ENTOMOLOGICAL Communications

Entomological Communications, 1, 2019: ec01013

Bioassay

Does corn phenological stage alter the attractiveness of herbivore-induced plant volatiles to the predatory lacewing *Chrysoperla externa* (Hagen, 1861)?

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Edited by: Clara Beatriz Hoffmann-Campo

Received: November 11, 2019. Accepted: November 21, 2019. Published: December 17, 2019.

Abstract. Plant chemical defenses can affect herbivores directly or indirectly through the emission of herbivore-induced plant volatiles (HIPVs) that recruit natural enemies. Corn seedlings have high concentrations of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) that deter aphids, but as concentration decreases over the course of plant phenology, plants become less resistant. We investigated whether corn phenological stage influences the attractiveness of *Rhopalosiphum maidis* (Fitch, 1856) - infested corn seedling volatiles to the predatory lacewing *Chrysoperla externa* (Hagen, 1861). In olfactometer, lacewings preferentially oriented to volatiles from aphid-infested over those by uninfested corn seedlings at V6 or V7 stages, but did not discriminate between volatiles from uninfested and aphid-infested V5-stage seedlings. Greater numbers of aphids died in V5 corn seedlings relative to those in V6 and V7 seedlings. Our results indicate that the lack of discrimination of the predatory lacewing to HIPVs emitted by V5 corn seedlings is due to insufficient induction given that they were more resistant to *R. maidis.*

Keywords: behavior, olfactory response, induced plant defenses, natural enemies, tritrophic interactions.

Understanding the role of chemical plant defenses to insect herbivory has advanced over the last decades. Plants have evolved different chemical defensive strategies to deter herbivore attack that are constitutive (i.e., always present in plant tissues) or induced (i.e., synthesized upon herbivore attack) (Fürstenberg-Hägg et al. 2013). Induced plant defenses comprise increased concentrations of constitutive defensive metabolites or novel compounds that directly act against herbivores by altering their physiology and behavior, or indirectly by attracting herbivore natural enemies (Dicke 1999, Turlings & Wäckers 2004).

The hydroxamic acid 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) is an example of secondary metabolite of Poaceae plants, such as corn (*Zea mays* L.), that act as a direct defense to herbivores (Niemeyer 1988). The inactive form of DIMBOA is stored in vacuoles and, after exogenous or endogenous damage, it is activated by enzymes (Czjzek et al. 2000, Von Rad et al. 2001, Park et al. 2004). DIMBOA in the corn seedling reaches the highest concentration around 24-36h after the germination (Ebisui et al. 2000) and then gradually reduces as the plant ages. This compound is well-known for its role in deterring herbivory by aphids, especially the corn aphid *Rhopalosiphum maidis* (Fitch, 1856) (Hemiptera: Aphididae), an important pest of corn crops, that is sensitive to DIMBOA and avoids feeding on corn genotypes with high levels of DIMBOA (Niemeyer et al. 1989, Givovich & Niemeyer 1995). As corn seedlings grow, the concentration of DIMBOA in the tissues decreases and plants become more susceptible to corn aphid infestations (Argandoña et al. 1981).

Herbivore-induced plant volatiles (HIPVs) are the main indirect plant defense against herbivores because they recruit several parasitoids and predators (Dicke et al. 1990, Turlings et al. 1990). Nevertheless, blend composition and abundance of HIPVs depend on the herbivore damage magnitude in a way that can affect plant attractiveness to natural enemies (Gouinguene et al. 2003). Therefore, direct defenses, such as DIMBOA, can alter the emission of HIPVs because of reduced damage, potentially affecting recruitment of natural enemies.

Here, we investigated whether phenological stage of corn seedlings affects the attraction of the predatory lacewing *Chrysoperla externa* (Hagen, 1861), which is a generalist natural predator of aphids, to the volatile emission of corn plants infested with *R. maidis*. We tested the olfactory response of lacewing larvae to volatiles emitted by V5 to V7 corn seedlings and registered the mortality of *R. maidis* on those plants.

Corn seeds (non-Bt hybrid P2530) (Pioneer Sementes, São Paulo, Brazil) were cultivated in plastic pots (2-L capacity) filled with soil (Haplortox) and no fertilization. Two seeds were sowed in each pot and 5 days after emergence, only one seedling was left/pot. Plants were watered daily and maintained in a greenhouse under natural light and no temperature control. When seedlings had 5, 6 or 7 expanded leaves, corresponding to V5, V6 and V7 stage, they were used in assays.

The lacewing and the corn aphid used in experiments were obtained from stock rearing maintained at the Department of Entomology (UFLA, Lavras, Brazil), according the methods described in Carvalho & Souza (2000) and Cabette (1992).

To obtain aphid-infested plants, V5, V6 or V7 corn seedlings were infested with 100 *R. maidis* nymph and adult aphids and covered with bags made of fine-mesh fabric to avoid insect scaping. Uninfested plants were also covered with the bags, although were not infested with insects. Both treatments were maintained at the same abiotic conditions for 72h, when they were used in assays.

Olfactory preference of *C. externa* third-instar larvae to corn seedling volatiles were assessed in olfactometry assays using a Y-tube olfactometer (18 cm long, 3 cm θ and angle of 120° between side arms). Aerial plant parts were covered in polyethylene bags (41 cm x 33 cm) (Wyda, Sorocaba, Brazil) with two openings, through which two hoses were connected for air inlet and outlet. Air flow was generated by an air compressor that pulled air from the room into the olfactometer system. Air was charcoal filtered and humidified before entering the polyethylene bags and then the Y-tube side arms. Airflow was adjusted to 0.8 L/min. A single lacewing larva was introduced in the central olfactometer arm and observed for up to 10 min. A choice was considered when the insect crossed a line traced at the mid third of the side arm and spent at least 30 sec there. If the insect did not choose a treatment within 10 min, it was considered non-responsive. Each larva was tested only once. Every 10 replicates, the plant pair was replaced and the Y-tube olfactometer cleaned with soap, rinsed with tap water and acetone, and dried in the oven at 180°C. Assays were conducted in a room under controlled conditions (25 ±2 °C, RH 60 ± 10 %, 10h of photophase) between 13:00 and 17:00h.

Olfactory response of lacewing larvae was tested when exposed to: *(i)* uninfested plant *vs.* aphid-infested plant at V5 stage; *(ii)* uninfested plant *vs.* aphid-infested plant at V6 stage; and *(iii)* uninfested plant *vs.* aphid-infested plant at V7 stage. Each assay consisted of 40 insect choices using 4 pairs of plants.The numbers of aphids on aphid-infested plants at V5, V6 and V7 stages (i.e., after about 72h of the initial infestation with 100 individuals) were counted just after tests. Choice proportions for treatments were analyzed by the binomial test at 5%, 1% and 0.1%. Aphid countings on V5 to V7 seedlings were analyzed by one-way ANOVA ($P < 0.05$) followed by Tukey's test.

C. externa larvae did not discriminate between volatiles emitted by uninfested and aphid-infested corn seedlings at V5 stage (Fig. 1, binomial test, $P = 0.080$). However, when *C. externa* were exposed to volatiles of aphid-infested and uninfested plants at V6 and V7, the predator was oriented preferentially to aphid-infested plants (Fig. 1, V6 stage: $P<0.01$; V7 stage: $P< 0.001$). Corn seedlings at V5 stage had about 60% less aphids after 72h from the initial infestation and two-fold less aphids than those at V7 and V6 stages (Fig. 2, one-way ANOVA $P < 0.05$, Tukey's test, $P < 0.01$).

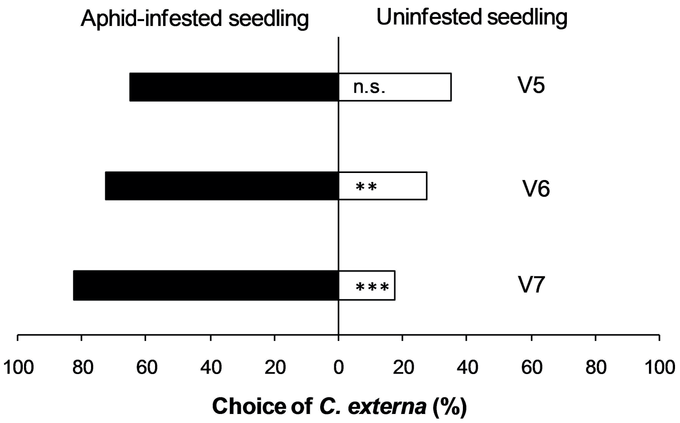

Figure 1. Olfactory preference of *Chrysoperla externa* third-instar larvae to aphid-infested corn seedlings and uninfested at V5, V6 and V7 phenological stages. Tests were conducted in a Y-tube olfactometer system. Corn seedlings were infested with 100 *Rhopalosiphum maidis* for 72h. * significant difference at 5% according to binomial test, ** significant difference at 1% *** significant difference at 0.1%; n.s= not significant.

The attraction of natural enemies to volatiles emitted by plants infested with their prey has been extensively shown in the literature (Dicke et al 1990, Mumm & Dicke 2010), including the lacewing *C. externa* (Zhu et al. 2005). However, herbivore density on the plant is an important biotic factor influencing the induced plant response as well as on tritrophic interactions mediated by HIPVs (Shiojiri et al. 2010, Cai et al. 2011). Here, we studied whether the phenological stage of corn seedlings influence the attraction of the predatory lacewing to volatiles emitted by seedlings infested with the corn aphid. As corn seedlings gradually become less resistant to the corn aphid due to reducing levels of DIMBOA, we hypothesized that later phenological stages of corn seedlings would emit more attractive volatiles to the predator since the aphid infestation is greater.

Our results confirmed our initial hypothesis that plant phenological stage interferes on the prey density, likely leading to consequences in the blend of HIPVs and the natural enemy behavior. The predatory lacewing *C. externa* was attracted to HIPVs emitted by corn seedlings older than V5 stage, which coincided with greater aphid colony settlement on the plant.

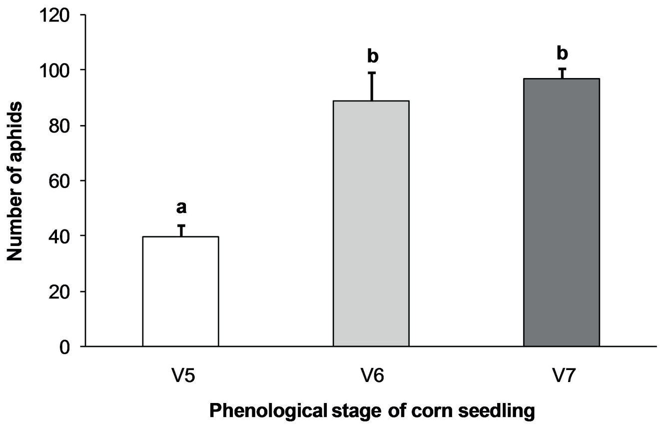

Figure 2. *Rhopalosiphum maidis* colony size (mean ± SE) on corn seedlings at V5, V6 and V7 phenological stages. Seedlings were initially infested with 100 aphids (varying ages) and left for 72h before counting.

Several works have demonstrated that concentration of DIMBOA is reduced along the course of corn plant development and, because of its feeding deterrent effect, aphid survival and reproduction are negatively affected (Argandoña et al. 1980, Corcuera et al. 1982, Bohidar et al. 1986, Hansen 2006, Ahmad et al. 2011). Although we did not measure DIMBOA concentration, our results suggest that the concentration in corn seedlings drastically reduces from V5 stage to V6 stage. Aphid initial population on corn seedlings at V5 stage decreased about 60% after 72h, while this reduction was of 10% in corn seedlings at V6 stage. The remnant aphids on corn seedlings at V5 stage might also feed less on the plant because of the antifeedant effect of DIMBOA.

Therefore, the lack of attraction of aphid-infested corn seedlings at V5 stage to the lacewing was likely due to the herbivore induction level, considering the low aphid population and the antifeedant effect of DIMBOA. As a result, aphid-infested corn seedlings at V5 stage may not have released HIPVs, or the blend composition of HIPVs emitted was not recognized by the predatory lacewing. In contrast, the two-fold larger aphid population on corn seedlings at V6 and V7 was sufficient for inducing the emission of an attractive blend of HIPVs to *C. externa* larvae. Future studies should measure DIMBOA levels and compare volatile chemical profiles of corn seedlings at V5 to V7 stages upon different levels of aphid infestations.

Authors' Contributions

MCO: experimental design, collected data and wrote the manuscript; PP: analysed and interpreted data and wrote the manuscript; MFGVP: experimental design and wrote the manuscript. All authors revised and approved the manuscript final version.

Acknowledgements

We thank Andrea Torres for the technical assistance. This study was financed by the Scientific Initiation Program of Lavras Federal University (UFLA) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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