representative non-target arthropods collected by Moericke (yellow pan) and Pitfall traps in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site	Year	Sampling method	
		Moericke traps	Pitfall traps
Castro	2012	0.89 ^a	1.46
	2013	1.66	2.13
Montividiu	2011	1.86	2.76
	2012	1.38	1.78
	2013	1.42	2.93
Uberlândia	2011	0.82	1.33
	2012	1.45	1.62

^aGradient lengths shorter than 3.0 indicate appropriate application of linear response models in redundancy analysis (Lepš and Šmilauer (2003)).

When the first canonical axis was statistically significant, a two-way repeated-measures analysis of variance (RM-ANOVA; $\alpha = 0.05$) was used to test the interaction of the effect of soybean treatment and sampling time for taxa that contributed most to the community response (bk values greater than 0.5 or less -0.5) in the PRC diagram (PROC MIXED; SAS Institute, 2008). RM-ANOVA was followed by Tukey's test ($\alpha = 0.05$) to separate means, when necessary.

4.4. Results

4.4.1. Non-Target Arthropod Collections

Across all years and sites, a total of 254,054 individuals from a mean of 190 taxa were collected by Moericke traps; and 29,813 individuals from a mean of 100 taxa were collected using pitfall traps. The number of individuals collected using Moericke traps was similar among treatments, where the percentages of total individuals collected in each treatment were 33.68% non-sprayed non-*Bt* (control), 33.21% sprayed non-*Bt* and 33.11% in DAS-81419-2. The percentages of total individuals collected using pitfall traps were also similar across treatments, with 35.4, 32.5 and 32.0% in the non-sprayed non-*Bt*, sprayed non-*Bt* and DAS-81419-2 treatments, respectively (S2 and S3 Tables).

Table S2. Abundance and faunistic analysis results for the most representative non-target arthropods collected by Moericke traps (yellow pan) in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site (year)	Rank	Taxon	Functional group ¹	No. individuals			Total	Faunistic indices ³			
				Non- <i>Bt</i>	Non- <i>Bt</i> sprayed	DAS-81419-2		D	A	F	C
Castro (2012)	1	<i>Astylus variegatus</i>	PHY	3480	3247	6392	13119	SD	SA	SF	W
	2	<i>Frankliniella schultzei</i>	PHY	3834	3013	3373	10220	SD	SA	SF	W
	3	<i>Frankliniella occidentalis</i>	PHY	2756	2156	2006	6918	SD	SA	SF	W
	4	<i>Condylostylus</i> sp.	PRE	1547	1166	2896	5609	SD	SA	SF	W
	5	<i>Elachiptera</i> spp.	PHY	630	567	456	1653	D	VA	VF	W
	6	Drosophilidae spp.	DET	373	378	289	1040	D	VA	VF	W
	7	Chrysomelidae spp.	PHY	284	179	341	804	D	VA	VF	W
	8	Sarcophagidae spp.	PAR	232	191	186	609	D	VA	VF	W
	9	<i>Megaselia</i> sp.	OMN	142	129	159	430	D	VA	VF	W
	10	<i>Diabrotica speciosa</i>	PHY	118	47	108	273	D	VA	VF	W
	11	Cicadellidae sp.	PHY	32	64	50	146	D	VA	VF	W
	12	Tachinidae spp.	PAR	26	38	71	135	D	VA	VF	W
	13	<i>Lebia concinna</i>	PRE	36	26	64	126	D	VA	VF	W
	14	<i>Aphis gossypii</i>	PHY	37	30	44	111	D	VA	VF	W
	15	<i>Musca domestica</i>	DET	14	20	73	107	D	VA	VF	W
	Total			13541	11251	16508	41300				
	Others ⁴			397	344	511	1252				
	Total individuals			13938	11595	17019	42552				

		Total taxa		86	83	107	133				
Castro (2013)	1	<i>Condylostylus</i> spp.	PRE	658	468	583	1709	D	VA	VF	W
	2	Sacophagidae spp.	PAR	416	543	388	1347	D	VA	VF	W
	3	<i>Elachiptera</i> spp.	PHY	519	362	351	1232	D	VA	VF	W
	4	<i>Caliothrips</i> spp.	PHY	153	134	157	444	D	VA	VF	W
	5	<i>Frankliniella occidentalis</i>	PHY	166	133	117	416	D	VA	VF	W
	6	Aphididae spp.	PHY	89	146	179	414	D	VA	VF	W
	7	<i>Diabrotica speciosa</i>	PHY	137	127	123	387	D	VA	VF	W
	8	Chloropidae spp.	PHY	107	117	129	353	D	VA	VF	W
	9	<i>Pheidole</i> spp.	OMN	62	49	231	342	D	VA	VF	W
	10	Sciaridae sp.	PHY	94	134	89	317	D	VA	VF	W
	11	Drosophilidae spp.	DET	110	121	77	308	D	VA	VF	W
	12	Collembola	DET	58	45	64	167	D	VA	VF	W
	13	Empidoidea spp.	PRE	66	41	49	156	D	VA	VF	W
	14	Pteromalidae spp.	PAR	57	41	46	144	D	VA	VF	W
	15	<i>Astylus variegatus</i>	PHY	59	25	41	125	D	VA	VF	W
	16	Miridae sp.	OMN	23	44	23	90	D	VA	VF	W
	17	<i>Megaselia scalaris</i>	OMN	48	22	19	89	D	VA	VF	W
	18	Cicadellidae spp.	PHY	27	26	29	82	D	VA	VF	W
		Total		2849	2578	2695	8122				
		Others		460	551	365	1376				
	Total individuals		3309	3129	3060	9498					
		Total taxa		135	148	112	207				
Montividiu (2011)	1	<i>Condylostylus</i> spp.	PRE	2308	2637	2271	7216	SD	SA	SF	W
	2	<i>Astylus variegatus</i>	PHY	370	391	335	1096	D	VA	VF	W

	3	Sciaridae spp.	PHY	208	179	155	542	D	VA	VF	W
	4	Drosophilidae spp.	DET	113	157	144	414	D	VA	VF	W
	5	Aphididae spp.	PHY	62	149	138	349	D	VA	VF	W
	6	<i>Elachiptera</i> sp.	PHY	76	135	70	281	D	VA	VF	W
	7	<i>Coenosia</i> sp.	PRE	93	102	82	277	D	VA	VF	W
	8	Cicadellidae spp.	PHY	20	44	39	103	D	VA	VF	W
	9	Stratiomyidae sp.	PHY	29	22	32	83	D	VA	VF	W
	10	Fulgoroidea sp.	PHY	22	15	29	66	D	VA	VF	W
	11	Tachinidae spp.	PAR	27	14	24	65	D	VA	VF	W
		Total		3328	3845	3319	10492				
		Others ⁴		637	578	580	1795				
		Total individuals		3965	4423	3899	12287				
		Total taxa		97	109	99	150				
Montividiu (2012)	1	<i>Bemisia tabaci</i>	PHY	3403	3587	6643	13633	SD	SA	SF	W
	2	<i>Condyllostylus</i> spp.	PRE	1857	1736	2877	6470	SD	SA	SF	W
	3	<i>Elachiptera</i> spp.	PHY	764	576	264	1604	D	VA	VF	W
	4	Drosophilidae spp.	DET	298	216	122	636	D	VA	VF	W
	5	<i>Megaselia</i> sp.	OMN	147	80	221	448	D	VA	VF	W
	6	Sarcophagidae spp.	PAR	84	107	91	282	D	VA	VF	W
	7	Sciaridae spp.	PHY	42	115	21	178	D	VA	VF	W
	8	Cicadellidae sp.	PHY	54	35	64	153	D	VA	VF	W
	9	<i>Euxesta</i> spp.	PHY	48	60	41	149	D	VA	VF	W
	10	Stratiomyidae sp.	PHY	43	30	11	84	D	VA	VF	W
	11	Tachinidae spp.	PAR	31	31	19	81	D	VA	VF	W
	12	<i>Dorymyrmex brunneus</i>	PRE	9	31	40	80	D	VA	VF	W

	Total			6780	6604	10414	23798				
	Others			378	377	340	1095				
	Total individuals			7158	6981	10754	24893				
	Total taxa			90	93	80	132				
Montividiu (2013)	1	<i>Condylostylus</i> spp.	PRE	2762	3072	3557	9391	SD	SA	SF	W
	2	Chloropidae spp.	PHY	337	318	289	944	D	VA	VF	W
	3	Sciaridae sp.	PHY	164	177	244	585	D	VA	VF	W
	4	Sarcophagidae spp.	PAR	192	188	143	523	D	VA	VF	W
	5	Cicadellidae spp.	PHY	136	148	149	433	D	VA	VF	W
	6	Tachinidae spp.	PAR	92	108	101	301	D	VA	VF	W
	7	<i>Caliothrips</i> sp.	PHY	97	72	67	236	D	VA	VF	W
	8	Drosophilidae spp.	DET	72	52	69	193	D	VA	VF	W
	9	Halictidae sp.	OMN	59	53	66	178	D	VA	VF	W
	10	Aphididae	PHY	41	45	55	141	D	VA	VF	W
	11	Stratiomydae spp.	PHY	38	42	49	129	D	VA	VF	W
	12	Chrysomelidae spp.	PHY	46	34	36	116	D	VA	VF	W
	13	Psyllidae spp. (nymph)	PHY	19	59	17	95	D	VA	VF	W
	14	<i>Coenosia</i> sp.	PRE	24	29	22	75	D	VA	VF	W
	15	Mycetophilidae spp.	DET	24	24	23	71	D	VA	VF	W
	16	Araneae spp.	PRE	19	25	25	69	D	VA	VF	W
	17	Fulgoroidea spp.	PHY	8	22	29	59	D	VA	VF	W
	18	Syrphidae spp.	PRE	20	15	21	56	D	VA	VF	W
	19	<i>Euschistus heros</i>	PHY	21	21	13	55	D	VA	VF	W
	20	Psyllidae spp. (adults)	PHY	10	19	17	46	D	VA	VF	W
	21	Staphilinidae spp.	PRE	12	8	25	45	D	VA	VF	W

	Total			4193	4531	5017	13741				
	Others			1746	1617	1896	5259				
	Total individuals			5939	6148	6913	19000				
	Total taxa			141	132	147	218				
Uberlândia (2011)	1	<i>Elachiptera</i> sp.	PHY	1730	1841	871	4442	D	VA	VF	W
	2	<i>Condylostylus</i> sp.	PRE	440	398	1108	1946	D	VA	VF	W
	3	<i>Selenophorus</i> sp.	OMN	222	595	322	1139	D	VA	VF	W
	4	Drosophilidae sp.	DET	427	437	212	1076	D	VA	VF	W
	5	Cicadellidae spp.	PHY	214	168	541	923	D	VA	VF	W
	6	<i>Coenosia</i> sp.	PRE	175	160	115	450	D	VA	VF	W
	7	Tachinidae spp.	PAR	49	45	54	148	D	VA	VF	W
	Total			3257	3644	3223	10124				
	Others ⁴			979	1041	1289	3309				
	Total individuals			4236	4685	4512	13433				
	Total taxa			149	149	137	222				
Uberlândia (2012)	1	<i>Bemisia tabaci</i>	PHY	34312	28957	26873	90142	SD	SA	SF	W
	2	<i>Condylostylus</i> sp.	PRE	3300	4578	5061	12939	SD	SA	SF	W
	3	<i>Elachiptera</i> sp.	PHY	4951	7344	540	12835	SD	SA	SF	W
	4	Chloropidae sp.	PHY	588	2754	205	3547	D	VA	VF	W
	5	<i>Frankliniella schultzei</i>	PHY	249	704	564	1517	D	VA	VF	W
	6	Sarcophagidae spp.	PAR	457	157	379	993	D	VA	VF	W
	7	Cicadellidae spp.	PHY	224	147	539	910	D	VA	VF	W
	8	Tachinidae spp.	PAR	312	140	262	714	D	VA	VF	W
	9	Drosophilidae spp.	DET	277	261	73	611	D	VA	VF	W
	10	<i>Coenosia</i> sp.	PRE	64	49	310	423	D	VA	VF	W

11	<i>Dorymyrmex brunneus</i>	PRE	193	63	153	409	D	VA	VF	W
12	<i>Eumecosomyia</i> sp.	PHY	78	120	188	386	D	VA	VF	W
13	<i>Euxesta</i> spp.	PHY	80	116	111	307	D	VA	VF	W
14	<i>Megaselia</i> sp.	OMN	43	74	166	283	D	VA	VF	W
15	Periscelididae sp.	PHY	93	9	165	267	D	VA	VF	W
16	<i>Allograpta</i> sp.	PHY	77	13	158	248	D	VA	VF	W
17	<i>Frankliniella occidentalis</i>	PHY	35	76	98	209	D	VA	VF	W
18	<i>Aphis gossypii</i>	PHY	51	37	117	205	D	VA	VF	W
19	Halictidae sp.	OMN	70	43	87	200	D	VA	VF	W
20	<i>Coenosia</i> sp.	PRE	71	60	57	188	D	VA	VF	W
21	Sciaridae spp.	PHY	127	27	7	161	D	VA	VF	W
22	<i>Diabrotica speciosa</i>	PHY	53	42	49	144	D	VA	VF	W
23	<i>Carpophilus</i> sp.	PHY	41	59	34	134	D	VA	VF	W
24	Fulgoroidea sp.	PHY	40	20	60	120	D	VA	VF	W
25	<i>Selenophorus</i> sp.	OMN	79	26	12	117	D	VA	VF	W
26	<i>Musca domestica</i>	DET	21	21	71	113	D	VA	VF	W
27	Pompilidae sp.	PRE	54	26	27	107	D	VA	VF	W
	Total		45940	45923	36366	128229				
	Others		1081	1490	1591	4162				
	Total individuals		47021	47413	37957	132391				
	Total taxa		193	175	209	271				

¹Detritivorous (DET), parasitoid (PAR), phytophagous (PHY), predator (PRE) or omnivorous (OMN).

²Total number of samples = 36.

³Faunistic indices: D = dominance (SD = super-dominant and D = dominant); A = abundance (SA = super-abundant and VA = very abundant); F = frequency (SF = super-frequent and VF = very frequent) and C = constancy (W = constant) according to the ANAFAU software (Moraes et al., 2003).

⁴Non-target arthropods that were not classified by the faunistic analysis as belonging to one of the classes cited above were added to 'others' and were not included in the Principal Response Curve (PRC) analysis.

Table S3. Abundance and faunistic analysis results for the most representative non-target arthropods collected by Pitfall traps in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site (year)	Rank	Taxon	Functional group ¹	No. individuals			Total	Faunistic indices ³			
				Non- <i>Bt</i>	Non- <i>Bt</i> sprayed	DAS-81419-2		D	A	F	C
Castro (2012)	1	Collembola	DET	393	166	199	758	D	VA	VF	W
	2	<i>Pheidole</i> spp.	OMN	100	171	147	418	D	VA	VF	W
	3	<i>Caliothrips</i> sp.	PHY	142	118	106	366	D	VA	VF	W
	4	Mollusca	PHY	88	40	18	146	D	VA	VF	W
	5	Araneae	PRE	36	45	34	115	D	VA	VF	W
	6	<i>Elachiptera</i> sp.	PHY	18	38	29	85	D	VA	VF	W
	7	Sciaridae sp.	PHY	18	29	29	76	D	VA	VF	W
	8	Annelida	DET	17	29	29	75	D	VA	VF	W
	9	<i>Frankliniella schultzie</i>	PHY	12	36	11	59	D	VA	VF	W
	10	Drosophilidae sp.	DET	3	8	10	21	D	VA	VF	W
Total				827	680	612	2119				
Others ⁴				70	98	122	290				
Total individuals				897	778	734	2409				
Total taxa				48	48	53	75				
Castro (2013)	1	Collembola sp.	DET	314	212	277	803	SD	SA	SF	W
	2	<i>Pheidole</i> spp.	OMN	206	107	167	480	SD	SA	SF	W
	3	Araneae spp.	PRE	108	140	68	316	SD	SA	SF	W
	4	Agromyzidae sp.	PHY	55	31	45	131	D	VA	VF	W
	5	Carabidae spp.	PRE	29	27	15	71	D	VA	VF	W
	6	Sarcophagidae spp.	PAR	18	25	22	65	D	VA	VF	W
	7	Sciaridae sp.	PHY	14	16	11	41	D	VA	VF	W

	8	<i>Elachiptera</i> sp.	PHY	13	12	15	40	D	VA	VF	W
	9	Ponerinae sp.	PRE	7	6	24	37	D	VA	VF	W
	10	Phoridae sp.	OMN	19	11	5	35	D	VA	VF	W
	11	Staphylinidae spp.	DET	16	8	11	35	D	VA	VF	W
	12	<i>Frankliniella schultzei</i>	PHY	9	18	6	33	D	VA	VF	W
	13	Drosophilidae spp.	DET	8	7	8	23	D	VA	VF	W
	14	<i>Diabrotica speciosa</i>	PHY	9	6	7	22	D	VA	VF	W
	15	Aphididae	PHY	9	4	6	19	D	VA	VF	W
	16	Diplopoda spp.	PHY	6	7	4	17	D	VA	VF	W
	17	Elateridae sp.	PRE	7	6	4	17	D	VA	VF	W
	18	<i>Condylostylus</i> sp.	PRE	6	6	3	15	D	VA	VF	W
	19	Cicadellidae spp.	PHY	3	5	4	12	D	VA	VF	W
	20	Pteromalidae spp.	PAR	5	5	2	12	D	VA	VF	W
	Total			861	659	704	2224				
	Others			91	80	73	244				
	Total individuals			952	739	777	2468				
	Total taxa			73	72	73	127				
Montividiu (2011)	1	<i>Pheidole</i> spp.	OMN	109	59	37	205	D	VA	VF	W
	2	Sciaridae spp.	PHY	22	43	44	109	D	VA	VF	W
	3	<i>Euschistus</i> sp.	PHY	31	22	7	60	D	VA	VF	W
	4	Drosophilidae sp.	DET	40	17	1	58	D	VA	VF	W
	5	Chloropidae sp.	PHY	20	13	22	55	D	VA	VF	W
	6	Phoridae sp.	OMN	13	21	17	51	D	VA	VF	W
	7	Formicidae sp.	OMN	33	12	6	51	D	VA	VF	W
	8	<i>Canthon</i> sp.	PRE	15	25	7	47	D	VA	VF	W
	9	<i>Gryllus assimilis</i>	PHY	2	4	25	31	D	VA	VF	W

Montividiu (2012)	10	<i>Piezodorus guildinii</i>	PHY	13	7	6	26	D	VA	VF	W
		Total		298	223	172	693				
		Others ⁴		99	88	83	270				
		Total individuals		397	311	255	963				
		Total taxa		46	47	45	72				
	1	<i>Bemisia tabaci</i>	PHY	369	149	98	616	D	VA	VF	W
	2	Sciaridae sp.	PHY	200	187	127	514	D	VA	VF	W
	3	<i>Dorymyrmex brunneus</i>	PRE	59	77	69	205	D	VA	VF	W
	4	<i>Elachiptera</i> sp.	PHY	89	89	14	192	D	VA	VF	W
	5	<i>Euxesta</i> sp.	PHY	40	107	5	152	D	VA	VF	W
Montividiu (2013)	6	<i>Condylostylus</i> spp.	PRE	138	3	2	143	D	VA	VF	W
	7	<i>Canthon</i> sp.	PRE	40	69	3	112	D	VA	VF	W
	8	<i>Labidura xanthopus</i>	PRE	51	41	16	108	D	VA	VF	W
	9	<i>Anurogryllus muticus</i>	PHY	31	32	20	83	D	VA	VF	W
	10	<i>Pheidole</i> spp.	OMN	2	55	19	76	D	VA	VF	W
		Total		1019	809	373	2201				
		Others		275	206	126	607				
		Total individuals		1294	1015	499	2808				
		Total taxa		77	63	56	104				
	1	<i>Pheidole</i> spp.	OMN	27	14	19	460	D	VA	VF	W
Montividiu (2013)	2	Sciaridae spp.	PHY	77	89	67	233	D	VA	VF	W
	3	Araneae spp.	PRE	25	23	24	72	D	VA	VF	W
	4	<i>Dorymyrmex brunneus</i>	PRE	14	32	0	46	D	VA	VF	W
	5	<i>Elachiptera</i> sp.	PHY	11	9	13	33	D	VA	VF	W
	6	<i>Anurogryllus muticus</i>	PHY	6	9	7	22	D	VA	VF	W
		Total		160	176	130	466				

			Others	98	99	98	295				
			Total individuals	258	275	228	761				
			Total taxa	57	47	48	88				
Uberlândia (2011)	1	<i>Selenophorus</i> sp.	OMN	72	314	302	688	D	VA	VF	W
	2	Chloropidae sp.	PHY	190	110	129	429	D	VA	VF	W
	3	Drosophilidae spp.	DET	118	209	61	388	D	VA	VF	W
	4	<i>Dorymyrmex brunneus</i>	PRE	151	77	145	373	D	VA	VF	W
	5	<i>Pheidole</i> sp.	OMN	81	80	204	365	D	VA	VF	W
	6	<i>Elachiptera</i> sp.	PHY	102	96	63	261	D	VA	VF	W
	7	<i>Ladibura xantophus</i>	PRE	70	90	66	226	D	VA	VF	W
	8	<i>Galerita collaris</i>	PRE	39	71	18	128	D	VA	VF	W
	9	<i>Condylostylus</i> spp.	PRE	24	11	73	108	D	VA	VF	W
	10	Sciaridae spp.	PHY	33	31	28	92	D	VA	VF	W
	Total			880	1089	1089	3058				
			Others ⁴	167	259	273	699				
			Total individuals	1047	1348	1362	3757				
			Total taxa	66	68	67	97				
Uberlândia (2012)	1	Collembola	DET	3302	1542	2819	7663	SD	SA	SF	W
	2	<i>Bemisia tabaci</i>	PHY	88	1218	1085	2391	SD	SA	SF	W
	3	<i>Pheidole</i> spp.	OMN	999	389	367	1755	SD	SA	SF	W
	4	<i>Elachiptera</i> sp.	PHY	347	342	97	786	D	VA	VF	W
	5	<i>Labidura xanthopus</i>	PRE	206	190	180	576	D	VA	VF	W
	6	Sciaridae spp.	PHY	168	164	169	501	D	VA	VF	W
	7	<i>Dorymyrmex brunneus</i>	PRE	113	101	275	489	D	VA	VF	W
	8	Araneae	PRE	43	260	39	342	D	VA	VF	W

9	<i>Drosophilidae</i> spp.	DET	52	169	18	239	D	VA	VF	W
10	<i>Sarcophagidae</i> spp.	PAR	7	122	70	199	D	VA	VF	W
11	<i>Euxesta</i> sp.	PHY	34	100	43	177	D	VA	VF	W
12	<i>Anurogryllus muticus</i>	PHY	34	44	34	112	D	VA	VF	W
13	<i>Cicadellidae</i> sp.	PHY	6	54	38	98	D	VA	VF	W
14	Orthoptera (nymph)	PHY	39	28	27	94	D	VA	VF	W
15	<i>Selenophorus</i> sp.	OMN	25	42	24	91	D	VA	VF	W
16	<i>Dichroplus</i> sp.	PHY	15	30	15	60	D	VA	VF	W
Total			5478	4795	5300	15573				
Others			231	443	400	1074				
Total individuals			5709	5238	5700	16647				
Total taxa			88	91	99	144				

¹Detritivorous (DET), parasitoid (PAR), phytophagous (PHY), predator (PRE) or omnivorous (OMN).

²Total number of samples = 36.

³Faunistic indices: D = dominance (SD = super-dominant and D = dominant); A = abundance (SA = super-abundant and VA = very abundant); F = frequency (SF = super-frequent and VF = very frequent) and C = constancy (W = constant) according to the ANAFAU software (Moraes et al., 2003).

⁴Non-target arthropods that were not classified by the faunistic analysis as belonging to one of the classes cited above were added to 'others' and were not included in the Principal Response Curve (PRC) analysis.

4.4.2. Effects on Aerial-Dwelling Non-Target Arthropods

The faunistic analysis showed a variable number of taxa selected as the most representative for each site and year (S2 Table). Via Moericke trapping, the number of taxa selected at the Castro location in 2012 and 2013 were 15 and 18 taxa, respectively. In Montividiu, the number of taxa selected by faunistic analysis were 11, 12 and 21 taxa in 2011, 2012 and 2013, respectively. In Uberlândia, the number of taxa selected were 7 and 27 in 2011 and 2012, respectively. In general, the most abundant NTAs sampled using Moericke trapping were the herbivores *Astylus variegatus* (Germar, 1824) (Coleoptera: Melyridae), *Bemisia tabaci* (Gennadius, 1889) (Hemiptera: Aleyrodidae), *Elachiptera* sp. (Diptera: Chloropidae) and the predator *Condyllostylus* spp. (Diptera: Dolichopodidae).

Overall, the results of diversity analyses for aerial dwelling arthropods did not identify significant differences among treatments. However, significant differences were observed among sampling times, as soybean crop phenology progressed (Table 2). The abundance (N) of NTAs was higher in DAS-81419-2 plots than in the non-sprayed non-*Bt* and sprayed non-*Bt* at Castro 2012 mainly during reproductive stages (S4 Table) whereas richness (S) associated with DAS-81419-2 was similar to the control at the same location in 2013. Arthropod diversity associated with DAS-81419-2, measured by Shannon's index (H') was similar to the non-sprayed non-*Bt* and significantly higher than the sprayed non-*Bt* in Uberlândia 2011 (Table 2 and S4 Table).

Table 2. Two-way repeated-measures ANOVA results for abundance (N), richness (S), Shannon's diversity index (H'), Simpson's diversity index (D) and Pielou's evenness index (J) of non-target arthropods collected by Moericke traps (yellow pan) in non-Bt (with and without insecticides) and Bt (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site	Year	Diversity indices	Soybean treatments			Two-way ANOVA					
			Non-Bt	Non-Bt sprayed	DAS-81419-2	Soybean treat.		Sampling time (B)		Interaction (A x B)	
						(A)					
						F	P	F	P	F	P
Castro	2012	N	1161.50 ± 122.52 b	966.25 ± 126.53 c	1418.25 ± 165.52 a	39.78	0.002	155.06	< 0.001	8.58	< 0.001
		S	28.08 ± 1.47	26.83 ± 1.27	32.42 ± 1.40	5.29	0.075	5.93	0.032	0.83	0.572
		H'	1.86 ± 0.05	1.89 ± 0.08	1.83 ± 0.10	0.76	0.525	481.13	< 0.001	7.49	0.002
		D	0.77 ± 0.01	0.78 ± 0.02	0.74 ± 0.02	2.66	0.184	129.84	< 0.001	8.35	0.001
		J	0.24 ± 0.02 ab	0.26 ± 0.02 a	0.20 ± 0.02 b	13.76	0.016	36.89	< 0.001	3.56	0.029
	2013	N	220.60 ± 25.07	208.60 ± 15.73	204.00 ± 22.47	0.61	0.588	6.38	0.013	0.23	0.980
		S	29.40 ± 1.77 ab	31.33 ± 1.78 a	25.93 ± 1.50 b	8.85	0.034	14.14	0.001	0.97	0.491
		H'	2.40 ± 0.05	2.49 ± 0.10	2.31 ± 0.06	4.34	0.099	7.91	0.007	2.03	0.109
		D	0.83 ± 0.01	0.84 ± 0.02	0.83 ± 0.01	0.55	0.614	6.62	0.012	1.78	0.156
		J	0.41 ± 0.04	0.41 ± 0.02	0.42 ± 0.03	0.02	0.979	5.18	0.023	0.23	0.980
Montividiu	2011	N	330.42 ± 76.83	368.58 ± 77.27	324.92 ± 61.71	1.59	0.311	84.23	< 0.001	4.55	0.012
		S	24.17 ± 1.35	24.17 ± 1.29	25.25 ± 1.22	0.44	0.674	18.17	0.002	0.58	0.737
		H'	1.74 ± 0.17	1.62 ± 0.10	1.71 ± 0.11	2.07	0.242	21.57	0.001	2.59	0.076
		D	0.67 ± 0.05	0.64 ± 0.03	0.64 ± 0.03	0.77	0.523	10.91	0.008	2.73	0.066
		J	0.26 ± 0.03	0.22 ± 0.02	0.23 ± 0.02	1.65	0.299	17.85	0.002	3.20	0.041
	2012	N	596.50 ± 227.99	581.75 ± 219.17	896.17 ± 441.55	0.35	0.723	83.34	< 0.001	2.34	0.099
		S	24.67 ± 2.57	24.25 ± 3.04	20.25 ± 2.35	4.94	0.083	10.72	0.008	1.64	0.219
		H'	1.67 ± 0.13	1.68 ± 0.14	1.59 ± 0.17	1.98	0.252	64.04	< 0.001	0.73	0.637
		D	0.67 ± 0.04	0.69 ± 0.05	0.65 ± 0.05	1.28	0.372	40.45	< 0.001	1.19	0.372
		J	0.26 ± 0.05	0.28 ± 0.05	0.32 ± 0.06	1.75	0.285	70.43	< 0.001	1.95	0.153
	2013	N	395.93 ± 81.60	409.87 ± 85.60	460.87 ± 85.34	0.96	0.458	43.43	< 0.001	0.92	0.524

		S	28.80 ± 2.53	29.27 ± 2.60	31.73 ± 2.32	4.86	0.085	60.57	< 0.001	4.89	0.003
		H'	1.72 ± 0.10	1.63 ± 0.15	1.62 ± 0.13	0.65	0.570	16.04	< 0.001	1.50	0.233
		D	0.63 ± 0.04	0.58 ± 0.05	0.57 ± 0.04	1.29	0.370	17.20	< 0.001	1.75	0.161
		J	0.23 ± 0.04	0.21 ± 0.04	0.19 ± 0.04	0.98	0.448	43.77	< 0.001	1.62	0.197
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		N	353.00 ± 53.59	390.42 ± 69.60	376.00 ± 61.37	0.20	0.826	29.37	< 0.001	5.18	0.008
		S	39.17 ± 2.38	39.33 ± 2.87	45.08 ± 4.77	2.94	0.164	21.59	0.001	5.91	0.004
	2011	H'	2.34 ± 0.06 ab	2.33 ± 0.13 b	2.51 ± 0.05 a	8.99	0.033	16.64	0.003	3.23	0.040
		D	0.80 ± 0.02	0.79 ± 0.02	0.83 ± 0.01	4.94	0.083	19.79	0.002	3.66	0.026
		J	0.29 ± 0.03	0.29 ± 0.04	0.31 ± 0.03	0.59	0.594	29.71	< 0.001	9.38	< 0.001
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Uberlândia		N	3918.50 ± 1045.77	3951.08 ± 858.45	3163.08 ± 950.32	0.99	0.446	39.60	< 0.001	5.76	0.005
		S	60.92 ± 3.50	54.42 ± 5.06	61.83 ± 4.42	0.77	0.520	68.16	< 0.001	2.36	0.096
	2012	H'	1.33 ± 0.18	1.33 ± 0.13	1.62 ± 0.20	5.35	0.074	58.33	< 0.001	3.38	0.035
		D	0.50 ± 0.07	0.56 ± 0.05	0.58 ± 0.07	6.43	0.056	53.29	< 0.001	1.82	0.178
		J	0.08 ± 0.02	0.10 ± 0.03	0.10 ± 0.02	5.39	0.073	59.40	< 0.001	5.93	0.004

P-values highlighted in bold are statistically significant ($\alpha = 0.05$).

Degrees of freedom: soybean treatments = 2; sampling time = 3 (2011 and 2012) and 4 (2013); interaction = 6 (2011 and 2012) and 8 (2013); residual = 12 (2011 and 2012) and 16 (2013).

Means ($\pm$ SE) followed by different letters were significantly different within rows (Tukey's test, $\alpha = 0.05$).

Abundance was $\log(x + 1)$ transformed prior to analysis. Non-transformed means are presented.

Table S4. Diversity index values for non-target arthropods collected by Moericke traps (yellow pan) in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site (year)	Diversity indices ¹	Soybean treatments	Sampling time (soybean growth stages)				
			V1	V4 – V5	R2	R4 – R5	R7 – R8
Castro (2012)	N	Non- <i>Bt</i>	-	1007.00 ± 33.20 a	1783.00 ± 65.28	1139.67 ± 123.47 b	716.33 ± 66.83 b
		Non- <i>Bt</i> sprayed	-	581.67 ± 22.30 b	1618.00 ± 18.77	1028.67 ± 54.82 b	636.67 ± 70.17 b
		DAS-81419-2	-	641.00 ± 7.23 b	1996.00 ± 226.89	1765.00 ± 49.90 a	1271.00 ± 85.94 a
	S	Non- <i>Bt</i>	-	32.33 ± 4.67	28.67 ± 1.76	26.67 ± 0.88	24.67 ± 2.40
		Non- <i>Bt</i> sprayed	-	30.00 ± 1.53	24.67 ± 1.67	30.00 ± 0.58	22.67 ± 2.96
		DAS-81419-2	-	37.67 ± 1.33	28.33 ± 1.45	33.00 ± 1.15	30.67 ± 3.76
	H'	Non- <i>Bt</i>	-	2.00 ± 0.10 b	1.61 ± 0.01	1.84 ± 0.01 a	1.98 ± 0.06
		Non- <i>Bt</i> sprayed	-	2.22 ± 0.03 a	1.48 ± 0.02	1.91 ± 0.03 a	1.93 ± 0.09
		DAS-81419-2	-	2.31 ± 0.05 a	1.55 ± 0.08	1.53 ± 0.05 b	1.94 ± 0.04
	D	Non- <i>Bt</i>	-	0.79 ± 0.02	0.71 ± 0.01	0.79 ± 0.01 a	0.81 ± 0.01
		Non- <i>Bt</i> sprayed	-	0.84 ± 0.01	0.67 ± 0.00	0.79 ± 0.01 a	0.80 ± 0.02
		DAS-81419-2	-	0.83 ± 0.02	0.68 ± 0.04	0.66 ± 0.03 b	0.79 ± 0.01
	J	Non- <i>Bt</i>	-	0.24 ± 0.02 b	0.17 ± 0.01	0.24 ± 0.01 a	0.30 ± 0.02 a
		Non- <i>Bt</i> sprayed	-	0.31 ± 0.02 a	0.18 ± 0.01	0.23 ± 0.01 a	0.31 ± 0.02 a
		DAS-81419-2	-	0.27 ± 0.00 ab	0.17 ± 0.02	0.14 ± 0.01 b	0.23 ± 0.02 b
Castro (2013)	N	Non- <i>Bt</i>	188.67 ± 68.79	157.33 ± 22.41	337.67 ± 26.26	219.67 ± 3.53	199.67 ± 89.24
		Non- <i>Bt</i> sprayed	224.33 ± 46.94	133.33 ± 26.67	264.33 ± 26.18	225.00 ± 11.02	196.00 ± 28.38
		DAS-81419-2	242.67 ± 47.61	123.00 ± 26.91	300.00 ± 13.01	182.33 ± 25.89	172.00 ± 75.29
	S	Non- <i>Bt</i>	29.33 ± 3.71	25.33 ± 2.19	37.67 ± 3.38	26.00 ± 2.08	28.67 ± 5.90
		Non- <i>Bt</i> sprayed	40.67 ± 1.76	25.00 ± 3.06	30.67 ± 0.67	25.33 ± 1.20	35.00 ± 4.00
		DAS-81419-2	26.00 ± 3.06	20.33 ± 3.53	30.33 ± 2.03	24.33 ± 2.03	28.67 ± 4.84
	H'	Non- <i>Bt</i>	2.55 ± 0.19	2.40 ± 0.08	2.41 ± 0.05	2.26 ± 0.07	2.41 ± 0.16
		Non- <i>Bt</i> sprayed	2.95 ± 0.11	2.46 ± 0.12	2.45 ± 0.05	1.89 ± 0.07	2.73 ± 0.09
		DAS-81419-2	2.32 ± 0.20	2.22 ± 0.08	2.34 ± 0.14	2.20 ± 0.15	2.46 ± 0.11

Montividiu (2011)	D	Non- <i>Bt</i>	0.86 ± 0.03	0.83 ± 0.01	0.83 ± 0.01	0.83 ± 0.01	0.83 ± 0.05
		Non- <i>Bt</i> sprayed	0.91 ± 0.01	0.85 ± 0.02	0.85 ± 0.02	0.71 ± 0.02	0.88 ± 0.01
		DAS-81419-2	0.83 ± 0.05	0.82 ± 0.00	0.83 ± 0.03	0.80 ± 0.03	0.85 ± 0.03
	J	Non- <i>Bt</i>	0.46 ± 0.07	0.44 ± 0.03	0.30 ± 0.02	0.37 ± 0.01	0.48 ± 0.21
		Non- <i>Bt</i> sprayed	0.47 ± 0.03	0.48 ± 0.06	0.38 ± 0.01	0.26 ± 0.02	0.44 ± 0.01
		DAS-81419-2	0.42 ± 0.09	0.47 ± 0.05	0.35 ± 0.03	0.38 ± 0.06	0.46 ± 0.15
	N	Non- <i>Bt</i>	-	266.67 ± 46.26	147.33 ± 11.89 b	207.67 ± 10.11	700.00 ± 12.29
		Non- <i>Bt</i> sprayed	-	296.00 ± 16.50	220.33 ± 51.60 ab	216.67 ± 10.74	741.33 ± 24.17
		DAS-81419-2	-	225.33 ± 23.68	297.33 ± 12.81 a	168.33 ± 14.71	608.67 ± 62.33
	S	Non- <i>Bt</i>	-	25.67 ± 2.73	27.33 ± 0.88	21.67 ± 2.19	22.00 ± 2.31
		Non- <i>Bt</i> sprayed	-	26.33 ± 0.88	23.33 ± 0.88	22.33 ± 1.86	24.67 ± 4.26
		DAS-81419-2	-	27.00 ± 1.00	29.33 ± 0.33	21.33 ± 1.76	23.33 ± 0.88
	H'	Non- <i>Bt</i>	-	1.96 ± 0.26	2.33 ± 0.06	1.49 ± 0.03	1.19 ± 0.09
		Non- <i>Bt</i> sprayed	-	1.81 ± 0.09	1.84 ± 0.19	1.60 ± 0.04	1.23 ± 0.05
		DAS-81419-2	-	2.12 ± 0.06	1.75 ± 0.13	1.62 ± 0.10	1.35 ± 0.04
	D	Non- <i>Bt</i>	-	0.73 ± 0.09	0.82 ± 0.01	0.60 ± 0.02	0.51 ± 0.04
		Non- <i>Bt</i> sprayed	-	0.67 ± 0.04	0.72 ± 0.06	0.63 ± 0.02	0.53 ± 0.02
		DAS-81419-2	-	0.74 ± 0.02	0.62 ± 0.06	0.63 ± 0.04	0.57 ± 0.02
	J	Non- <i>Bt</i>	-	0.30 ± 0.07	0.38 ± 0.01 a	0.21 ± 0.02	0.15 ± 0.01
		Non- <i>Bt</i> sprayed	-	0.24 ± 0.02	0.28 ± 0.04 b	0.22 ± 0.01	0.14 ± 0.02
		DAS-81419-2	-	0.31 ± 0.01	0.20 ± 0.02 c	0.24 ± 0.01	0.17 ± 0.01
Montividiu (2012)	N	Non- <i>Bt</i>	-	280.00 ± 94.17	174.00 ± 41.51	1481.33 ± 547.31	450.67 ± 63.56
		Non- <i>Bt</i> sprayed	-	185.67 ± 36.29	180.67 ± 13.17	1590.67 ± 312.86	370.00 ± 33.15
		DAS-81419-2	-	118.33 ± 20.51	83.33 ± 8.19	2940.67 ± 618.31	442.33 ± 28.17
	S	Non- <i>Bt</i>	-	24.67 ± 2.96	15.33 ± 0.88	26.00 ± 4.73	32.67 ± 0.88
		Non- <i>Bt</i> sprayed	-	19.00 ± 3.46	17.00 ± 3.00	35.00 ± 5.29	26.00 ± 2.00
		DAS-81419-2	-	18.33 ± 2.33	11.00 ± 0.58	25.33 ± 1.33	26.33 ± 2.73
	H'	Non- <i>Bt</i>	-	2.05 ± 0.17	1.69 ± 0.11	1.15 ± 0.07	1.78 ± 0.12
		Non- <i>Bt</i> sprayed	-	2.08 ± 0.16	1.72 ± 0.07	1.07 ± 0.04	1.86 ± 0.15
		DAS-81419-2	-	2.12 ± 0.08	1.74 ± 0.05	0.83 ± 0.05	1.67 ± 0.02

Montividiu (2013)	D	Non- <i>Bt</i>	-	0.79 ± 0.04	0.73 ± 0.06	0.52 ± 0.03	0.66 ± 0.07
		Non- <i>Bt</i> sprayed	-	0.82 ± 0.03	0.72 ± 0.00	0.47 ± 0.03	0.72 ± 0.03
		DAS-81419-2	-	0.81 ± 0.01	0.76 ± 0.02	0.42 ± 0.02	0.63 ± 0.02
	J	Non- <i>Bt</i>	-	0.34 ± 0.10	0.36 ± 0.06	0.14 ± 0.04	0.18 ± 0.02
		Non- <i>Bt</i> sprayed	-	0.44 ± 0.03	0.34 ± 0.03	0.09 ± 0.02	0.25 ± 0.02
		DAS-81419-2	-	0.47 ± 0.04	0.53 ± 0.05	0.09 ± 0.00	0.21 ± 0.02
	N	Non- <i>Bt</i>	83.00 ± 5.00	199.00 ± 40.20	589.33 ± 60.08	393.33 ± 56.70	715.00 ± 53.95
		Non- <i>Bt</i> sprayed	121.33 ± 35.38	238.67 ± 55.95	483.67 ± 85.23	429.00 ± 25.74	776.67 ± 159.99
		DAS-81419-2	85.33 ± 8.41	361.33 ± 44.85	598.67 ± 78.12	470.67 ± 69.86	788.33 ± 107.98
	S	Non- <i>Bt</i>	20.00 ± 1.73	24.33 ± 2.03 b	28.00 ± 2.89 a	30.67 ± 0.33	41.00 ± 1.73
		Non- <i>Bt</i> sprayed	23.00 ± 1.00	28.67 ± 1.20 ab	21.33 ± 2.73 b	32.00 ± 3.46	41.33 ± 1.20
		DAS-81419-2	22.00 ± 1.15	33.33 ± 1.20 a	29.33 ± 2.33 a	32.33 ± 1.20	41.67 ± 3.18
	H'	Non- <i>Bt</i>	2.12 ± 0.02	1.84 ± 0.06	1.36 ± 0.05	1.83 ± 0.06	1.44 ± 0.14
		Non- <i>Bt</i> sprayed	1.92 ± 0.34	1.97 ± 0.17	1.05 ± 0.10	1.77 ± 0.07	1.43 ± 0.19
		DAS-81419-2	2.23 ± 0.04	1.75 ± 0.10	1.21 ± 0.03	1.50 ± 0.23	1.40 ± 0.12
	D	Non- <i>Bt</i>	0.77 ± 0.01	0.69 ± 0.02	0.52 ± 0.02	0.66 ± 0.01	0.49 ± 0.05
		Non- <i>Bt</i> sprayed	0.67 ± 0.12	0.69 ± 0.06	0.40 ± 0.04	0.65 ± 0.02	0.50 ± 0.07
		DAS-81419-2	0.79 ± 0.02	0.60 ± 0.04	0.45 ± 0.01	0.53 ± 0.09	0.49 ± 0.05
	J	Non- <i>Bt</i>	0.42 ± 0.03	0.27 ± 0.04	0.14 ± 0.01	0.20 ± 0.01	0.10 ± 0.01
		Non- <i>Bt</i> sprayed	0.33 ± 0.10	0.26 ± 0.05	0.14 ± 0.03	0.19 ± 0.01	0.11 ± 0.02
		DAS-81419-2	0.43 ± 0.04	0.17 ± 0.02	0.12 ± 0.01	0.14 ± 0.03	0.10 ± 0.02
Uberlândia (2011)	N	Non- <i>Bt</i>	-	262.67 ± 79.64 ab	612.67 ± 83.10	274.00 ± 60.21	262.67 ± 34.09 b
		Non- <i>Bt</i> sprayed	-	344.67 ± 16.80 a	731.00 ± 129.95	169.67 ± 5.24	316.33 ± 54.57 b
		DAS-81419-2	-	161.00 ± 29.60 b	562.33 ± 50.45	203.00 ± 16.50	577.67 ± 56.58 a
	S	Non- <i>Bt</i>	-	36.67 ± 5.24	45.00 ± 5.03	39.00 ± 6.03	36.00 ± 3.06 c
		Non- <i>Bt</i> sprayed	-	30.33 ± 2.03	45.33 ± 2.85	31.00 ± 0.58	50.67 ± 3.38 b
		DAS-81419-2	-	26.33 ± 0.67	54.33 ± 4.91	35.00 ± 2.52	64.67 ± 2.73 a
	H'	Non- <i>Bt</i>	-	2.35 ± 0.08 a	2.09 ± 0.04 b	2.35 ± 0.08	2.58 ± 0.10
		Non- <i>Bt</i> sprayed	-	1.93 ± 0.14 b	1.98 ± 0.09 b	2.68 ± 0.01	2.73 ± 0.28
		DAS-81419-2	-	2.40 ± 0.08 a	2.49 ± 0.10 a	2.66 ± 0.09	2.50 ± 0.10

Uberlândia (2012)	D	Non- <i>Bt</i>	-	0.81 ± 0.02 ab	0.72 ± 0.02 b	0.81 ± 0.03	0.86 ± 0.01
		Non- <i>Bt</i> sprayed	-	0.73 ± 0.03 b	0.72 ± 0.02 b	0.88 ± 0.00	0.83 ± 0.05
		DAS-81419-2	-	0.85 ± 0.02 a	0.82 ± 0.02 a	0.88 ± 0.01	0.79 ± 0.03
	J	Non- <i>Bt</i>	-	0.30 ± 0.03 b	0.18 ± 0.02	0.29 ± 0.06 b	0.37 ± 0.03 a
		Non- <i>Bt</i> sprayed	-	0.23 ± 0.02 b	0.16 ± 0.01	0.47 ± 0.00 a	0.32 ± 0.07 a
		DAS-81419-2	-	0.42 ± 0.03 a	0.22 ± 0.01	0.41 ± 0.01 a	0.19 ± 0.01 b
	N	Non- <i>Bt</i>	-	1289.00 ± 202.33	1902.67 ± 282.14 b	6960.67 ± 1856.06	5521.67 ± 3005.98 ab
		Non- <i>Bt</i> sprayed	-	468.00 ± 48.42	4484.33 ± 1391.63 a	3610.33 ± 1008.11	7241.67 ± 1240.49 a
		DAS-81419-2	-	1002.33 ± 338.68	1266.33 ± 146.03 b	8081.33 ± 1710.17	2302.33 ± 403.19 b
	S	Non- <i>Bt</i>	-	48.67 ± 4.81	65.33 ± 9.74	63.67 ± 5.04	66.00 ± 4.93
		Non- <i>Bt</i> sprayed	-	29.00 ± 4.51	52.00 ± 3.46	67.33 ± 1.33	69.33 ± 2.73
		DAS-81419-2	-	44.33 ± 6.44	64.33 ± 6.44	59.67 ± 1.76	79.00 ± 6.03
	H'	Non- <i>Bt</i>	-	2.09 ± 0.21	1.43 ± 0.15 ab	0.56 ± 0.13	1.24 ± 0.27 b
		Non- <i>Bt</i> sprayed	-	1.96 ± 0.17	1.03 ± 0.15 b	1.11 ± 0.21	1.20 ± 0.08 b
		DAS-81419-2	-	2.14 ± 0.24	1.90 ± 0.22 a	0.55 ± 0.03	1.90 ± 0.04 a
	D	Non- <i>Bt</i>	-	0.76 ± 0.06	0.53 ± 0.06	0.18 ± 0.05	0.51 ± 0.11
		Non- <i>Bt</i> sprayed	-	0.75 ± 0.03	0.48 ± 0.07	0.43 ± 0.11	0.55 ± 0.02
		DAS-81419-2	-	0.76 ± 0.07	0.65 ± 0.05	0.20 ± 0.03	0.71 ± 0.01
	J	Non- <i>Bt</i>	-	0.17 ± 0.03 b	0.07 ± 0.01 b	0.03 ± 0.00	0.06 ± 0.02
		Non- <i>Bt</i> sprayed	-	0.25 ± 0.03 a	0.06 ± 0.01 b	0.05 ± 0.01	0.05 ± 0.01
		DAS-81419-2	-	0.20 ± 0.02 b	0.11 ± 0.01 a	0.03 ± 0.00	0.09 ± 0.01

¹Diversity indices: abundance (N), richness (S), Shannon's diversity index (H'), Simpson's diversity index (D) and Pielou's evenness index (J).

Means (± SE) of each site/year within sampling time followed by different letters are significantly different (Tukey's test, $\alpha = 0.05$).

Dashes indicate no data.

The redundancy analysis (RDA) results for the most representative NTAs collected by Moericke trapping in Castro (2012) was significant for the first canonical axis ($F = 6.49$; $P = 0.001$) (S5 Table, Fig 1A). The first axis explained 37.5% of the variation of the aerial NTA community sampled. Of all the variance, 56.3% was attributed to the sampling time and 8.8% to the soybean treatments (S5 Table). Abundance of several taxa was higher in DAS-81419-2 plots compared with the non-sprayed non-*Bt* and sprayed non-*Bt* treatments as crop phenology progressed (Fig 1A). In 2013, no significant patterns were observed across treatments at Castro (first axis: $F = 1.36$; $P = 0.994$) (S5 Table, Fig 1B).

In Montividiu during 2011 and 2012 the first axis of RDA analysis was not significant (2011: $F = 3.09$; $P = 0.082$; 2012: $F = 4.71$; $P = 0.091$) (S5 Table, Figs 1C and 1D). The Principal Response Curve (PRC) analysis identified changes in the aerial NTA community structure where 67.3% of the variation was explained by sampling time and 5.8% by soybean treatment (S5 Table). The sprayed non-*Bt* and DAS-81419-2 treatments presented different canonical coefficients (C_{dt}) over sampling periods, where taxa in the sprayed non-*Bt* treatment showed a negative response and the taxa response in the DAS-81419-2 treatment was positive compared with the non-sprayed non-*Bt* treatment. In 2013, the first axis ($F = 3.09$; $P = 0.161$) was not significant (S5 Table, Fig 1E).

In Uberlândia, during 2011, the RDA results indicated a significant first axis ($F = 9.97$; $P = 0.001$) (S5 Table, Fig 1F). The first axis explained 62.3% of the variation of the aerial NTA community structure, wherein 59.3% was explained by sampling time and 10.9% by soybean treatment (S5 Table). In this case, the PRC response for DAS-81419-2 was positive over the sampling time whereas the sprayed non-*Bt* was negative compared with the non-sprayed non-*Bt* treatment. The NTAs that followed the observed trends were Cicadellidae spp. and *Condylostylus* sp., whereas *Coenosia* sp. (Diptera: Muscidae), Drosophilidae sp. (Diptera), *Elachiptera* sp. and *Selenophorus* sp. showed negative weights and responded oppositely to the trends (Fig 1F). In 2012, the first axis was not significant ($F = 4.11$; $P = 0.084$) (S5 Table, Fig 1G). We observed higher abundance for most of NTA in the DAS-81419-2 plots except for some Diptera (*Elachiptera* sp., Chloropidae sp., Drosophilidae spp.), and the carabid beetle, *Selenophorus* sp. (Fig 1G).

Table S5. Summary of the redundancy analysis (RDA) of abundance of the most representative non-target arthropods collected by Moericke traps (yellow pan) in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site (year)		Axes				Total variance
		First	Second	Third	Fourth	
Castro (2012)	Eigenvalues	0.093	0.053	0.038	0.027	1.000
	Species-environment correlations	0.968	0.842	0.827	0.806	
	Cumulative percentage variance					
	of species data	21.3	33.5	42.3	48.5	
	of species-environment relation	37.5	58.9	74.4	85.3	
	Sum of all eigenvalues					0.436
	Sum of all canonical eigenvalues					0.248
	----- Partition of variability (%) -----					
	Sampling time	56.3				
	Soybean treatments	8.8				
	Permutation test for canonical axes ¹	F-ratio	P-value			
	First axis	6.49	0.001			
	All axes	3.95	0.001			

Castro (2013)	Eigenvalues	0.016	0.014	0.010	0.007	1.000
	Species-environment correlations	0.673	0.536	0.533	0.477	
	Cumulative percentage variance					
	of species data	4.3	8.1	10.8	12.7	
	of species-environment relation	24.3	45.2	60.6	71.0	
	Sum of all eigenvalues					0.370
	Sum of all canonical eigenvalues					0.066
	----- Partition of variability (%) -----					
	Sampling time	63.0				
	Soybean treatments	1.3				
	Permutation test for canonical axes	F-ratio	P-value			
	First axis	1.36	0.994			

Montividiu (2011)	All axes	0.65	0.981			
	Eigenvalues	0.021	0.013	0.010	0.007	1.000
	Species-environment correlations	0.756	0.668	0.623	0.582	
	Cumulative percentage variance					
	of species data	11.4	18.1	23.2	27.2	
	of species-environment relation	37.2	59.0	75.5	88.5	
	Sum of all eigenvalues					0.187
	Sum of all canonical eigenvalues					0.058
	Partition of variability (%)					
	Sampling time	81.3				
	Soybean treatments	1.5				
	Permutation test for canonical axes ¹	F-ratio	P-value			
	First axis	3.09	0.082			
	All axes	1.33	0.057			
Montividiu (2012)	Eigenvalues	0.054	0.027	0.015	0.010	1.000
	Species-environment correlations	0.698	0.750	0.615	0.706	
	Cumulative percentage variance					
	of species data	16.4	24.6	29.3	32.2	
	of species-environment relation	45.5	68.2	81.2	89.5	
	Sum of all eigenvalues					0.327
	Sum of all canonical eigenvalues					0.118
	Partition of variability (%)					
	Sampling time	67.3				
	Soybean treatments	5.8				
Montividiu (2013)	Permutation test for canonical axes	F-ratio	P-value			
	First axis	4.71	0.091			
	All axes	1.69	0.020			
	Eigenvalues	0.025	0.013	0.011	0.010	
	Species-environment correlations	0.766	0.822	0.754	0.675	
	Cumulative percentage variance					

Uberlândia (2011)	of species data	9.3	14.4	18.6	22.4	
	of species-environment relation	31.0	47.8	61.8	74.5	
	Sum of all eigenvalues					0.263
	Sum of all canonical eigenvalues					0.079

	Partition of variability (%)					
	Sampling time	73.7				
	Soybean treatments	1.0				

	Permutation test for canonical axes	F-ratio	P-value			
	First axis	3.09	0.161			
	All axes	1.29	0.056			

	Eigenvalues	0.120	0.045	0.014	0.008	1.000
	Species-environment correlations	0.833	0.772	0.634	0.538	
	Cumulative percentage variance					
	of species data	29.4	40.5	43.9	45.9	
	of species-environment relation	62.3	85.9	93.1	97.4	
	Sum of all eigenvalues					0.407
	Sum of all canonical eigenvalues					0.192
Uberlândia (2012)	-----					
	Partition of variability (%)					
	Sampling time	59.3				
	Soybean treatments	10.9				

	Permutation test for canonical axes ¹	F-ratio	P-value			
	First axis	9.97	0.001			
	All axes	2.67	0.003			

	Eigenvalues	0.070	0.052	0.029	0.019	1.000
	Species-environment correlations	0.860	0.780	0.740	0.827	
	Cumulative percentage variance					
	of species data	14.6	25.5	31.6	35.4	
	of species-environment relation	34.4	60.0	74.3	83.4	
	Sum of all eigenvalues					0.481
	Sum of all canonical eigenvalues					0.204

Partition of variability (%)		
Sampling time	51.9	
Soybean treatments	8.4	
Permutation test for canonical axes	F-ratio	<i>P</i> -value
First axis	4.11	0.084
All axes	2.22	0.005

¹Permutation test by 999 Monte Carlo permutations ($\alpha = 0.05$).

P-values highlighted in bold are statistically significant.

The abundance of non-target arthropods was $\log(x + 1)$ transformed before analysis.

Fig. 1. Principal Response Curves (PRC) indicating the effect of non-Bt soybean with insecticides sprayed and Bt soybean (DAS-81419-2) on the most representative non-target arthropods collected by Moericke traps (yellow pan) at three sites over two to three years in Brazil. Dotted line indicates the community response to the control treatment (non-Bt without insecticides). The non-target arthropods weight (b_k) can be interpreted as the affinity of the taxon with the Principal Response Curves (C_{dt}). Only taxa with b_k values greater than 0.5 and less than -0.5 are shown in the diagram. Following Monte Carlo permutation tests, P -values less than 0.05 indicate a statistically significant difference in community response between at least one of the treatments and the control. Down arrows ($\downarrow$) indicate the time of insecticide application in the sprayed non-Bt treatment.

The first axis was significant only for the field trials at Castro in 2012 and Uberlândia in 2011. Applying the step-wise data analysis for these trials, we investigated the effect of soybean treatments and sampling times (two-way RM-ANOVA) for NTA that contributed the most to the community response (weights greater than 0.5 or less -0.5). For Castro in 2012, the abundance of *A. variegatus* was higher in DAS-81419-2 plots compared with the other treatments. The abundance of *Condylostylus* sp. and *Lebia concinna* Brullé, 1838 (Coleoptera: Carabidae) were also higher in the DAS-81419-2 treatment compared with the sprayed non-*Bt* treatment. Only the abundances of *Musca domestica* (Linnaeus, 1758) (Diptera: Muscidae), and other Diptera (Tachinidae sp., *Megaselia* sp.) were not influenced by sampling time (Table 3). Significant interactions were observed for soybean treatment and sampling time for *A. variegatus*, *M. domestica*, Tachinidae sp., *Condylostylus* sp., *Diabrotica speciosa* (Germar, 1824) (Coleoptera: Chrysomelidae), *Megaselia* sp. and *L. concinna* (Table 3, Figs 2A - 2G).

In Uberlândia in 2011, the abundances of *Condylostylus* sp. and Cicadellidae spp. (Hemiptera) were higher in the DAS-81419-2 treatment, whereas the abundance of *Elachiptera* sp. was lower compared to the other treatments. In this trial, only the abundances of Cicadellidae spp. and *Selenophorus* sp. were not influenced by sampling time (Table 3). Significant interactions between soybean treatment and sampling time were observed for *Condylostylus* sp., Cicadellidae spp. and Drosophilidae sp. (Table 3, Figs 2H - 2J), where abundances in DAS-81419-2 plots were also generally similar to or greater than those of non-*Bt* soybean.

Table 3. Two-way repeated-measures ANOVA results for abundance of non-target arthropods with values weights greater than 0.5 and less -0.5 in the Principal Response Curves (PRC) analysis with first axis significant collected by Moericke traps (yellow pan) in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at two sites over two to three years in Brazil.

Site (year)	Non-target arthropods	Soybean treatments			Two-way ANOVA					
		Non- <i>Bt</i>	Non- <i>Bt</i> sprayed	DAS-81419-2	Soybean treat. (A)		Sampling time (B)		Interaction (A x B)	
					<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Castro (2012)	<i>Astylus variegatus</i>	290.00 ± 99.45 b	270.58 ± 97.21 b	532.67 ± 140.85 a	12.58	0.019	114.25	< 0.001	7.58	0.002
	<i>Musca domestica</i>	1.17 ± 0.55	1.67 ± 0.61	6.08 ± 1.82	6.97	0.050	2.84	0.128	5.21	0.007
	Tachinidae sp.	2.17 ± 0.83	3.17 ± 1.01	5.92 ± 1.54	6.17	0.060	1.99	0.217	4.81	0.010
	<i>Condylostylus</i> sp.	128.92 ± 11.15 ab	97.17 ± 10.18 b	241.33 ± 39.95 a	10.57	0.025	45.83	< 0.001	20.28	< 0.001
	Chrysomelidae spp.	23.67 ± 6.16	14.92 ± 3.50	28.41 ± 7.19	5.54	0.070	65.00	< 0.001	2.78	0.062
	<i>Diabrotica speciosa</i>	9.83 ± 2.73	3.92 ± 1.04	9.00 ± 2.60	3.43	0.135	4.93	0.047	4.15	0.017
	<i>Megaselia</i> sp.	11.83 ± 2.50	10.75 ± 2.22	13.25 ± 1.63	0.26	0.780	3.89	0.074	3.95	0.020
	<i>Lebia concinna</i>	3.00 ± 0.89 ab	2.17 ± 0.56 b	5.33 ± 1.46 a	7.92	0.041	13.48	0.004	5.63	0.005
Uberlândia (2011)	<i>Condylostylus</i> sp.	36.67 ± 5.82 b	33.17 ± 7.41 b	92.33 ± 23.36 a	27.02	0.005	96.81	< 0.001	18.37	< 0.001
	Cicadellidae spp.	17.83 ± 4.41 b	14.00 ± 2.79 b	45.08 ± 12.88 a	26.05	0.005	3.93	0.073	5.95	0.004
	<i>Coenosia</i> sp.	14.58 ± 2.79	13.33 ± 2.72	9.58 ± 2.57	3.79	0.119	12.77	0.005	2.24	0.111
	Drosophilidae sp.	35.58 ± 8.13	36.42 ± 7.77	17.67 ± 3.35	4.69	0.089	19.63	0.002	3.48	0.031
	<i>Selenophorus</i> sp.	18.50 ± 7.57	49.58 ± 17.24	26.83 ± 18.46	0.89	0.477	4.08	0.067	0.92	0.516
	<i>Elachiptera</i> sp.	144.17 ± 32.61 a	153.42 ± 28.69 a	72.58 ± 18.69 b	53.59	0.001	47.01	< 0.001	2.08	0.132

P-values highlighted in bold are statistically significant ($\alpha = 0.05$).

Degrees of freedom: soybean fields = 2; sampling time = 3; interaction = 6; residual = 12.

Means ($\pm$ SE) followed by different letters were significantly different within rows (Tukey's test, $\alpha = 0.05$).

Fig. 2. Population dynamics for non-target arthropod groups collected by Moericke traps (yellow pan) in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields. Means ($\pm$ SE) within sampling time followed by different letters are significantly different (Tukey's test, $\alpha = 0.05$). Taxa shown include those with taxon weights greater than 0.5 or less -0.5 which were associated with statistically significant Monte Carlo tests. Down arrows ($\downarrow$) indicate the time of insecticide application in the sprayed non-*Bt* treatment.

4.4.3. Effects on Surface-Dwelling Non-Target Arthropods

Via pitfall trapping in the Castro trials, the number of representative taxa selected by faunistic analysis was 10 in 2012 and 20 in 2013 (S3 Table). In Montividiu, the number of taxa selected was 10 in 2011 and 2012, and 6 in 2013. In the locality of Uberlândia, the number of taxa selected was 10 in 2011 and 16 in 2012. In general, the most abundant surface-dwelling NTAs collected by pitfall traps were detritivores (*Collembola*), omnivores (*Pheidole* spp. [Hymenoptera: Formicidae] and *Selenophorus* sp. [Coleoptera: Carabidae]), and herbivores (*B. tabaci*).

Similar to the results from the aerial dwelling arthropod survey, diversity analyses for surface dwelling arthropods did not identify significant differences among treatments; but significant differences were observed among sampling times (Table 4). Shannon's diversity index (H'), Simpson's diversity index (D), and Pielou's evenness index (J) measures for surface-dwelling arthropods were higher for the DAS-81419-2 treatment than the non-sprayed non-*Bt* treatment at Castro in 2012 and Montividiu in 2011. For other measures, abundance (N) and richness (S) were lower in the DAS-81419-2 treatment than the non-sprayed non-*Bt* treatment for Montividiu in 2012 (Table 4). In addition, the abundance and diversity of surface-dwelling arthropods varied with sampling dates, but in few cases was there an interaction between sampling dates and soybean treatments (S6 Table). Simpson's diversity index (D) values for arthropods in these cases were higher in the DAS-81419-2 treatment than in the non-sprayed non-*Bt* treatment, mainly during the reproductive stages R4-R5 and R7-R8 at Castro in 2012. Similarly, DAS-81419-2 plots were associated with higher richness (S) than non-sprayed non-*Bt* plots during the reproductive stages R2 and R4-R5 at Uberlândia in 2011 (S6 Table). Thus, considering all growing seasons (years) and sites, the community of surface-dwelling insects was similar across treatments and, in some cases, DAS-81419-2 supported a higher abundance and diversity than the non-*Bt* treatments (Table 4 and S6 Table).

Table 4. Two-way repeated-measures ANOVA results for abundance (N), richness (S), Shannon's diversity index (H'), Simpson's diversity index (D) and Pielou's evenness index (J) of non-target arthropods collected by Pitfall traps in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site	Year	Diversity indices	Soybean treatments			Two-way ANOVA					
			Non- <i>Bt</i>	Non- <i>Bt</i> sprayed	DAS-81419-2	Soybean treat. (A)		Sampling time (B)		Interaction (A x B)	
						<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Castro	2012	N	74.75 ± 15.05	64.83 ± 10.59	61.17 ± 9.91	0.23	0.809	87.09	< 0.001	0.88	0.537
		S	11.33 ± 1.14	12.67 ± 0.92	14.00 ± 1.50	4.41	0.097	5.57	0.036	0.37	0.884
		H'	1.63 ± 0.10 b	1.90 ± 0.07 a	2.00 ± 0.09 a	34.27	0.003	3.42	0.093	2.23	0.112
		D	0.71 ± 0.03 b	0.77 ± 0.02 a	0.80 ± 0.02 a	18.70	0.009	8.18	0.015	4.89	0.009
		J	0.52 ± 0.06	0.55 ± 0.02	0.57 ± 0.03	0.71	0.544	15.23	0.003	3.39	0.034
	2013	N	63.47 ± 16.17	49.27 ± 12.77	51.80 ± 9.88	0.54	0.616	18.69	< 0.001	0.33	0.945
		S	13.93 ± 1.42	12.93 ± 1.07	12.13 ± 1.09	1.03	0.440	4.12	0.042	0.37	0.924
		H'	1.91 ± 0.12	2.05 ± 0.12	1.80 ± 0.08	1.39	0.346	2.73	0.106	1.38	0.276
		D	0.75 ± 0.04	0.81 ± 0.03	0.74 ± 0.03	0.60	0.594	3.53	0.061	1.37	0.282
		J	0.59 ± 0.06	0.69 ± 0.06	0.59 ± 0.06	0.76	0.525	13.82	0.001	0.79	0.616
Montividiu	2011	N	33.08 ± 4.57	25.92 ± 5.96	21.25 ± 4.61	3.13	0.152	4.14	0.066	0.74	0.627
		S	9.92 ± 0.76	10.17 ± 0.98	7.83 ± 1.21	4.27	0.102	3.57	0.086	0.73	0.637
		H'	1.83 ± 0.09	1.97 ± 0.12	1.69 ± 0.16	3.61	0.127	1.38	0.337	1.88	0.166
		D	0.77 ± 0.03	0.81 ± 0.03	0.75 ± 0.05	2.31	0.215	0.99	0.459	2.60	0.075
		J	0.66 ± 0.05 b	0.76 ± 0.06 a	0.78 ± 0.04 a	17.61	0.010	4.15	0.065	1.96	0.151
	2012	N	107.83 ± 45.82 a	84.58 ± 18.29 ab	41.58 ± 7.85 b	8.91	0.034	12.34	0.006	0.36	0.891
		S	17.25 ± 1.39 a	16.50 ± 1.54 a	11.75 ± 1.17 b	17.55	0.010	22.43	0.001	0.75	0.621
		H'	2.21 ± 0.15	2.11 ± 0.09	1.93 ± 0.13	2.08	0.241	8.59	0.014	1.11	0.412
		D	0.82 ± 0.03	0.81 ± 0.02	0.79 ± 0.02	0.36	0.719	4.54	0.055	0.74	0.625
		J	0.58 ± 0.16	0.54 ± 0.06	0.63 ± 0.05	1.18	0.395	11.02	0.007	0.10	0.995
	2013	N	17.20 ± 3.96	18.33 ± 4.51	15.20 ± 4.20	0.57	0.603	8.11	0.006	0.53	0.813
		S	7.87 ± 1.06	7.67 ± 0.89	7.27 ± 0.85	0.22	0.812	3.38	0.067	0.47	0.863
		H'	1.71 ± 0.15	1.71 ± 0.13	1.67 ± 0.10	0.14	0.871	4.35	0.037	0.451	0.873
		D	0.75 ± 0.04	0.76 ± 0.03	0.75 ± 0.03	0.21	0.818	5.83	0.017	0.45	0.872
		J	0.80 ± 0.07	0.79 ± 0.05	0.80 ± 0.06	0.04	0.965	9.55	0.004	0.36	0.927

Uberlândia	2011	N	87.25 ± 11.06	112.33 ± 28.21	113.50 ± 18.97		0.69	0.551		15.17	0.003		1.02	0.458
		S	17.25 ± 1.14	18.75 ± 1.40	19.83 ± 1.51		4.30	0.101		1.86	0.238		6.74	0.003
		H'	2.18 ± 0.09	2.24 ± 0.05	2.17 ± 0.13		0.37	0.712		0.03	0.994		2.47	0.086
		D	0.82 ± 0.02	0.84 ± 0.01	0.80 ± 0.03		1.44	0.339		0.03	0.993		0.96	0.492
		J	0.53 ± 0.03	0.53 ± 0.04	0.48 ± 0.05		0.72	0.543		2.35	0.172		0.691	0.662
	2012	N	475.75 ± 79.51	436.50 ± 73.25	475.00 ± 125.25		0.48	0.650		67.13	< 0.001		1.76	0.191
		S	21.58 ± 1.36	23.67 ± 1.78	23.58 ± 2.06		1.51	0.325		1.59	0.286		1.68	0.208
		H'	1.44 ± 0.16 b	1.86 ± 0.12 a	1.60 ± 0.15 ab		12.79	0.018		27.48	< 0.001		2.43	0.090
		D	0.57 ± 0.06 b	0.71 ± 0.04 a	0.61 ± 0.06 ab		8.24	0.038		34.97	< 0.001		2.84	0.059
		J	0.22 ± 0.03	0.30 ± 0.03	0.25 ± 0.04		3.88	0.116		16.14	0.003		1.62	0.225

P-values highlighted in bold are statistically significant ($\alpha = 0.05$).

Degrees of freedom: soybean treatments = 2; sampling time = 3 (2011 and 2012) and 4 (2013); interaction = 6 (2011 and 2012) and 8 (2013); residual = 12 (2011 and 2012) and 16 (2013).

Means ($\pm$ SE) followed by different letters were significantly different within rows (Tukey's test, $\alpha = 0.05$).

Abundance was $\log(x + 1)$ transformed prior to analysis. Non-transformed means are presented.

Table S6. Diversity index values for non-target arthropods collected by Pitfall traps in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site (year)	Diversity indices ¹	Soybean treatments	Sampling time (soybean growth stages)				
			V1	V4 – V5	R2	R4 – R5	R7 – R8
Castro (2012)	N	Non- <i>Bt</i>	-	28.67 ± 7.51	31.00 ± 10.60	122.67 ± 20.95	116.67 ± 17.80
		Non- <i>Bt</i> sprayed	-	35.00 ± 4.04	30.00 ± 4.04	104.67 ± 6.64	89.67 ± 15.17
		DAS-81419-2	-	30.00 ± 8.19	38.33 ± 1.45	95.00 ± 16.64	81.33 ± 16.97
	S	Non- <i>Bt</i>	-	10.33 ± 1.20	8.67 ± 3.18	14.00 ± 2.31	12.33 ± 1.86
		Non- <i>Bt</i> sprayed	-	11.33 ± 0.88	9.00 ± 0.00	16.33 ± 1.45	14.00 ± 0.58
		DAS-81419-2	-	10.00 ± 1.15	11.00 ± 1.00	17.33 ± 2.60	17.67 ± 3.93
	H'	Non- <i>Bt</i>	-	1.95 ± 0.04	1.65 ± 0.20	1.63 ± 0.16	1.31 ± 0.19
		Non- <i>Bt</i> sprayed	-	1.87 ± 0.02	1.61 ± 0.14	2.18 ± 0.03	1.96 ± 0.08
		DAS-81419-2	-	1.91 ± 0.12	1.76 ± 0.04	2.15 ± 0.15	2.18 ± 0.24
	D	Non- <i>Bt</i>	-	0.81 ± 0.01	0.74 ± 0.04	0.72 ± 0.03 b	0.56 ± 0.05 b
		Non- <i>Bt</i> sprayed	-	0.76 ± 0.01	0.68 ± 0.06	0.84 ± 0.00 a	0.78 ± 0.02 a
		DAS-81419-2	-	0.80 ± 0.04	0.73 ± 0.00	0.83 ± 0.02 a	0.82 ± 0.04 a
	J	Non- <i>Bt</i>	-	0.70 ± 0.07	0.69 ± 0.11	0.38 ± 0.04	0.31 ± 0.02 b
		Non- <i>Bt</i> sprayed	-	0.58 ± 0.03	0.57 ± 0.08	0.55 ± 0.04	0.51 ± 0.05 a
		DAS-81419-2	-	0.70 ± 0.12	0.53 ± 0.03	0.51 ± 0.03	0.53 ± 0.03 a
Castro (2013)	N	Non- <i>Bt</i>	97.00 ± 13.32	137.33 ± 64.24	18.00 ± 6.24	33.33 ± 9.26	31.67 ± 8.11
		Non- <i>Bt</i> sprayed	117.33 ± 48.89	60.67 ± 12.55	14.00 ± 1.00	31.33 ± 6.01	23.00 ± 1.15
		DAS-81419-2	77.33 ± 17.98	99.00 ± 14.93	16.00 ± 5.13	39.33 ± 17.89	27.33 ± 17.37
	S	Non- <i>Bt</i>	18.00 ± 2.00	17.33 ± 3.76	10.33 ± 3.38	12.33 ± 3.33	11.67 ± 3.28
		Non- <i>Bt</i> sprayed	13.00 ± 4.16	16.33 ± 2.60	9.67 ± 1.20	11.00 ± 1.53	14.67 ± 0.67
		DAS-81419-2	15.33 ± 2.03	16.00 ± 1.73	8.33 ± 2.40	12.00 ± 1.73	9.00 ± 2.08
	H'	Non- <i>Bt</i>	1.68 ± 0.12	1.88 ± 0.15	2.14 ± 0.29	2.04 ± 0.29	1.80 ± 0.47
		Non- <i>Bt</i> sprayed	1.26 ± 0.14	2.27 ± 0.05	2.18 ± 0.13	2.09 ± 0.19	2.47 ± 0.03
		DAS-81419-2	1.83 ± 0.20	1.72 ± 0.23	1.89 ± 0.29	1.88 ± 0.22	1.67 ± 0.10
	D	Non- <i>Bt</i>	0.65 ± 0.07	0.75 ± 0.04	0.86 ± 0.03	0.80 ± 0.07	0.69 ± 0.16

Montividiu (2011)		Non- <i>Bt</i> sprayed	0.58 ± 0.05	0.85 ± 0.01	0.88 ± 0.02	0.84 ± 0.03	0.89 ± 0.01
		DAS-81419-2	0.73 ± 0.04	0.69 ± 0.09	0.81 ± 0.05	0.74 ± 0.11	0.73 ± 0.07
	J	Non- <i>Bt</i>	0.32 ± 0.07	0.42 ± 0.09	0.91 ± 0.02	0.69 ± 0.11	0.59 ± 0.13
		Non- <i>Bt</i> sprayed	0.34 ± 0.10	0.62 ± 0.09	0.93 ± 0.01	0.75 ± 0.04	0.81 ± 0.05
		DAS-81419-2	0.42 ± 0.05	0.37 ± 0.08	0.86 ± 0.04	0.62 ± 0.18	0.68 ± 0.18
	N	Non- <i>Bt</i>	-	45.00 ± 8.50	23.00 ± 1.73	22.33 ± 0.67	42.00 ± 7.02
		Non- <i>Bt</i> sprayed	-	24.67 ± 3.76	12.67 ± 4.81	29.33 ± 6.84	37.00 ± 18.56
		DAS-81419-2	-	28.33 ± 8.09	10.33 ± 2.40	18.67 ± 7.88	27.67 ± 10.37
	S	Non- <i>Bt</i>	-	12.33 ± 0.88	7.67 ± 0.33	8.67 ± 0.88	11.00 ± 1.00
		Non- <i>Bt</i> sprayed	-	12.00 ± 0.58	7.33 ± 1.86	10.00 ± 1.53	11.33 ± 1.86
		DAS-81419-2	-	10.67 ± 2.60	6.67 ± 0.67	4.67 ± 1.67	9.33 ± 1.86
	H'	Non- <i>Bt</i>	-	1.94 ± 0.24	1.76 ± 0.11	1.90 ± 0.11	1.71 ± 0.13
		Non- <i>Bt</i> sprayed	-	2.23 ± 0.10	1.85 ± 0.23	1.79 ± 0.31	2.00 ± 0.11
		DAS-81419-2	-	1.96 ± 0.24	1.79 ± 0.08	1.12 ± 0.34	1.91 ± 0.15
	D	Non- <i>Bt</i>	-	0.76 ± 0.08	0.79 ± 0.03	0.82 ± 0.02	0.72 ± 0.05
		Non- <i>Bt</i> sprayed	-	0.86 ± 0.02	0.82 ± 0.04	0.73 ± 0.11	0.80 ± 0.04
		DAS-81419-2	-	0.81 ± 0.05	0.81 ± 0.01	0.57 ± 0.13	0.81 ± 0.02
	J	Non- <i>Bt</i>	-	0.59 ± 0.11	0.76 ± 0.05	0.78 ± 0.02	0.51 ± 0.02
		Non- <i>Bt</i> sprayed	-	0.78 ± 0.06	0.92 ± 0.01	0.64 ± 0.11	0.69 ± 0.11
		DAS-81419-2	-	0.71 ± 0.06	0.90 ± 0.03	0.75 ± 0.10	0.75 ± 0.03
Montividiu (2012)	N	Non- <i>Bt</i>	-	48.33 ± 10.41	47.33 ± 4.81	245.67 ± 145.44	90.00 ± 2.08
		Non- <i>Bt</i> sprayed	-	58.67 ± 16.60	38.00 ± 16.17	121.33 ± 9.91	120.33 ± 46.25
		DAS-81419-2	-	37.00 ± 5.20	18.00 ± 4.51	59.67 ± 19.22	51.67 ± 9.74
	S	Non- <i>Bt</i>	-	13.67 ± 0.33	18.00 ± 0.58	16.00 ± 2.00	21.33 ± 3.38
		Non- <i>Bt</i> sprayed	-	16.00 ± 2.08	14.00 ± 2.00	15.33 ± 3.33	20.67 ± 2.60
		DAS-81419-2	-	14.00 ± 1.00	8.33 ± 1.76	9.67 ± 0.33	15.00 ± 1.53
	H'	Non- <i>Bt</i>	-	1.91 ± 0.13	2.58 ± 0.08	1.80 ± 0.29	2.55 ± 0.16
		Non- <i>Bt</i> sprayed	-	2.09 ± 0.12	2.22 ± 0.06	1.79 ± 0.21	2.35 ± 0.06
		DAS-81419-2	-	2.08 ± 0.15	1.86 ± 0.23	1.58 ± 0.14	2.21 ± 0.23
	D	Non- <i>Bt</i>	-	0.74 ± 0.05	0.90 ± 0.01	0.73 ± 0.09	0.89 ± 0.02

Montividiu (2013)	J	Non- <i>Bt</i> sprayed	-	0.80 ± 0.03	0.85 ± 0.02	0.74 ± 0.05	0.85 ± 0.02
		DAS-81419-2	-	0.79 ± 0.04	0.80 ± 0.05	0.73 ± 0.04	0.84 ± 0.04
		Non- <i>Bt</i>	-	0.51 ± 0.08	0.73 ± 0.04	0.44 ± 0.15	0.62 ± 0.03
		Non- <i>Bt</i> sprayed	-	0.53 ± 0.10	0.70 ± 0.12	0.41 ± 0.04	0.52 ± 0.07
		DAS-81419-2	-	0.58 ± 0.04	0.81 ± 0.02	0.52 ± 0.10	0.62 ± 0.09
		Non- <i>Bt</i>	7.00 ± 2.31	13.33 ± 3.84	13.33 ± 4.37	17.00 ± 8.74	35.33 ± 1.86
	N	Non- <i>Bt</i> sprayed	12.00 ± 2.00	7.67 ± 1.20	14.67 ± 1.67	20.33 ± 4.70	37.00 ± 12.90
		DAS-81419-2	11.00 ± 1.15	8.00 ± 1.53	11.33 ± 1.45	16.67 ± 9.67	29.00 ± 12.53
		Non- <i>Bt</i>	4.67 ± 0.88	7.67 ± 0.33	9.00 ± 1.00	8.00 ± 2.89	10.00 ± 2.65
	S	Non- <i>Bt</i> sprayed	5.33 ± 1.20	5.00 ± 0.58	10.00 ± 0.00	8.33 ± 1.45	9.67 ± 1.67
		DAS-81419-2	6.67 ± 1.20	6.33 ± 0.88	7.67 ± 1.20	7.33 ± 2.85	8.33 ± 1.86
		Non- <i>Bt</i>	1.44 ± 0.19	1.83 ± 0.14	2.02 ± 0.03	1.70 ± 0.38	1.55 ± 0.45
	H'	Non- <i>Bt</i> sprayed	1.42 ± 0.27	1.44 ± 0.11	2.16 ± 0.05	1.84 ± 0.24	1.67 ± 0.19
		DAS-81419-2	1.68 ± 0.15	1.76 ± 0.12	1.91 ± 0.19	1.66 ± 0.26	1.34 ± 0.08
		Non- <i>Bt</i>	0.74 ± 0.04	0.80 ± 0.06	0.84 ± 0.02	0.75 ± 0.09	0.62 ± 0.14
	D	Non- <i>Bt</i> sprayed	0.70 ± 0.08	0.72 ± 0.03	0.86 ± 0.01	0.79 ± 0.07	0.72 ± 0.06
		DAS-81419-2	0.77 ± 0.03	0.81 ± 0.02	0.83 ± 0.04	0.77 ± 0.04	0.59 ± 0.03
		Non- <i>Bt</i>	0.94 ± 0.04	0.84 ± 0.12	0.86 ± 0.09	0.82 ± 0.09	0.54 ± 0.13
	J	Non- <i>Bt</i> sprayed	0.83 ± 0.02	0.86 ± 0.01	0.87 ± 0.04	0.79 ± 0.08	0.60 ± 0.15
		DAS-81419-2	0.83 ± 0.03	0.94 ± 0.02	0.90 ± 0.04	0.84 ± 0.08	0.49 ± 0.08
		Non- <i>Bt</i>	-	86.00 ± 11.53	136.00 ± 15.31	70.00 ± 21.78	57.00 ± 6.81
Uberlândia (2011)	N	Non- <i>Bt</i> sprayed	-	87.33 ± 23.56	253.00 ± 55.43	45.00 ± 8.66	64.00 ± 10.82
		DAS-81419-2	-	89.67 ± 26.42	191.00 ± 47.09	87.00 ± 26.85	86.33 ± 15.90
		Non- <i>Bt</i>	-	21.00 ± 2.00 a	18.00 ± 1.00 b	12.00 ± 1.00 b	18.00 ± 1.15
	S	Non- <i>Bt</i> sprayed	-	17.33 ± 3.33 ab	25.00 ± 1.00 a	15.67 ± 2.03 b	17.00 ± 0.00
		DAS-81419-2	-	15.67 ± 3.18 b	24.67 ± 1.45 a	21.00 ± 3.79 a	18.00 ± 1.00
		Non- <i>Bt</i>	-	2.42 ± 0.14	2.11 ± 0.13	1.85 ± 0.11	2.33 ± 0.11
	H'	Non- <i>Bt</i> sprayed	-	2.20 ± 0.10	2.15 ± 0.12	2.30 ± 0.12	2.30 ± 0.10
		DAS-81419-2	-	1.92 ± 0.13	2.37 ± 0.20	2.37 ± 0.47	2.03 ± 0.11
		Non- <i>Bt</i>	-	0.86 ± 0.02	0.82 ± 0.04	0.77 ± 0.03	0.85 ± 0.02
	D	Non- <i>Bt</i>	-	0.86 ± 0.02	0.82 ± 0.04	0.77 ± 0.03	0.85 ± 0.02

Uberlândia (2012)		Non- <i>Bt</i> sprayed	-	0.84 ± 0.02	0.80 ± 0.03	0.86 ± 0.02	0.85 ± 0.02
		DAS-81419-2	-	0.77 ± 0.04	0.83 ± 0.04	0.81 ± 0.11	0.78 ± 0.03
	J	Non- <i>Bt</i>	-	0.55 ± 0.06	0.47 ± 0.07	0.54 ± 0.07	0.58 ± 0.04
		Non- <i>Bt</i> sprayed	-	0.54 ± 0.04	0.35 ± 0.05	0.65 ± 0.05	0.59 ± 0.06
		DAS-81419-2	-	0.48 ± 0.12	0.45 ± 0.09	0.57 ± 0.14	0.43 ± 0.07
	N	Non- <i>Bt</i>	-	848.33 ± 168.50	431.67 ± 56.53	353.33 ± 78.83	269.67 ± 42.94
		Non- <i>Bt</i> sprayed	-	747.00 ± 160.82	480.00 ± 73.58	321.33 ± 43.85	197.67 ± 16.60
		DAS-81419-2	-	1098.00 ± 266.07	362.67 ± 55.92	183.67 ± 49.10	255.67 ± 33.15
	S	Non- <i>Bt</i>	-	18.00 ± 1.00	21.67 ± 3.18	24.00 ± 1.00	22.67 ± 4.33
		Non- <i>Bt</i> sprayed	-	32.00 ± 3.79	19.33 ± 1.76	22.33 ± 1.86	21.00 ± 0.58
		DAS-81419-2	-	28.67 ± 6.49	24.33 ± 4.06	21.33 ± 2.03	20.00 ± 2.89
	H'	Non- <i>Bt</i>	-	0.74 ± 0.13	1.45 ± 0.13	1.91 ± 0.27	1.68 ± 0.19
		Non- <i>Bt</i> sprayed	-	1.87 ± 0.18	1.34 ± 0.07	2.03 ± 0.22	2.20 ± 0.09
		DAS-81419-2	-	1.02 ± 0.27	1.49 ± 0.17	2.09 ± 0.10	1.80 ± 0.25
	D	Non- <i>Bt</i>	-	0.29 ± 0.05	0.61 ± 0.05	0.73 ± 0.10	0.64 ± 0.07
		Non- <i>Bt</i> sprayed	-	0.71 ± 0.07	0.55 ± 0.03	0.77 ± 0.08	0.81 ± 0.03
		DAS-81419-2	-	0.37 ± 0.10	0.56 ± 0.04	0.80 ± 0.03	0.72 ± 0.06
	J	Non- <i>Bt</i>	-	0.12 ± 0.02	0.21 ± 0.03	0.30 ± 0.06	0.26 ± 0.06
		Non- <i>Bt</i> sprayed	-	0.21 ± 0.04	0.20 ± 0.03	0.35 ± 0.05	0.43 ± 0.03
		DAS-81419-2	-	0.10 ± 0.01	0.19 ± 0.02	0.39 ± 0.05	0.32 ± 0.06

¹Diversity indices: abundance (N), richness (S), Shannon's diversity index (H'), Simpson's diversity index (D) and Pielou's evenness index (J).

Means (± SE) of each site/year within sampling time followed by different letters are significantly different (Tukey's test, $\alpha = 0.05$).

Dashes indicate no data.

The redundancy analysis (RDA) of NTA pitfall trap data collected in Castro, followed by Monte Carlo tests, did not identify significant differences for either of the years in the study: 2012 (first axis: $F = 3.63$; $P = 0.052$) and 2013 (first axis: $F = 1.98$; $P = 0.878$) (S7 Table, Figs 3A - 3B). These results indicated that the soybean treatments did not affect non-target surface-dwelling groups within or across sampling time.

In Montividiu, the first axis was not significant in 2011 ($F = 3.40$; $P = 0.264$) (S7 Table, Fig 3C). Phorid and chloropid species (Diptera) tended to decrease in abundance over time, whereas *Euschistus* sp. (Hemiptera: Pentatomidae) and *Pheidole* spp. tended to increase (Fig 3C). In 2012, the first axis ($F = 5.77$; $P = 0.001$) was significant (S7 Table, Fig 3D). The first axis explained 47.9% of the variation of the surface-dwelling NTA community structure. Wherein 41.0% was explained by sampling time and 10.1% by soybean treatments (S7 Table). The PRC curves for sprayed non-*Bt* and DAS-81419-2 treatments were similar until the R4-R5 stages. In the R7-R8 stages, the DAS-81419-2 treatment presented a canonical coefficient higher than sprayed non-*Bt* treatment. Only the species *Dorymyrmex brunneus* (Forel, 1908) (Hymenoptera: Formicidae) did not follow the pattern of PRC curves (negative weight) of sprayed non-*Bt* and DAS-81419-2 treatments compared to the control (Fig 3D). In 2013, none of the axes were significant (first axis: $F = 2.7$; $P = 0.763$; all axes: $F = 0.97$; $P = 0.547$) (S7 Table, Fig 3E) indicating that the soybean treatments did not affect the surface-dwelling NTA community structure.

The analysis for the 2011 Uberlândia trial revealed significance only for first axis (first axis: $F = 3.52$; $P = 0.025$) (S7 Table, Fig 3F). During that year, the first axis explained 41.3% of the variation of the surface-dwelling NTA community structure, where 48.9% was explained by sampling time and 5.0% by soybean treatments (S7 Table). The PRC diagram revealed a late season increase in the abundance of *Condylostylus* sp. and *Pheidole* sp. in DAS-81419-2 plots and lower or variable abundances for *D. brunneus*, *Selenophorus* sp., *Elachiptera* sp. and Drosophilidae sp. in *Bt* and sprayed non-*Bt* treatments (Fig 3F). In 2012, the first axis was not significant ($F = 7.59$; $P = 0.080$) (S7 Table, Fig 3G). The analysis of organism weight indicated an expected increase in abundance of *D. brunneus*, *Labidura xanthopus* (Stal, 1855) (Dermaptera: Labiduridae) and Collembola in the treatments (Fig 3G).

Table S7. Summary of the redundancy analysis (RDA) of abundance of the most representative non-target arthropods collected by Pitfall traps in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at three sites over two to three years in Brazil.

Site (year)		Axes				Total variance
		First	Second	Third	Fourth	
Castro (2012)	Eigenvalues	0.061	0.028	0.016	0.012	1.000
	Species-environment correlations	0.867	0.729	0.561	0.521	
	Cumulative percentage variance					
	of species data	13.1	19.2	22.6	25.3	
	of species-environment relation	44.8	65.5	77.1	86.1	
	Sum of all eigenvalues					0.463
	Sum of all canonical eigenvalues					0.136
	Partition of variability (%)					
	Sampling time	53.7				
	Soybean treatments	6.3				
	Permutation test for canonical axes ¹	F-ratio	<i>P</i> -value			
	First axis	3.63	0.052			
	All axes	1.25	0.117			
Castro (2013)	Eigenvalues	0.030	0.018	0.012	0.011	1.000
	Species-environment correlations	0.722	0.501	0.608	0.497	
	Cumulative percentage variance					
	of species data	6.2	10.0	12.6	14.9	
	of species-environment relation	29.8	48.4	60.5	71.8	
	Sum of all eigenvalues					0.477
	Sum of all canonical eigenvalues					0.099
	Partition of variability (%)					
	Sampling time	63.0				
	Soybean treatments	1.3				
	Permutation test for canonical axes	F-ratio	<i>P</i> -value			
	First axis	1.98	0.878			
	All axes	0.78	0.932			

Montividiu (2011)	Eigenvalues	0.079	0.060	0.032	0.021	1.000
	Species-environment correlations	0.761	0.659	0.678	0.645	
	Cumulative percentage variance					
	of species data	12.4	21.9	27.0	30.3	
	of species-environment relation	36.4	64.2	79.2	88.7	
	Sum of all eigenvalues					0.633
	Sum of all canonical eigenvalues					0.216
	Partition of variability (%)					
	Sampling time	36.7				
	Soybean treatments	8.5				
	Permutation test for canonical axes ¹	F-ratio	<i>P</i> -value			
	First axis	3.40	0.264			
Montividiu (2012)	All axes	1.55	0.039			
	Eigenvalues	0.114	0.039	0.032	0.025	1.000
	Species-environment correlations	0.837	0.672	0.737	0.669	
	Cumulative percentage variance					
	of species data	19.4	26.0	31.4	35.6	
	of species-environment relation	47.9	64.2	77.4	87.8	
	Sum of all eigenvalues					0.589
	Sum of all canonical eigenvalues					0.239
	Partition of variability (%)					
	Sampling time	41.0				
	Soybean treatments	10.1				
	Permutation test for canonical axes	F-ratio	<i>P</i> -value			
	First axis	5.77	0.001			
	All axes	2.04	0.001			

Montividiu (2013)	Eigenvalues	0.048	0.037	0.023	0.014	1.000
	Species-environment correlations	0.584	0.546	0.516	0.507	
	Cumulative percentage variance					
	of species data	9.0	16.0	20.3	22.9	
	of species-environment relation	36.8	65.4	82.8	93.5	
	Sum of all eigenvalues					0.535
	Sum of all canonical eigenvalues					0.131
	Partition of variability (%)					
	Sampling time	46.5				
	Soybean treatments	3.8				
Uberlândia (2011)	Permutation test for canonical axes	F-ratio	<i>P</i> -value			
	First axis	2.97	0.763			
	All axes	0.97	0.547			
	Eigenvalues	0.065	0.039	0.024	0.014	1.000
	Species-environment correlations	0.816	0.736	0.692	0.557	
	Cumulative percentage variance					
	of species data	12.8	20.4	25.1	27.8	
	of species-environment relation	41.3	65.7	80.8	89.5	
	Sum of all eigenvalues					0.511
	Sum of all canonical eigenvalues					0.159
	Partition of variability (%)					
	Sampling time	48.9				
	Soybean treatments	5.0				
	Permutation test for canonical axes ¹	F-ratio	<i>P</i> -value			
	First axis	3.52	0.025			
	All axes	1.35	0.055			

Uberlândia (2012)	Eigenvalues	0.151	0.059	0.025	0.014	1.000
	Species-environment correlations	0.812	0.869	0.747	0.735	
	Cumulative percentage variance					
	of species data	24.0	33.3	37.4	39.6	
	of species-environment relation	55.8	77.5	86.8	92.0	
	Sum of all eigenvalues					0.630
	Sum of all canonical eigenvalues					0.271
	Partition of variability (%)					
	Sampling time	37.0				
	Soybean treatments	9.3				
	Permutation test for canonical axes	F-ratio	<i>P</i> -value			
	First axis	7.59	0.080			
	All axes	2.27	0.021			

¹Permutation test by 999 Monte Carlo permutations ($\alpha = 0.05$).

P-values highlighted in bold are statistically significant.

The abundance of non-target arthropods was $\log(x + 1)$ transformed before analysis.

Fig. 3. Principal Response Curves (PRC) indicating the effect of non-Bt soybean with insecticides sprayed and Bt soybean (DAS-81419-2) on the most representative non-target arthropods collected by Pitfall traps at three sites over two to three years in Brazil. Dotted line indicates the community response to the control treatment (non-Bt without insecticides). The non-target arthropods weight (b_k) can be interpreted as the affinity of the taxon with the Principal Response Curves (C_{dt}). Only taxa with b_k values greater than 0.5 and less than -0.5 are shown in the diagram. Following Monte Carlo permutation tests, P-values less than 0.05 indicate a statistically significant difference in community response between at least one of the treatments and the control. Down arrows (↓) indicate the time of insecticide application in the sprayed non-Bt treatment.

The RM-ANOVA analysis was performed only for the sites and years in which the first axis was identified as significant. The results for Montividiu in 2012 demonstrated that the abundances of *Canthon* sp. (Coleoptera: Scarabaeidae) and *Elachiptera* sp. were higher in the non-sprayed non-*Bt* and sprayed non-*Bt* treatments compared with DAS-81419-2. Differences in abundance were observed for *Euxesta* sp. (Diptera: Otitidae) and *D. brunneus* among sampling times (Table 5). The interaction of sampling time and soybean treatment was significant only for *Euxesta* sp. where its abundance during the R7-R8 soybean stages was higher in the sprayed non-*Bt* treatment compared to the other treatments (Table 5, Fig 4A). The results for Uberlândia in 2011 identified higher abundance of *Condylostylus* spp. in the DAS-81419-2 treatment compared with the other treatments. Only *Pheidole* sp. was not influenced by sampling time (Table 5). Significant interactions of sampling time and soybean treatment were observed for *Condylostylus* spp. and Drosophilidae spp. (Table 5). The abundance of *Condylostylus* spp. during the R7-R8 soybean stages was higher in DAS-81419-2 plots compared with the other treatments (Fig 4B). The higher abundance of Drosophilidae spp. was observed at the R2 soybean stage in the sprayed non-*Bt* treatment (Fig 4C).

Table 5. Two-way repeated-measures ANOVA results for abundance of non-target arthropods with values weights greater than 0.5 and less -0.5 in the Principal Response Curves (PRC) analysis with first axis significant collected by Pitfall traps in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields at two sites over two to three years in Brazil.

Site (year)	Non-target arthropods	Soybean treatments			Two-way ANOVA					
		Non- <i>Bt</i>	Non- <i>Bt</i> sprayed	DAS-81419-2	Soybean treat. (A)		Sampling time (B)		Interaction (A x B)	
					<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>	<i>F</i>	<i>P</i>
Montividiu (2012)	<i>Canthon</i> sp.	3.33 ± 0.99 a	5.75 ± 1.98 a	0.25 ± 0.18 b	17.01	0.011	1.22	0.381	2.67	0.070
	<i>Elachiptera</i> sp.	7.42 ± 1.60 a	7.42 ± 2.86 a	1.17 ± 0.36 b	46.87	0.002	1.97	0.220	1.13	0.400
	<i>Labidura xanthopus</i>	4.25 ± 1.19	3.42 ± 1.02	1.33 ± 0.39	3.99	0.112	2.10	0.202	1.82	0.177
	<i>Euxesta</i> sp.	3.33 ± 2.10	8.92 ± 3.95	0.42 ± 0.28	6.80	0.052	5.66	0.035	5.51	0.006
	<i>Dorymyrmex brunneus</i>	4.92 ± 1.92	6.42 ± 3.46	5.75 ± 2.29	0.24	0.795	8.97	0.012	2.22	0.113
Uberlândia (2011)	<i>Condylostylus</i> spp.	2.00 ± 0.63 b	0.92 ± 0.31 b	6.08 ± 1.99 a	19.44	0.009	5.57	0.036	9.56	< 0.001
	<i>Pheidole</i> sp.	6.75 ± 1.95	6.67 ± 1.50	17.00 ± 5.82	2.96	0.163	0.58	0.650	0.774	0.605
	<i>Dorymyrmex brunneus</i>	12.58 ± 3.91	6.42 ± 2.03	12.08 ± 3.60	0.99	0.445	5.04	0.044	1.45	0.276
	<i>Selenophorus</i> sp.	6.00 ± 1.54	26.17 ± 12.40	25.17 ± 11.97	1.13	0.409	25.89	< 0.001	2.57	0.077
	<i>Elachiptera</i> sp.	8.50 ± 2.55	8.00 ± 2.63	5.25 ± 2.61	0.51	0.635	8.48	0.014	0.48	0.809
	Drosophilidae spp.	9.83 ± 3.83	17.42 ± 8.65	5.08 ± 2.05	3.71	0.123	12.83	0.005	3.77	0.024

P-values highlighted in bold are statistically significant ($\alpha = 0.05$).

Degrees of freedom: soybean fields = 2; sampling time = 3; interaction = 6; residual = 12.

Means ($\pm$ SE) followed by different letters were significantly different within rows (Tukey's test, $\alpha = 0.05$).

Fig. 4. Population dynamics for non-target arthropod groups collected by Pitfall traps in non-*Bt* (with and without insecticides) and *Bt* (DAS-81419-2) soybean fields. Means ($\pm$ SE) within sampling time followed by different letters are significantly different (Tukey's test, $\alpha = 0.05$). Taxa shown include those with taxon weights greater than 0.5 or less -0.5 which were associated with statistically significant Monte Carlo tests. Down arrows (↓) indicate the time of insecticide application in the sprayed non-*Bt* treatment.

4.5. Discussion

The results obtained from field trials conducted over three years at multiple locations within key soybean producing regions in Brazil demonstrated that the NTA community was not adversely affected by *Bt* soybean event DAS-81419-2 and that the major differences among field treatments were mostly related to sampling date through the effect of soybean developmental stages on arthropod activity. In agricultural systems, the predominant factors that shape arthropod community composition include: regional climate and weather; cropping system, crop species and phenology (time effects); soil management practices such as tillage; crop management through the application of fertilizer or broad spectrum pesticides; and surrounding landscape diversity that influences NTAs directly and indirectly through prey/food-mediated effects (Kromp, 1999). Within the context of these factors, the effects of *Bt* proteins and other narrow-spectrum insect resistance traits on NTAs have been shown to be negligible (Kromp, 1999; Dively, 2005; Hatten et al., 2007; Mumm & Dicke, 2010; Roger-Estrade et al., 2010; Rusch et al., 2010; Storer et al., 2008). Anthropogenic stress agents, like pesticides, are frequent components of agriculture ecosystems that potentially affect community structure and population dynamics through imparting lethal and/or sublethal effects on dominant species (Guedes et al., 2016). While lethality will deplete a species population directly via reducing its abundance, at least temporarily, sublethal effects are more difficult to detect in the field, and may lead to a range of subtle changes in the associated community, including impairment of species interactions, and eventual pest outbreaks, among others (Guedes & Cutler, 2014; Guedes et al., 2016, 2017; Cutler & Guedes, 2017). The same rationale is valid when assessing genetically modified crop plants that express insecticidal proteins, such as those including toxins derived from *Bacillus thuringiensis* (*Bt*) (Guedes et al., 2016, 2017). Both lethal and sublethal effects on sensitive species are expected, primarily for target insect pest species, but also potentially for non-targeted species. Because the mode of action for *Bt* toxins is highly specific, requiring alkaline midgut pH and the presence of specific receptors on midgut epithelial cells, the range of potentially sensitive species has been shown to be very limited (Piggot & Ellar, 2007). Indirect effects of *Bt* plants are expected due to a reduction in number or quality of target pests of the *Bt* proteins that serve as prey and/or hosts that attract predators and parasitoids (Tian et al., 2014). Indirect effects may also arise if the *Bt* plants are more attractive or suitable for herbivores in the

absence of significant damage from the target pests (Marvier et al., 2007; Wolfenbarger et al., 2008; Naranjo, 2009).

The number of studies evaluating the overall environmental impact of pesticides or *Bt* plants on arthropod assemblages (i.e., co-existing species of a given environment) and communities (i.e., interacting species of an assemblage) available under realistic field conditions is limited. Most available studies are short-term encompassing no more than a season or two and focus on a limited number of non-target species whose ecological benefits have been characterized, and thus are less likely to detect population or community-wide effects since these usually take longer to manifest and are strongly influenced by variation in environmental conditions beyond the presence of pesticides (Guedes et al., 2017). Here we reported a three-year study in three areas in different representative soybean-producing regions of Neotropical America designed to assess the potential impact of a *Bt* soybean variety, expressing the Cry1Ac and Cry1F proteins, as compared with its near (non-*Bt*) isoline with and without insecticide applications. The initial expectation was of a higher stress imposed by sprayed insecticides rather than by the *Bt* toxins based on previous studies on other crops (Pékar, 1999; Torres & Ruberson, 2005; Badji et al., 2004, 2006, 2007; Nash et al., 2010; Monzo et al., 2014). Curiously, the effects of both *Bt* crops and sprayed insecticides were negligible in most of the instances investigated.

Results from diversity analyses were largely indistinguishable when the effects of the *Bt* proteins and insecticide applications were compared with non-sprayed non-*Bt* fields. This result was expected, as such generally low-resolution indices are less likely to provide evidence of environmental impact for insecticidal compounds (Guedes et al., 2017). Additionally, the time-dependent multivariate approach was used (i.e. Principal Response Curve method), and applied to higher taxa, focusing mainly at the genus and family levels. This multivariate approach indicated a statistically significant effect only in two out of seven instances where Moericke (aerial) traps collected arthropods associated with the soybean canopy, and in only two out of seven instances where pitfall traps collected epigeic arthropods (i.e., associated with the soil surface). In those instances, the *Bt* soybean treatment was associated with higher abundance of aerial non-target species both in Castro (2011) and Uberlândia (2011), and lower abundance in epigeic non-target arthropods in Montividiu (2012) and Uberlândia (2011), although in this last case an initial reduction in abundance was followed by an increase during the late reproductive period of the soybean crop, thus no consistent pattern was observed. Examination of successive sampling

periods in each time-series demonstrated no sustained patterns. Furthermore, cross-referencing results with the complementing sampling method indicated no significant trends. Finally, abundance patterns were also not observed at all locations, indicating the few differences detected were not likely due to treatment effects.

Interpretation of results from multivariate analyses, such as the Principal Response Curve method, are benefitted when an optimal taxonomic resolution (species-, genus-, and/or family-level data) is achieved. The depth of taxonomic determinations may be guided by the potential for effects based on the known activity spectrum of the GM traits present in the crop, where family level data may suffice for most groups, and sub-family level determinations may aid in instances where taxonomic determinations for specific groups are practical and those groups are present in sufficient abundance to enable assessment.

The lack of insecticide effects in short-term studies of arthropod communities has also been detected in other ecosystems, particularly tropical agroecosystems (Chong et al., 2007; Guedes et al., 2016), where the cultivation system itself usually exhibits a more prominent effect and may buffer the potential insecticide impact (Araújo et al., 2004; Badji et al., 2004, 2006, 2007). While *Bt* proteins are constitutively expressed in the crop throughout the growing season, there is a lack of significant short- and long-term impacts on arthropod assemblages, as reported for maize (Dutton et al., 2003; Dively, 2005), cotton (Naranjo, 2005; Marvier et al., 2007; Torres & Ruberson, 2005; Yao et al., 2016) and soybean (Yu et al., 2014). Yu et al. (2014) reported non-significant impact of *Bt*-soybean, but using general faunistic indexes rather than multivariate analyses with higher taxonomic resolution. Furthermore, Szénási et al. (2014) also reported a lack of effect of *Bt* maize in central Europe using food-web analysis (Guedes et al., 2017). Thus, the relative lack of impact on the overall arthropod community or specific assemblages by *Bt* crops, as reported here, is in agreement with previous studies. Similar to previous studies, some statistically significant effects were detected in the *Bt* crop and separately in the non-*Bt* following insecticide application. The non-target arthropod herbivores cucurbit rootworm *D. speciosa*, the pollen beetle *A. variegatus*, the corn silk fly *Euxesta* sp., and leafhoppers were the main species for which differences were observed between *Bt* soybean and non-*Bt* soybean plots. Curcubit rootworm, a secondary pest species of soybean in Neotropical America (Hoffmann-Campos et al., 2000), was reduced by insecticide application during the vegetative period up to the early reproductive stage. In contrast, the corn silk fly was more abundant in the insecticide treated plot

in the late soybean reproductive period, while the pollen beetle and leafhoppers were more abundant in the *Bt* treatment during the mid and late soybean reproductive period, respectively. These species are highly mobile, and are not likely associated closely with treatment effects. None of these species are considered economic pests in the region (Hoffmann-Campos et al., 2000), and their late increase is unlikely to affect the soybean arthropod community and more specifically the soybean-based food web. Rootworms (*D. speciosa*) are minor pests of soybean in Brazil and were not impacted by *Bt* soybean, but its suppression by insecticides may benefit soybean yield. Rather curious is the low incidence of stink bugs in the samples, a likely shortcoming from the sampling methods used, which efficiently capture a majority of aerial species but are not optimized for this non-target pest. This group is currently the major soybean pest in Brazil, Argentina, and Paraguay (Hoffmann-Campos et al., 2000; Panizzi, 2013) and it is not susceptible to current *Bt* soybean events, which only target lepidopteran pests. Thus, outbreaks of stink bugs are likely to occur even with the use of this technology and may be actually favored by its extensive adoption if stink-bug specific management tactics are not employed simultaneously.

Drosophilids and house flies were also collected in aerial traps. The number of drosophilids were significantly reduced in the *Bt* soybean plots at the early reproductive plant stage. The number of house flies were higher in *Bt* soybean early and late during the plant reproductive period. This outcome is unlikely to affect soybean yield and the associated arthropod community since these species are not relevant components in the soybean-based food web and given the effect was detected only in one field each, and in a single year. Examining natural enemies, where the *Bt* soybean was associated with higher incidence of parasitic tachinid flies, the long-legged fly *Condylostylus* sp., and the predatory ground beetle *L. concinna*. However, the incrementally higher abundance of these natural enemies were likely too late in the soybean reproductive period to impart any substantial benefit on soybean yield via biological control of economic pests (Hoffmann-Campos et al., 2000).

In summary, a multi-year survey of the arthropod assemblage associated with soybean in three representative production regions indicated negligible short-term impact of *Bt* technology and sprayed insecticides. An effect was detected in only two instances and in isolated years restricted to few species of low relevance in the soybean-based food web which were unlikely to affect ecosystem processes or soybean yield. These findings are consistent with previous studies

performed in cotton and soybean in the US (Torres & Ruberson, 2005; Yu et al., 2014), maize in central Europe (Szénási et al., 2014), China (Guo et al., 2016) and Brazil (Fernandes et al., 2007). Use of *Bt* crops has reduced the need for insecticide applications in several cropping systems and survival of non-target arthropods has increased (Naranjo, 2009). These non-target arthropods can include both beneficial species and other pest species. While an increase in predator, parasitoid, and pollinator populations' benefits agriculture (Lu et al., 2012), an increased impact of pest hemipteran species has been documented in certain *Bt* cropping systems as a result of decreased broad-spectrum insecticide applications (Lu et al., 2010). While such changes are predictable from the pre-commercial risk assessment of a *Bt* crop, their extent may take several cropping seasons to emerge and can be impacted by unrelated changes in the agricultural landscape that occur over time. In any case, insecticide applications targeted at emergent pests do not alter the environmental benefits of the *Bt* crops themselves. Although field-level research into non-target effects such as the present study can provide information about the ecology of GM fields, there is a considerable body of literature (Romeis et al., 2006; Rose, 2007; Garcia-Alonso et al., 2006; Duan et al., 2009; Naranjo, 2009; Wach et al., 2016; Li et al., 2014) that argues that such research is not necessary to perform regulatory risk assessment for GM crops. The present research supports that position in finding no unexpected adverse effects on NTA communities, a finding that was anticipated from lower tier NTA experiments conducted with higher concentrations of *Bt* proteins in controlled laboratory conditions (ILSI, 2011, 2013). Assessments for GM crops that contain insect resistance traits could be expected to require field studies only when the results from lower tier hazard testing suggest the potential for adverse effects (Garcia-Alonso et al., 2006). When applicable, post-commercialization assessments for *Bt* crops are expected to confirm the results of the research presented here, and offer additional opportunities to document the environmental safety of *Bt* crops and their contribution as a pest management tool (Lu et al., 2012). Similarly, the results of ERAs that conclude no adverse effects of a novel trait(s) on non-target arthropods in one crop could be extended to other crops with the same or similar trait in a similar production system (Nakai et al., 2015). In the context of a tiered testing framework, field studies enable refinement of the risk assessment to address specific or unique considerations.

4.6. References

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5. SUMMARY AND CONCLUSIONS

This study aimed at a better understanding of the efficacy of genetically modified soybean with transgenic event DAS-81419-2 (ConkestaTM technology)⁵, which expresses two *Bacillus thuringiensis* (*Bt*) derived proteins, Cry1Ac and Cry1F, to manage primary and secondary lepidopteran pests. This work also evaluated potential adverse effects of this technology on non-target arthropods (NTAs) under field conditions. Field experiments conducted across several seasons in southern and central Brazil demonstrated that DAS-81419-2 soybean is significantly more efficient in controlling *Anticarsia gemmatilis*, *Chrysodeixis includens*, *Chloridea virescens*, and *Spodoptera cosmioides*, compared to the non-*Bt* isogenic variety managed with commercial foliar insecticide program during vegetative and reproductive crop developmental stages. Also, results from research trials across nine localities showed that the event DAS-81419-2 significantly reduced seedling stage plant mortality caused by *Elasmopalpus lignosellus* and the feeding damage caused by *Helicoverpa armigera* compared to a non-*Bt* isolate. On the other hand, this technology showed only moderate activity against *Agrotis ipsilon*, suggesting that additional control tactics, such as chemical seed treatment or foliar applications, may be needed to manage this insect pest. In addition, a three-year in-field study on the impact on NTAs revealed that total abundance and diversity were not significantly affected by *Bt* soybean with event DAS-81419-2 and only showed a negligible short-term impact. These results suggest that this technology is unlikely to affect soybean yield and interfere with its arthropod community structure.

Crop biotechnology must pass strict regulatory tests before it is approved for commercial planting. After more than 20 years of being commercially available, genetically modified plants expressing *Bt* proteins has demonstrated its efficacy at protecting crops from the impact of pests. However, the potential of biotech crops to contribute, in a sustainable manner, to feed a growing population, will largely depend on our ability to manage resistant development. This technology should not alter the proper balance of sustainable agroecosystems. In addition, to be of a larger global impact, the benefits of this technology must be properly communicated to all stakeholders. DAS-81419-2 soybeans will be a valuable and highly effective new tool to manage primary and secondary lepidopteran infestations in soybeans. This product should be used within Integrated Pest Management programs. Strong Insect Resistance Management plans should be also implemented to maintain the benefits and to extend the durability of DAS-81419-2 soybean efficacy against key and secondary pests.

⁵TMConkesta is trademark of The Dow Chemical Company (“DOW”) or an affiliated company of Dow.