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Multiple infestations induce direct defense of maize to *Tetranychus urticae* (Acari: Tetranychidae)

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and Simone Martins Mendes²

Abstract

Plants present constitutive or induced defense mechanisms against herbivory. In addition, studies show that there are interactions between these different defense mechanisms when multiple species infestations occur. This study investigated the interaction between maize defensive mechanisms to control two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) under single and double species infestations with this spider mite and fall armyworm, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). The experiment was carried out in a greenhouse with the following treatments: uninfested Bt (*Bacillus thuringiensis* Berliner; Bacillaceae) and conventional maize, single infestation with spider mite on Bt and conventional maize, and both maize types with infestations of spider mite and fall armyworm. Two d after infestation, all treatments were re-infested with spider mite. The number of eggs, immatures, and adult females (alive and dead) were recorded for re-infested populations. In addition, a leaf sample was taken from all maize plants for spectrometric analysis. There was no significant difference of biological variables of spider mite between Bt and conventional plants, as well as the ones from pre-infested and non-infested plants. The same chemical pattern of ions was observed on plants in these 4 groups. In the conventional pre-infested plants with spider mite and fall armyworm, the population of re-infested mite showed reduced survival and fertility. Defensive compounds detected were HMBOA-Glc, Linoleoyl-GPI, and kaempferol rutinose. It was suggested that there is direct induced defense against spider mite in conventional maize in multiple infestations with spider mite and fall armyworm.

Key Words: plant resistance; plant defense mechanisms; constitutive defense; *Spodoptera frugiperda*

Resumo

As plantas podem apresentar mecanismos de defesa constitutivos ou induzidos contra herbivoria. Estudos mostram que há interações entre esses diferentes mecanismos de defesa quando ocorrem infestações múltiplas. Este estudo investigou a interação entre os mecanismos de defesa do milho contra o ácaro-rajado *Tetranychus urticae* Koch (Acari: Tetranychidae) sob infestações de uma e várias espécies com este ácaro e lagarta-do-cartucho, *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). O experimento foi conduzido em casa de vegetação, com os seguintes tratamentos: milho Bt não infestado e milho convencional, infestação única com ácaro em milho Bt e convencional e milho com infestações de espécies de ácaro-rajado e lagarta-do-cartucho. Dois dias após a infestação, todos os tratamentos foram infestados com ácaros. O número de ovos, indivíduos jovens e fêmeas adultas (vivas e mortas) foram registrados para populações reinfestadas. Além disso, foi retirada uma amostra de folhas de milho de todas as plantas para análise espectrométrica. Não houve diferença significativa de variáveis biológicas entre ácaros, e plantas Bt e convencionais, bem como de plantas pré-infestadas e não infestadas. Nas plantas convencionais pré-infestadas, com ácaro-rajado e a lagarta-do-cartucho, a população de ácaros reinfestada mostrou redução na sobrevivência e fertilidade. Os compostos de defesa observados foram HMBOA-Glc, Linoleoyl-GPI e kaempferol rutinose. Foi sugerido que há defesa induzida direta contra *T. urticae* em milho convencional em infestação múltipla com ácaro-rajado e lagarta-do-cartucho.

Palavras Chaves: resistência de plantas; mecanismos de defesa de plantas; defesa constitutiva; *Spodoptera frugiperda*

Plants use mechanisms to defend themselves against herbivorous attack (Price et al. 1980; Kessler & Baldwin 2002; Schaller 2008). Direct induced defenses are activated after an herbivore attack (Chen 2008) and reduced the herbivore survival and fertil-

ity (Karban & Myers 1989; Fürstenberg-Hägg et al. 2013). After being attacked by herbivores, plant sensors perceive the physical and chemical signals induced by herbivore feeding, such as elicitors present in the saliva of herbivores (Wu & Baldwin 2009). These

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elicitors link to putative receptors on plant plasma membranes and the induced defense signaling process is activated, producing defense chemical compounds (Wu & Baldwin 2009) through chemical changes in plants by the routes of salicylic acid and jasmonic acid. These signaling processes vary with the attacking herbivore species (Walling 2000; Vos et al. 2005). Usually, salicylic acid induces resistance to phloem feeding insects and jasmonic acid induces resistance to chewing herbivores (Thaler et al. 2002, 2012). However, plants are commonly attacked by multiple species of herbivores and defenses induced by multiple species may differ from those induced by each species separately (Rodríguez-Saona et al. 2010; Thaler et al. 2012; Oliveira et al. 2016). Plant defenses can be constitutive,

always expressed, which can complicate herbivore feeding (Karban & Myers 1989; Mello & Silva Filho 2002). For example, cuticular deposits and thickened epidermis increases the time for feeding, reducing growth and survival of herbivores (Becerra 1994). Another example of constitutive defense mechanism is the genetically modified insect-resistant crops such as maize with the Bt gene (Macintosh et al. 1990; Maagd et al. 1999).

Plants produce a range of chemical compounds that are systemically increased by the amount of damage caused by tissue feeders (Alborn et al. 1996; McAuslane et al. 1997). As the incidence of pest tissue feeders decreases in Bt maize, direct induced defense compounds could be changed by the Bt maize protein affecting the non-targeted organism.

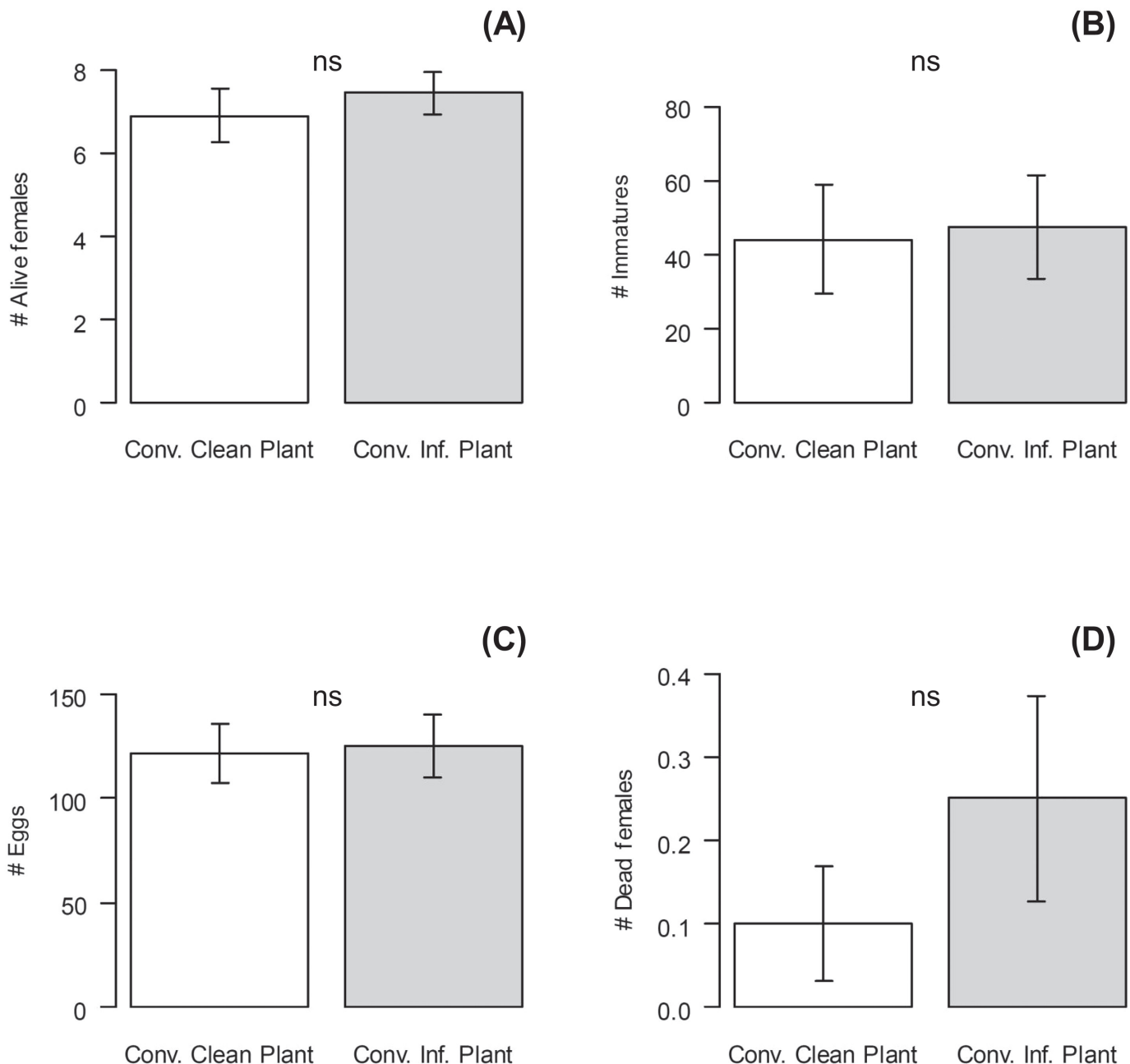


Fig. 1. Number (mean \pm SE) of live females (A, $\chi^2 = 45.2$; df = 36; $P = 0.861$), immature individuals (B, $\chi^2 = 38.7$; df = 37; $P = 0.608$), eggs (C, $\chi^2 = 38.1$; df = 36; $P = 0.624$), and dead females (D, $\chi^2 = 29.7$; df = 37; $P = 0.203$) of the two-spotted spider mite *Tetranychus urticae* on clean (white bars) and co-specific re-infested (gray bars) conventional maize plants (30F35). ns = statistically insignificant.

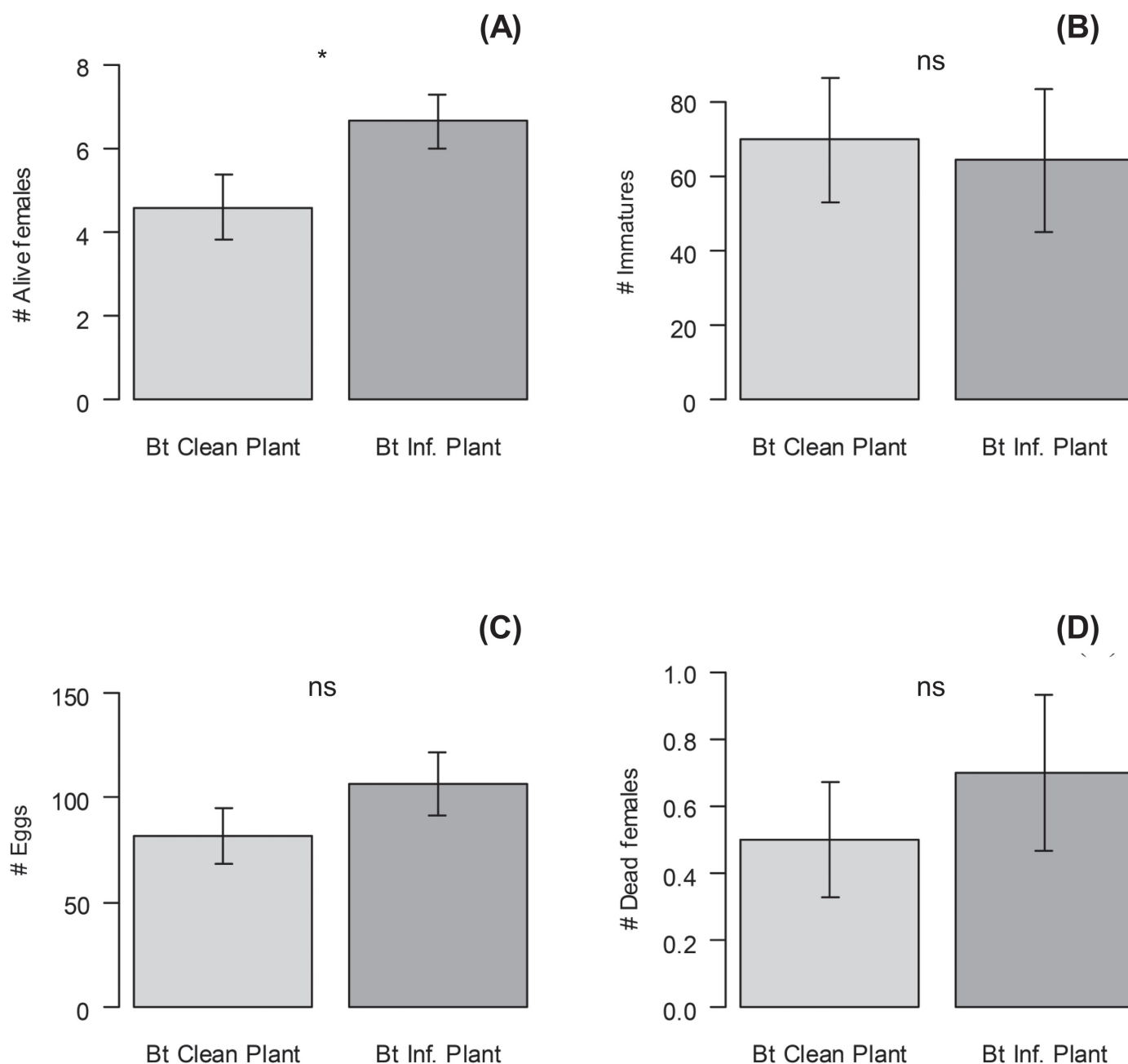


Fig. 2. Number (mean \pm SE) of live females (A, $\chi^2 = 21.9$; df = 37; $P = 0.02$), immature individuals (B, $\chi^2 = 40.9$; df = 37; $P = 0.698$), eggs (C, $\chi^2 = 30.5$; df = 37; $P = 0.235$), and dead females (D, $\chi^2 = 21.9$; df = 37; $P = 0.383$) of the two-spotted spider mite, *Tetranychus urticae*, on clean (light gray bars) and co-specific re-infested (dark gray bars) Bt maize plants (30F35Hx). ns = statistically insignificant, * = statistically significant ($P < 0.05$).

For example, the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae), feeds on Bt maize. However, *T. urticae* is a non-targeted organism to Bt maize protein, and perhaps it should be considered the risk of spider mites to become a more relevant pest to the Bt crops (Paulo et al. 2018). On another hand, the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is a primary pest of maize plants. It can significantly reduce the maize yield if not managed. Therefore, *T. urticae* and *S. frugiperda* have a co-occurrence in vegetative phenology of maize with potential interaction.

There are physiological costs associated with the induction of defenses. A question that arises is, in what situation do plants produce those defense mechanisms, such as when plants are attacked

by secondary pests? In a study conducted by Paulo et al. (2018) to determine if maize plants would develop direct resistance to *T. urticae*, results showed that the conspecific survival of adult *T. urticae* females on infested maize plants was reduced, thus suggesting direct induced maize resistance to *T. urticae*. However, at this time there is no conclusive information regarding induced defenses in maize plants attacked by multiple herbivores.

In this paper, we investigated whether Bt and conventional maize can induce direct resistance to *T. urticae* after single infestation with *T. urticae* and dual species infestation with *T. urticae* plus *S. frugiperda*. We also evaluated the chemical compounds induced by maize plants after herbivore attack.

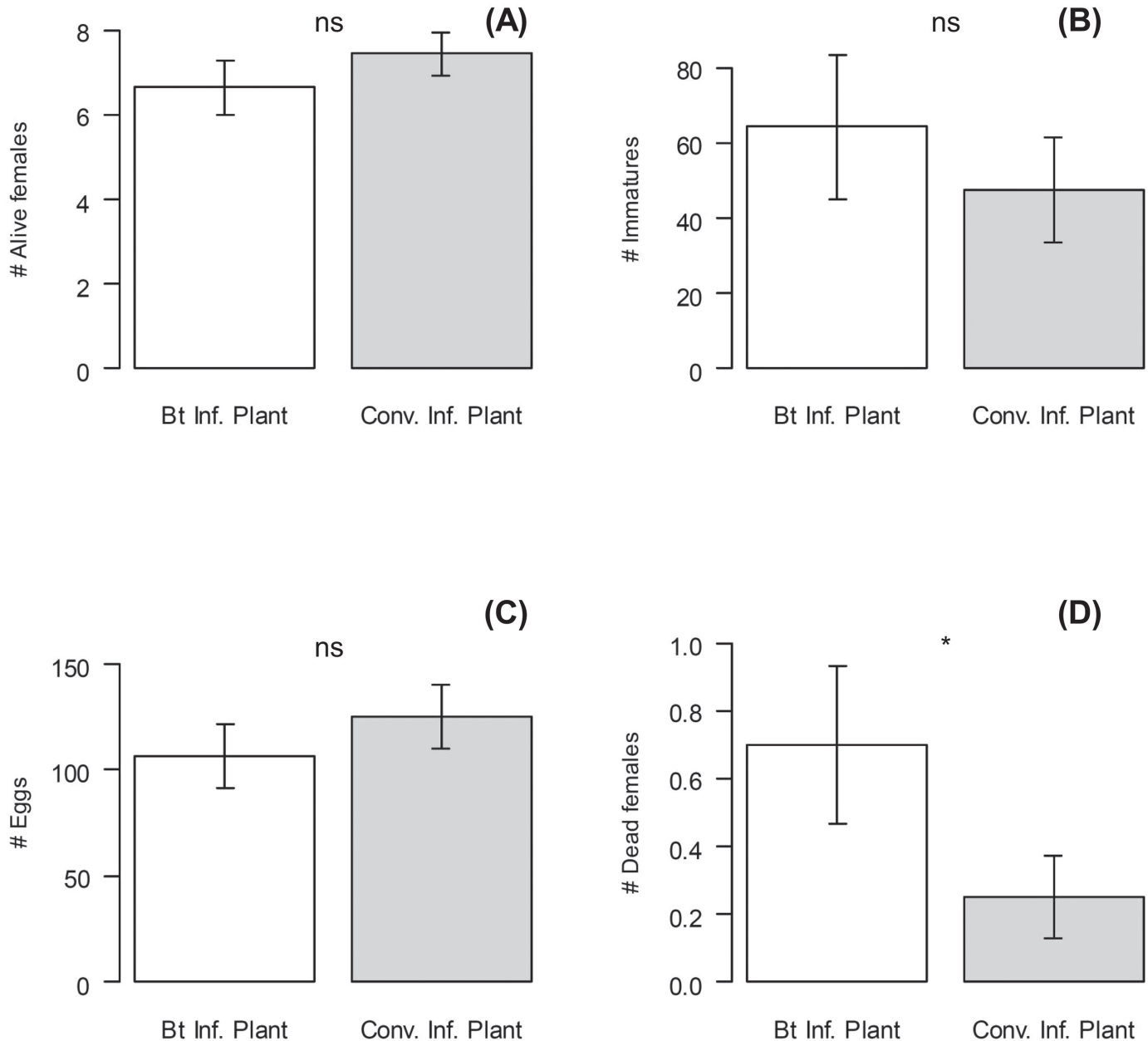


Fig. 3. Number (mean \pm SE) of live females (A, $\chi^2 = 31.11$; df = 37; $P = 0.259$), immature individuals (B, $\chi^2 = 27.67$; df = 37; $P = 0.133$), eggs (C, $\chi^2 = 27.67$; df = 36; $P = 0.240$), and dead females (D, $\chi^2 = 23.58$; df = 37; $P = 0.042$) of the two-spotted spider mite *Tetranychus urticae* on infested conventional (30F35) (white bars) and Bt (30F35Hx) (gray bars) maize plants. ns = statistically insignificant, * = statistically significant ($P < 0.05$).

Material and Methods

HERBIVORES

Specimens of *T. urticae* were obtained from sorghum, *Sorghum bicolor* (L.) Moench (Poaceae), cultivated in a greenhouse. In the laboratory, sorghum leaves were examined with a stereomicroscope (Zeiss, Oberkochen, Germany) and mites were transferred individually with a hair brush to seedlings of jack bean, *Canavalia ensiformis* (L.) DC (Fabaceae), cultivated in plastic pots (6.3 L) (Nutriplan, Cascavel, Paraná, Brazil) using Terral Solo® (Inhaúma, Minas Gerais, Brazil) as the substrate. Infested plants were protected individually with screened cages and maintained in a greenhouse at 25 ± 5 °C,

and were watered as necessary. Uninfested plants were added to the colony every 7 d to maintain the increasing mite population 15 d after planting. Third instar larvae of *S. frugiperda* were obtained from a laboratory colony maintained at Embrapa Milho e Sorgo, in Sete Lagoas, Minas Gerais State, Brazil.

PLANTS

The maize seeds used were the hybrid 30F35 Pioneer® in 2 versions, Bt (Herculex-Hx), which expresses the Cry1F (Bt) protein, and its respective isohybrid (conventional). The maize seeds were planted in plastic pots (1 L) using Terral Solo® as the substrate and kept in screened cages. Three seeds were sown per pot and after 2 wk, thinned to only 1 maize seedling per pot. Individual plants were

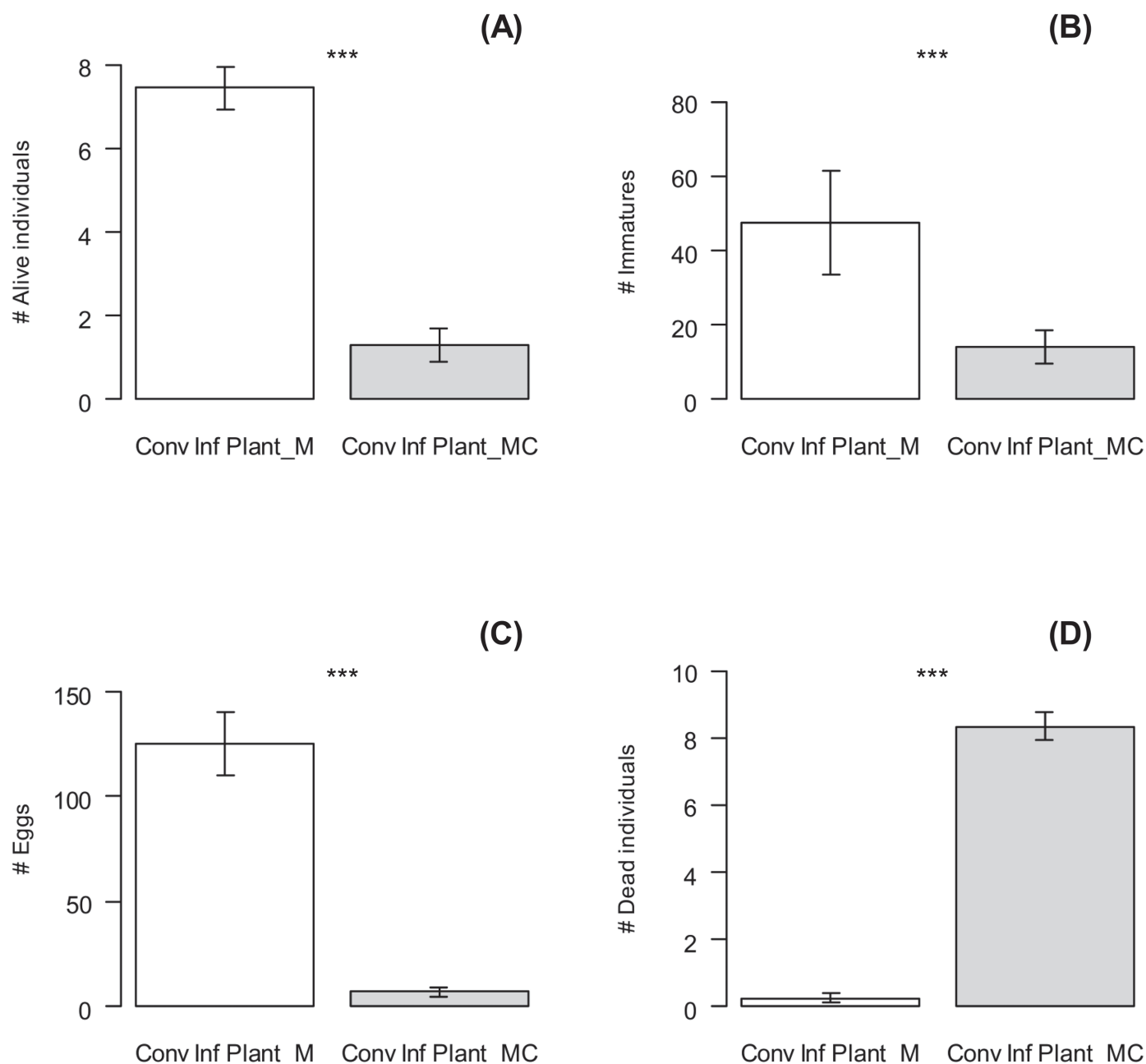


Fig. 4. Number (mean \pm SE) of live females (A, $\chi^2 = 7.65$; df = 37; $P < 0.001$), immature individuals (B, $\chi^2 = 2.60$; df = 36; $P < 0.001$), eggs (C, $\chi^2 = 2.14$; df = 36; $P < 0.001$), and dead females (D, $\chi^2 = 5.38$; df = 37; $P < 0.001$) of the two-spotted spider mite *Tetranychus urticae* on conspecific (white bars) and conspecific plus *Spodoptera frugiperda* infested (gray bars) conventional maize plants. M = mite, C = caterpillar. *** = statistically significant ($P < 0.01$).

fertilized with 0.2 g of ammonium sulfate $[(\text{NH}_4)_2\text{SO}_4]$ every 15 d. Watering was done as required. Maize plants were allowed to reach 40 d after planting, before the start of the experiments.

INFESTATION OF PLANTS AND BIOLOGICAL ANALYSIS

The experiment was carried out in a greenhouse with 6 treatments. Each treatment had a group of 22 plants that were separated in screened cages. Two plants without any type of infestation were used as control and for spectrometry analysis. The treatments were 2 versions of Bt maize and non Bt; uninfested (= clean) plants; single infestation with spider mite; and dual species infestation with spider mite and fall armyworm for each maize version. Two d after the first infestation,

all plants were re-infested with spider mite to evaluate the impact of previous infestations, because 1 d of infestation is sufficient for *T. urticae* to induce defense in other plants (Kant et al. 2004; Oliveira et al. 2016; Oliveira et al. 2017). For initial infestation or re-infestation with *T. urticae*, 10 female *T. urticae* were introduced to the abaxial surface of a leaf within a barrier made with entomological glue Biocontrol® (Indaiatúba, São Paulo, Brazil) to avoid escape or wandering off. In addition, a single *S. frugiperda* at the third instar was introduced to the adaxial surface of the leaf, confined in a “clip-cage” (Smith et al. 1994). The leaves that were infested only once were marked with yellow ribbon and the re-infested leaves were marked with red ribbon for future identification. Evaluations started at 24 h after re-infestation and lasted for 10 d. To evaluate each treatment, plants were sampled

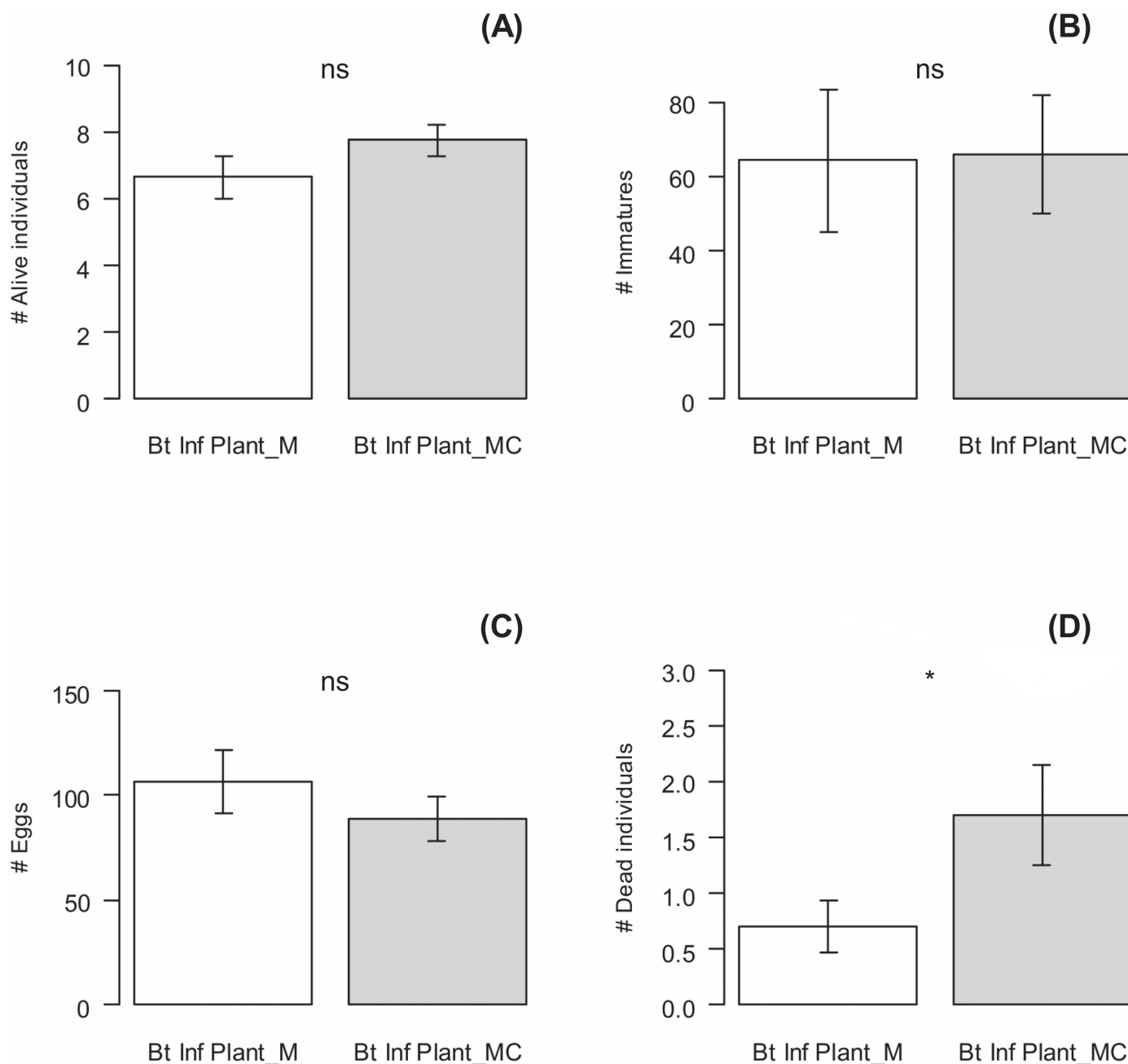


Fig. 5. Number (mean \pm SE) of live females (A, $\chi^2 = 29.5$; df = 37; $P = 0.195$), immature individuals (B, $\chi^2 = 39.1$; df = 36; $P = 0.670$), eggs (C, $\chi^2 = 31.7$; df = 36; $P = 0.326$), and dead females (D, $\chi^2 = 22.0$; df = 37; $P = 0.024$) of the two-spotted spider mite *Tetranychus urticae* on conspecific (white bars) and conspecific plus *Spodoptera frugiperda* infested (gray bars) Bt maize plants. M = mite, C = caterpillar. ns = statistically insignificant, * = statistically significant ($P < 0.05$).

randomly. The plants, numbered from 1 to 20, were used for just 1 record, so each maize plant became an independent sampling unit. The daily mite counting was done by removing the re-infested leaves of 2 plants in each treatment. Using scissors, the leaves were cut at their extreme, and taken directly to the laboratory for mite counting under a 50 \times stereomicroscope (Zeiss, Oberkochen, Germany). The number of eggs, immature stages (larva, protonymph, and deutonymph) and adult (alive and dead) were recorded for re-infested populations.

SPECTROMETRY ANALYSIS

Leaf samples weighing 500 mg were used for the analyses from maize plants at 1, 3, 5, and 10 d after re-infestation, and from control

plants, all for spectrometry analysis. The samples were prepared by macerating leaf sample weighing 500 mg from individual plants separately in a beaker (50 mL) with a glass stick. After that, 5 mL of methanol was added and sealed with aluminum foil and PVC film, and left for 4 h to extract the leaf compounds. After extraction, the supernatant was removed with a 10 mL disposable syringe coupled to a pre-cleaned welded syringe filter, PVDF membrane 25 mm \times 0.45 μ m, and transferred to an Eppendorf tube (1.5 mL). Then, 10 μ L of the solution was transferred to another Eppendorf tube and 1 mL of methanol was added.

The solutions were injected in the electrospray ionization mass spectrometry (Thermo Scientific, San Jose, California, USA) apparatus as described by Catharino et al. (2005). Then gaseous ions were generated and separated from the sample, according to their mass-to-charge ratios

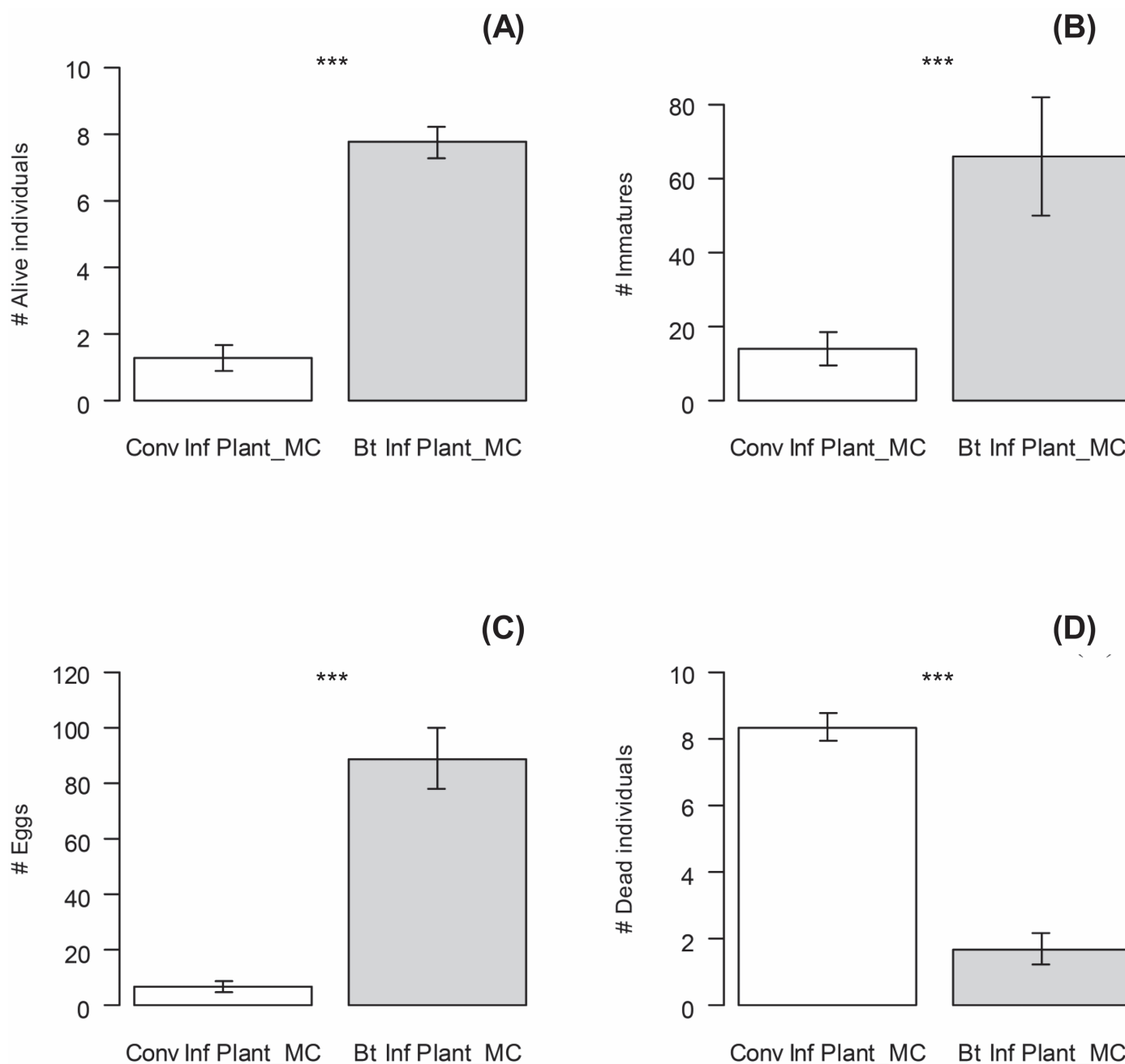


Fig. 6. Number (mean \pm SE) of live females (A, $\chi^2 = 7.39$; df = 37; $P < 0.001$), immature individuals (B, $\chi^2 = 2.14$; df = 36; $P < 0.001$), eggs (C, $\chi^2 = 2.14$; df = 36; $P < 0.001$), and dead females (D, $\chi^2 = 7.7$; df = 36; $P < 0.001$) of the two-spotted spider mite *Tetranychus urticae* on conspecific plus *Spodoptera frugiperda* infested conventional (white bars) and Bt (gray bars) maize plants. M = mite, C = caterpillar. *** = statistically significant ($P < 0.01$).

(Silverstein et al. 2006). The mass spectra obtained was characterized by the presence of a few fragment of ions and relatively strong precursor ions. Hence, few or no structural information can be obtained, given the reduced number of fragment of ions formed (Yamashita & Fenn 1984).

STATISTICAL ANALYSIS

The experimental design was completely randomized. Data were analyzed using the generalized linear models with Poisson distribution for the count. Independent variable (x) is the plant condition (i.e., Bt and conventional: cleaned or pre-infested with two-spotted spider mite or fall armyworm), and dependent variables (y) are biological parameters of the re-infested mite population (i.e., number of alive and dead fe-

males, number of immature stages, and number of eggs). The data were submitted to normality tests and residual analysis to evaluate the assumptions and the adjusted models adequacy (Crawley 2013). To relate the spectrometry analysis to the direct induced defense, 4 plant groups were based on ions average in each pair of plant. The data were subjected to a principal component analysis. R (R Development Core Team 2014) software was used for exploratory and statistical and data analysis.

Results

The initial plant infestation by *T. urticae* in conventional and Bt maize did not affect the conspecific re-infested population (Figs.

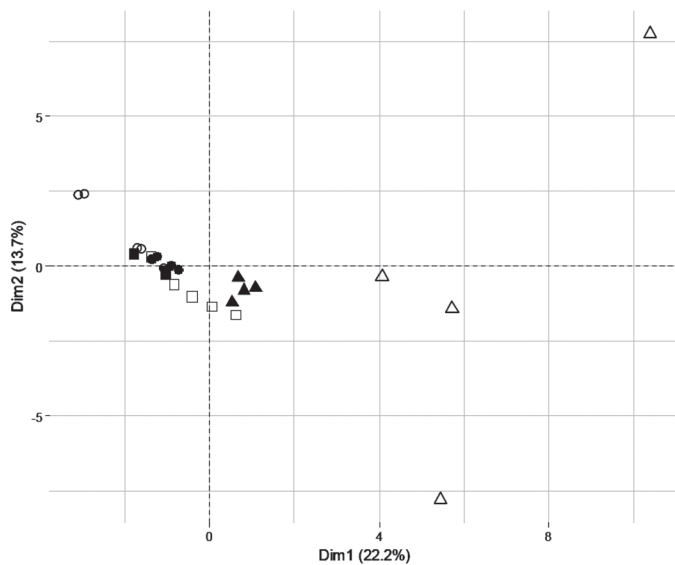


Fig. 7. Projection to principal component analysis based on the ions detected by electrospray ionization mass spectrometry of uninfested (C = ○) and infested with *Tetranychus urticae* (C + Tu = ●) conventional maize; uninfested (Bt = □) and infested with *T. urticae* (Bt + Tu = ■) Bt maize; infested with *T. urticae* and *Spodoptera frugiperda* (C + Tu + Sf = △) conventional maize; and infested with *T. urticae* and *S. frugiperda* (Bt + Tu + Sf = ▲) Bt maize, using the first 2 principal components (Dim) with explained variance in brackets

1, 2). The number of surviving females, immature individuals, and eggs did not differ between Bt and conventional plants pre-infested with *T. urticae*, but the number of dead females was significantly higher in Bt maize (Fig. 3).

Conventional maize plants pre-infested with fall armyworm and *T. urticae* negatively affected the re-infested spider mite *T. urticae* compared with the ones on conventional maize plants with mites only (Fig. 4). However, there was no significant difference between the *T. urticae* on re-infested plants of Bt maize pre-infested with fall armyworm plus *T. urticae* and Bt maize previously infested only with *T. urticae*, except to the variable number of dead females, which was higher on pre-infested Bt maize with fall armyworm plus *T. urticae* (Fig. 5). The number of surviving females, eggs, and immatures of *T. urticae* on conventional maize pre-infested with fall armyworm was significantly lower than the ones on Bt maize infested with fall armyworm only (Fig. 6).

The 2 principal components of principal component analysis cumulatively account for 33.9% of variation (Fig. 7). No differences were observed among ions of the uninfested Bt and conventional maize, single infestation with spider mite on Bt and conventional maize, Bt maize with multiple infestations of spider mite and fall armyworm. Furthermore, all plants in the principal component analysis gathered in the same plot quadrant have formed a group in the principal component analysis (Fig. 7). However, there was a clear discrimination between these treatments and conventional maize with dual infestations of spider mite and fall armyworm that formed another group in the principal component analysis (Fig. 7).

Thirty-five ions were detected in the electrospray ionization mass spectrometry, the ions 318.37 mass-to-charge ratio and 871.47 mass-to-charge ratio were present in all the plants. The ions 274.36 mass-to-charge ratio, 701.53 mass-to-charge ratio, and 959.51 mass-to-charge ratio were not detected on conventional maize pre-infested by spider mite plus fall armyworm. In conventional maize pre-infested with spider mite plus fall armyworm, a total of 18 ions were detected. Furthermore, we identified compounds related to

induced defenses. The ion 279.06 mass-to-charge ratio was identified as Linoleoil-GPI (Marti et al. 2013). The ion 356.08 mass-to-charge ratio was identified as HMBOA-Glc (2-hidroxi-7-metoxi-1,4 (2H)-benzoxazin-3(4H)-on)-β-d-glucopiranosido) (Oikawa et al. 2001; Marti et al. 2013; Wouters et al. 2016a), and the ion 593.4 mass-to-charge ratio as kaempferol rutinose (Oikawa et al. 2001).

Discussion

The pre-infestation of conventional and Bt maize plants with *T. urticae* did not affect the conspecific re-infestation, so the plants previously infested with *T. urticae* were not able to induce direct defenses. Furthermore, the biological results are a fit with the spectrometry analysis, because there was no difference among the ions detected in Bt and conventional maize uninfested and pre-infested with spider mite. Therefore, these results show that pre-infestation with *T. urticae* cannot induce expression of direct defense compounds in Bt or conventional maize.

Paulo et al. (2018) found that infestation of conventional maize plants by *T. urticae* reduced the conspecific adult survival. However, they suggested that additional spectrometry analysis on infested and uninfested plants by *T. urticae* is necessary to confirm the hypothesis of induction of direct defenses in conventional maize. Thus, the hypothesis that the infestation period by *T. urticae* on conventional maize was not long enough for induction of direct defenses. Many studies show that duration of infestation and other factors may influence the rate at which a plant responds defensively to insect attack (Rhoades 1979; Sabelis & Dicke 1985; Dicke et al. 1990; Brown et al. 1991; Takabayashi et al. 1994; Nachappa et al. 2006).

In Bt maize, plants were unable to induce defenses. Because these plants were able to express constitutive defense mechanisms, it is possible they did not allocate resources to induce direct defense mechanisms. Hagenbucher et al. (2013) found an effective suppression of Bt-sensitive herbivores with Bt cotton expressing reduced levels of induced terpenoids.

Multiple pre-infestations with *T. urticae* plus fall armyworm did not affect the biology of two-spotted spider mite re-infested on Bt maize, but it was affected on conventional maize. This finding can be attributed to the difference in the injury intensity on leaf tissue, caused by the pre-infestation with fall armyworm (Brown et al. 1991; Nachappa et al. 2006). It suggests that small injuries of fall armyworm on Bt maize is insufficient to induce defense compounds. However, multiple species infestations in conventional maize are able to induce direct defense, because the initial infestation with fall armyworm plus *T. urticae* reduced the survival and reproduction of *T. urticae* from the second infestation.

The spectrometry analysis did not indicate the presence of defense compounds, so we hypothesize that *T. urticae* cannot induce direct defense in maize. Thus, the ecological interactions between maize and *T. urticae* may be insufficient to make selection pressure to input evaluation of plant defense mechanisms. Furthermore, previous studies showed that 1 d of infestation is sufficient for the *T. urticae* to induce plant defenses (Kant et al. 2004; Oliveira et al. 2016, 2017).

Furthermore, compounds Linoleoil-GPI, HMBOA-Glc, and kaempferol rutinose were detected and can be related to plant defenses. The HMBOA-Glc has toxic and anti-feeding effects toward arthropods (Wouters et al. 2016b). The effects of Linoleoil-GPI and kaempferol rutinose were unknown, but these compounds were detected in plants related to direct induced defense (Wouters et al. 2016a).

This is the first report regarding the induction of direct defense mechanism in conventional maize promoted by fall armyworm plus *T. urticae* infestation demonstrated with biological and spectrometry data.

Acknowledgments

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