Research Article

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Plant volatiles guide the new pest *Dichelops furcatus* to feed on corn seedlings

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Abstract

BACKGROUND: Recently, in temperate and neotropical regions of South America the generalist stink bug *Dichelops furcatus* (Hemiptera: Pentatomidae) became a new pest of corn (*Zea mays*) seedlings. Implementation of no-tillage cultivation system left organic matter covering the soil, which shelters adults of stink bugs during winter. In spring, corn is sowed under soybean stubble and *D. furcatus* adults start to feed on seedlings. To determine corn-derived volatile organic compounds (VOCs) that attract this stink bug species, we evaluated stink bug preferences from two corn hybrids with contrast germplasm backgrounds, a temperate and a tropical hybrid.

RESULTS: Stink bugs preferred to feed on temperate seedlings rather than on the tropical ones. GC–MS and PCA analysis of VOCs suggested that hybrids emitted contrasting blends. Linalool represented 68% of total VOCs emitted from temperate corn, while in the tropical hybrid this compound represented 48%. Olfactometer experiments demonstrated that linalool was attractive to stink bugs. However, 2 h of *D. furcatus* attack induced emission of 14 additional VOCs in temperate seedlings, and olfactometer bioassay and blend of VOCs emission suggested that perceived volatiles by stink bugs induced feeding avoidance. The increment of VOCs emission was associated with the induction of JA, JA-Ile, ABA, and IAA, and decreasing of SA concentrations.

CONCLUSION: This is the first time showing a complete profile of defensive phytohormones induced by stink bugs feeding on corn, and further demonstrating that a blend of corn seedling-associated VOCs, mainly composed by linalool, modulates *D. furcatus* adults' behavior and feeding preferences.

Supporting information may be found in the online version of this article.

Keywords: stink bugs; *Dichelops furcatus*; corn seedlings; organic volatile compounds; Phytohormone regulation; linalool

1 INTRODUCTION

Corn (*Zea mays* L.) is one of the most important commodities worldwide. Only Argentina and Brazil allocate more than 20 million hectares to field corn, providing almost 10% of world production.[1] Frequently different insect pests attack corn and decrease crop yield. In temperate and neotropical regions of South America, the generalist stink bug *Dichelops furcatus* F. has recently become a

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new pest of corn at seedling stage. Stink bugs feed on plants by inserting toxic saliva and sucking fluids from damaged tissues.[2,3] Adults of *D. furcatus* feed on seedlings by inserting their stylets near the coleoptile, mechanically and/or chemically macerating plant tissues. Consequences of corn damage ranged from leaves with symmetric punctiform holes to aborted seedlings.[4]

Although some decades ago in Argentina and Brazil *D. furcatus* was considered a secondary pest of soybean (*Glycine max* L. Merr.) crops at reproductive stage.[5,6] In recent years pest status of this stink bug species has progressively changed. Adults of *D. furcatus* have become a serious problem not only to soybean, but also recently to field crops, such as corn.[4] It has been suggested that the implementation of a no-tillage cultivation system by Argentinean and Brazilian farmers has changed the pest status of *D. furcatus*.[6] A no-tillage system avoids soil disturbance and leaves organic matter covering the soil to control soil erosion,[7] but plant stubble left by this system is used as shelter by adult *D. furcatus*. Consequently, once soybean crops are harvested, overwintering adults are able to spend the colder months in the field under crop stubble. In spring, corn is sowed under soybean stubble and higher temperature increases the activity of *D. furcatus* adults that start feeding on corn seedlings. Understanding how stink bugs find hosts, such as corn seedlings in spring can help to control this pest.

Host-plant recognition by insects is based on detection of a particular blend of host-associated volatile organic compounds (VOCs) in a complex background constituted by non-host plants.[8] VOCs blends are a combination of fatty acid-derived green leaf volatiles (GLVs), terpenoids, and phenolic compounds.[8-10] Herbivores are capable of recognizing not only the presence or absence of specific compounds, but also the ratios of ubiquitous VOCs emitted by host plants.[11] Corn seedlings displayed a specific volatile blend depending on genetic background[12] and abiotic factors.[13] However, there are several VOCs that overlap through blends among contrasting corn germplasms, as myrcene, linalool, hexenyl acetate, and caryophyllene.[12] Moreover, corn seedlings respond to herbivory damage by altering their volatile profile, by synthesizing new compounds and changing the amounts of constitutive VOCs.[14-16] Feeding injury produced by the stink bug species *Nezara viridula* L. increased two-fold emission of linalool, (E)-β-caryophyllene, α-trans-bergamotene, and (E,E)-β-farnesene, but not GLVs in corn seedlings.[17] Stink bug-induced plant volatiles may repel herbivores and/or attract their natural enemies.

VOCs emission is regulated by different pathways that allow plants to perceive herbivore attack through mechanical damage and elicitors present in oral secretions.[3,18-20] Stink bug-attacked plants can respond with distinctive signaling pathways, by triggering a series of fine-tuning signaling. Developing seeds of field-grown soybean triggered an early response to *N. viridula* herbivory, by increasing simultaneously jasmonic acid (JA), and salicylic acid (SA) levels and the emission of ethylene (ET).[20] These plant responses are mediated by mitogen-activated protein kinases (MAPK) signaling, and induced chemical defenses against herbivores.[20] Moreover, soybean seeds restructured their cell walls after stink bug attack and accumulated isoflavonoids and protease inhibitors, affecting insect feeding preference.[20–23] Although some studies have identified and quantified constitutive and herbivore-induced volatiles from corn seedlings and phytohormonal crosstalk, evidence comes from plants damaged by chewing herbivores, as lepidopterans larvae,[19,24,25] or aphids with piercing-sucking mouthparts that feed directly from the phloem.[24,26] However, the feeding strategy of stink bugs provokes a different kind of tissue damage in comparison with those triggered by aphids or chewing insects. Stink bugs use stylets to pierce and inject saliva with digestive activity and organic compounds that induced plant responses.[3] Whereas corn seedlings treated with regurgitant of lepidopterans larvae increased JA, decreased SA levels and trigger VOCs emission in corn seedlings,[16] effectors present in aphid saliva have opposite effects, triggering SA pathway[27] and reminding VOCs constitutive levels.[24] However, there is no information about corn seedling phytohormonal responses to stink bug herbivory. Recognizing VOCs from corn seedlings and hormonal regulation that modulate *D. furcatus* adults' behavior and feeding preferences will help farmers to find ways to manage this new and important pest.

Plant VOCs allow insects to identify appropriate host plant species.[28] To study the orientation of *D. furactus* adults by VOCs, we compared stink bug feeding preference and olfactory orientation through free choice tests in olfactometer between two contrasting commercial hybrids of corn (temperate and tropical) with different susceptibility to herbivory. Moreover, we analyzed VOCs emitted by undamaged and damaged corn hybrids and identified changes in emission profiles. To study the regulation of VOCs emission, we quantified phytohormones' content in damaged and undamaged plant tissue. Corn hybrids showed different VOCs profile emission and stink bugs were able to discriminate between them. Additionally by using pure linalool we identified this compound as a main cue for stink bug orientation. We found that VOCs' emission after damaging is regulated by JA in corn seedlings.

2 MATERIALS AND METHODS

2.1 Plant material

Two corn hybrids, one temperate (P1780YR) and one tropical (P30B39HR) origin, were used for the experiments. Since previous studies showed different VOCs emission and preference of feeding by specialist herbivores between the tropical and temperate hybrids,[29] we used these hybrids to identify VOCs that allow *D. furcatus* adults to find and feed on corn seedlings. Corn seedlings were grown in plastic pots (180 mL) with commercial soil and under natural conditions of light, temperature and humidity (average temperature: 24 °C, average humidity 65%) at the experimental open field established at Agronomy School at the University of Buenos Aires, Argentina. Plants used for the experiments were 14 days old and possessed three fully developed leaves (V3 stage of corn).[30]

2.2 Insects

D. furcatus colony was established by individuals collected in rural areas near Buenos Aires. Adult stink bugs were placed in breeding plastic cages (37 × 28 × 21cm), with the upper side covered with nylon mesh (organdy type), under laboratory conditions (24 ± 2 °C, 60 ± 5% RH, 14:10 L:D, 50 μmol m^{-1} s^{-1}) for at least 7 days. Stink bugs were reared on raw peanuts (*Arachis hypogaea* L.), soybean and sunflower seeds (*Helianthus annuus* L.), fresh green beans, and water supplemented with 0,5% w/v ascorbic acid. Two cotton balls per cage were used as oviposition substrate. All materials were renewed three times per week. Equal number of adult males and females of *D. furcatus* were starved for 48 h before bioassays.

2.3 Plant treatments

Single V3 corn seedlings were placed inside a plastic cage (25.5 × 13.5 × 37 cm, clear, polypropylene) with a nylon mesh on the upper side, and used for bioassays. Cages were kept under laboratory conditions (24 ± 2 °C, 60 ± 5% RH, 14:10 L: D, 350 μmol m^{-1} s^{-1}). Treatments (feeding damaged, mechanical damaged, and undamaged) were randomly assigned to each experimental unit. To perform feeding damage, two adult insects per cage were placed on the seedling and feeding punctures were confirmed by visual observation. The insects were allowed to feed for 2 and 24 h. For mechanical damage, we used a dissection needle to produce punctures on corn seedlings mimicking stink bugs. Undamaged seedlings were kept under the same environmental conditions to be used as a control. Replicates number varied according to each bioassay requirement: Two choice bioassays = 20 replicates; Olfactometer bioassays = between 20 and 37 replicates; Volatile collection and chemical analysis = 10 replicates; Phytohormone determination = from four to five replicates; and Linalool bioassays = 16 replicates.

2.4 Two-choice bioassays

To assess herbivore preference between the two corn hybrids, two V3 potted corn seedlings (one from each hybrid) were offered to a single *D. furcatus* adult in the plastic cage (25.5 × 13.5 × 37 cm). Corn seedlings were separated by 13 cm apart. Stink bugs were allowed to feed for 24 h. Insect position and feeding behavior were recorded each 30 min for 7 h, and at the end of the assay (24 h; 20 replicates).

2.5 Olfactometer bioassays

The attractiveness of volatile compounds from V3 corn seedlings on *D. furcatus* (adults) was evaluated in olfactory two-choice tests using a stationary phase olfactometer (Supporting Information, Fig. S1). The olfactometer consisted of the same plastic cage mentioned before but vertically divided into three chambers by partition walls of double mesh, made of voile fabric and a plastic net, which allows orientation by volatiles while avoiding the use of visual cues. The experimental seedlings or a cardboard mummy were offered in the opposite chambers. Bioassays were performed during the day, between 10:00 and 16:00 h under the same laboratory conditions mentioned before. Insects were released in the central chamber (Supporting Information, Fig. S1) and left during 10 min for acclimatization. After 30 min, stink bugs that contacted one of the two mesh walls for at least 10 min were recorded as a choice, otherwise it was scored as 'no choice'. The olfactometer was cleaned with ethanol after each measurement (n = 20). The 'Y' olfatometer was not used for the experiments because the stink bugs did not walk well along the arms.

2.6 Volatile collection and chemical analysis

Headspace samples were taken by enclosing intact V3 potted corn seedlings into 2 L glass containers (one seedling per pot), maintained under laboratory conditions (25 ± 2 °C, 60 ± 5% RH). Plants were illuminated from above with blue and red LED lamps and high-pressure metal halide lamps, light intensity 350 μmol m^{-1} s^{-1}. A pump pushed and pulled air through the container at a constant rate of 500 mL min^{-1}, and the air was cleaned by activated charcoal-filter. Volatiles were collected into a trap (35 mg HayeSep Q, 80–100 mesh) for 6 h (from 10:00 a.m. to 16:00 p.m.). After the sampling period, volatile traps were eluted with 150 μL of dichloromethane containing dodecane (5 ng μL^{-1}) as an internal standard. Volatile samples (n = 10) were analyzed by coupling gas chromatography–mass spectrometry (GC/MS) (Agilent 7890A coupled to Agilent 5977 selective mass detector). A HP5ms capillary column was used (0.25-mm i.d., film thickness 0.25 μm). Samples (1 μL) were injected at 240 °C in a splitless mode. The carrier gas used was Helium at a rate of 0.75 mL min^{-1}. The column temperature was held at 35 °C for 1 min, and the temperature ramp consists on increasing 5 °C per min until it reached 100 °C, then the increase rate changed to 12 °C per min. When the temperature raised 230 °C, it was held for 10 min. Compounds were identified by comparing the mass spectra to those provided by NIST (National Institute of Standards and Technology), by comparison of retention times through the estimation of the Kovats retention index and by comparison with available analytical standards (α-pinene, 98%; β-pinene, 99%; Linalool oxide; 97% and farnesene mix of isomers provided by Sigma-Aldrich; 3-hexenyl acetate, 98%; eucalyptol, 99%; cis/-trans-ocimene solution; γ-terpinene, 98.5%; linalool, 99% and β-caryophyllene, 98% provided by Supelco).

2.7 Phytohormone determination

10 mg dry weight (DW; mg $plant^{-1}$) of seedling was used for determinations. Plant material was shaken at room temperature for 30 min in the Starlab shaker, with 1 mL of MeOH:Water (7:3) solution containing, 20 μg of d4-SA, and d5-IAA, and 10 μg of d6-JA and d6-ABA, as internal standards. Samples were centrifuged at 16 000 *g* at 4 °C for 5 min. The supernatant was transferred to a new 1.5 mL Eppendorf tube. The supernatant mixture was evaporated in a speed vacuum program at 45 °C for 2.5 h. The dried extracts were dissolved in 100 μL of methanol: water (1:1) solution with 0.05% formic acid and then were vortexed and centrifuged at 16 000 *g* at 4 °C for 10 min. The supernatant was transferred to labeled HPLC vials using a piece of cotton and stored at −20 °C until measurements. The assay used at least four biological replicates. The quantification of phytohormones was performed by HPLC-MS/MS according to Almeida Trapp *et al.*[31] in an Agilent 1100 HPLC system coupled to Ion trap mass spectrometer (Thermo Scientific, Bremen, Germany).

2.8 Bioassay with linalool

To test if linalool can be a cue in host recognition by *D. furcatus*, three different olfactory two-choice tests were performed by using the previously mentioned stationary phase olfactometer (Supporting Information Fig. S1). Since temperate V3 seedlings (fresh weight 87 ± 2 mg) released near 60 ng of linalool per hour (Supporting Information, Table S1), the concentration was calculated by referring to the internal standard dodecane. Thus, we used 30 ng (the quantity released in 30 min) for the behavioral assays. We did not correct this quantity by the dry weight of the samples. We prepared a stock solution with 6 μL of the synthetic standard of linalool in 1 mL of dichloromethane (5.15 μg μL^{-1}.). We took 1 μL from the stock solution and we diluted in 1 mL of dichloromethane to prepare the working solution (5 ng μL^{-1}). We used 6 μL (which correspond to 30 ng of linalool) from the working solution for each linalool bioassay repetition. In all cases linalool diluted in CH_2Cl_2 (5 ng μL^{-1}) or the solvent alone was applied onto a filter paper (1 × 2.5 cm) over a microscope slide. Linalool (6 μL) was applied by mimicking the headspace amount released by temperate hybrids over 30 min. Filter papers and corn seedlings were replaced and the olfactometers were cleaned with ethanol after each repetition. A single stink bug was released in the olfactometer central chamber. After 30 min a choice was

scored when the stink bug contacted a wall mesh for more than 10 min. Stink bug preference was tested in the following comparisons: (i) Linalool *vs* solvent; (ii) Linalool *vs* temperate seedling + solvent; and (iii) Linalool + tropical seedling *vs* temperate seedling + solvent. At least 16 replicates per contrast were conducted.

2.9 Statistical analyses

Stink bug choices observed in the two-choice assay, the olfactometer bioassay and the bioassay with linalool were analysed by Chi-square goodness-of-fit test (χ^2) ($P < 0.05$). For a correlation comparison of VOCs blends, Principal Component Analysis (PCA) was performed by using all the compound classes collected in measurable rates *via* vegan R package (Oksanen *et al*., 2018). Individual VOCs and phytohormone data were analyzed by two-way and one-way analysis of variance (ANOVA) respectively. Data were normalized by logarithmic transformation and pairwise comparisons were conducted using Duncan test ($P < 0.05$). We used Kruskal-Wallis H test if ANOVA assumptions were not met.

3 RESULTS

3.1 Two choice bioassays in undamaged plants

During the first 2 h of the preference choice experiment, a higher number of *D. furcatus* adults preferred to feed on temperate than on tropical hybrids ($P < 0.05$; Fig. 1). After that period, stink bugs remained out of the plants for the next 22 h.

3.2 Undamaged seedlings

3.2.1 Olfactometer bioassays

A higher proportion of *D. furcatus* adults preferred to move toward either temperate or tropical corn seedling rather than the control made of cardboard (χ^2, $P < 0.05$; Fig. 2(A)). However, adults of *D. furcatus* showed a higher preference for temperate (67%) than to tropical (33%) hybrids ($P < 0.05$; Fig. 2(A)).

3.2.2 Volatile analysis

Seedlings of both hybrids emitted at least 8 different constitutive VOCs (Supporting Information, Table S1). Quantitatively, seedlings of temperate corn emitted three times more volatiles than the tropical ones (ANOVA, $P < 0.05$; Supporting Information, Table S1). While high emission levels of β-pinene, linalool, ethyl benzoate and (E)-β-farnesene were found from seedlings of the temperate hybrids, emission of (+) cyclosativene was found from the tropical ones (ANOVA, $P < 0.05$; Supporting Information, Table S1). To analyze VOCs correlation between hybrids, the quantitative data of constitutive VOCs were subjected to the multivariate technique principal component analysis (PCA; Fig. 2(B)). PCA analysis explained 52.7% of the total variation of VOCs emission between hybrids and samples taken from seedlings grouped separated, suggesting that each type of hybrid emits different blends. While the main compounds that contribute to the first axis (PC1, Eigenvalue 3.11) were linalool, (E)-β-farnesene, ethyl benzoate, nerolidol acetate, β-pinene, (2E,6E)-farnesyl acetate and methyl salicylate, β-ocimene and (+) cyclosativene contributed to the second axis (PC2, Eigenvalue 2.15; Supporting Information, Fig. S2).

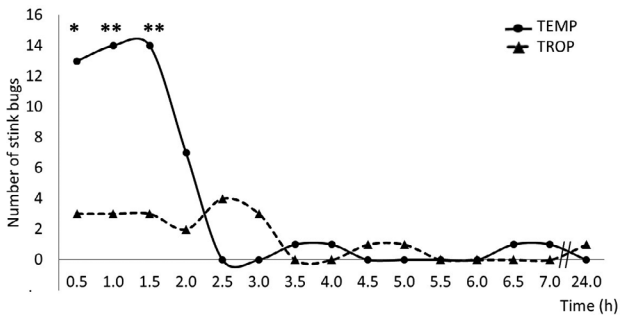

Figure 1. Dichelops furcatus preference between tropical (TROP) and temperate (TEMP) corn seedlings. Two choice bioassay between TEMP and TROP seedlings. Total number of stink bugs feeding on one of both commercial hybrids along time. Chi-square goodness-of-fit (χ^2), * $\chi^2 = 6.25$, $P = 0.012$; ** $\chi^2 = 7.12$, $P = 0.008$.

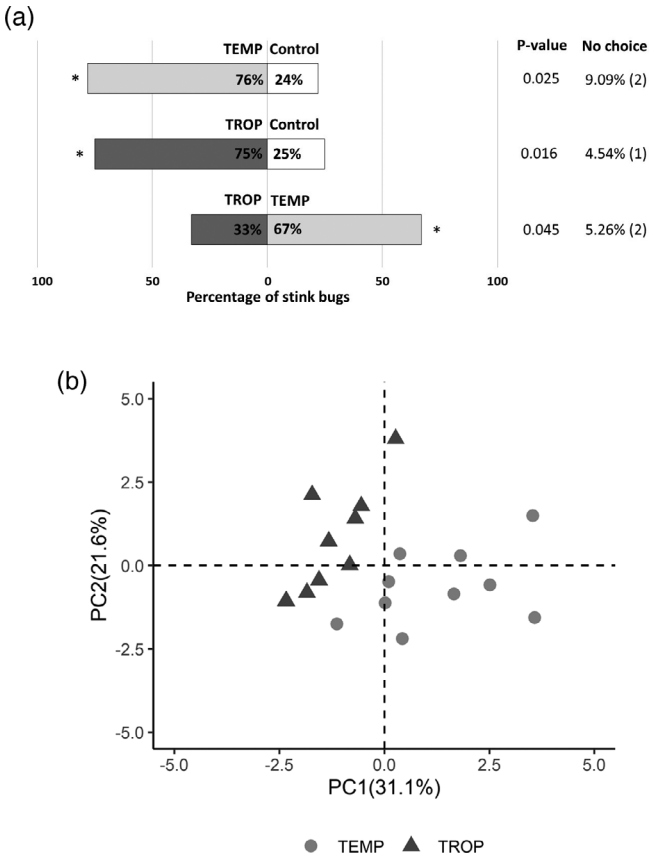

Figure 2. Dichelops furcatus olfactory orientation and volatile analysis in undamaged temperate (TEMP) and tropical (TROP) corn seedlings. (A) Percentage of *D. furcatus* adults contacting one side of the olfactometer for at least 10 min. Chi-square goodness-of-fit (χ^2) *$P < 0.05$ (Statistical details: Supporting Information, Table S2). (B) Principal Component Analysis (PCA) showing separation of genotypes according to constitutive VOCs (Factors 1, PC1 and 2, PC2). Percentage of the variance explained by each axis is shown between parentheses. The data represents the datasets of both hybrids: TEMP, grey dots; TROP, dark grey triangles. N = 10 in all cases.

3.3 Seedlings damaged by stink bugs

3.3.1 Olfactometer bioassays

A significantly higher percentage of *D. furcatus* adults were attracted to undamaged temperate seedlings than to those previously damaged by stink bugs ($P < 0.05$; Fig. 3(A)). However, 2 h of stink bug damage did not significantly affect adult's preference between undamaged and damaged tropical seedlings (Fig. 3 (A)). Interestingly, 24 h after stink bug damage there were no differences in adult's preferences between undamaged and damaged seedlings of either tropical or temperate hybrids (Fig. 3(A)).

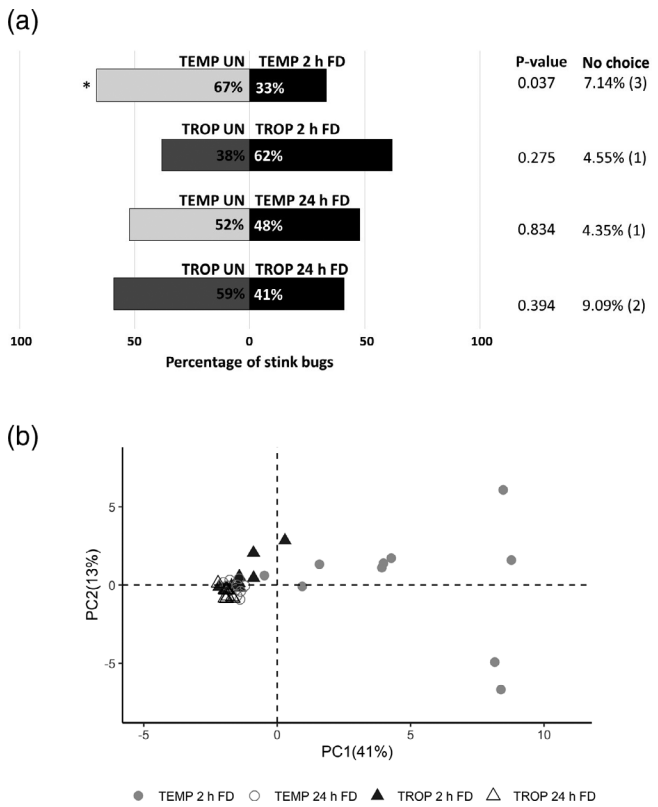

Figure 3. Dichelops furcatus olfactory orientation and volatile analysis in feeding damaged temperate (TEMP) and tropical (TROP) corn seedlings. (A) Percentage of *D. furcatus* adults contacting one side of the olfactometer in the following choices: TEMP seedlings undamaged (TEMP UN) *versus* feeding damage (FD) treatments: 2 h (TEMP 2 h FD) and 24 h (TEMP 24 h FD) after feeding, and TROP seedlings undamaged (TROP UN) *versus* 2 h (TROP 2 h FD) and 24 h (TROP 24 h FD) after feeding. Chi-square goodness-of-fit (χ^2) *$P < 0.05$ (Statistical details: Supporting Information, Table S2). (B) Principal Component Analysis (PCA) showing separation of genotypes according to *D. furcatus* feeding treatment (Factors 1, PC1 and 2, PC2). Percentage of the variance explained by each axis is shown between parentheses. The data represents the datasets of both treatments to the two hybrids: TEMP 2 h after FD, grey dots; TEMP 24 h after FD, empty dots; TROP 2 h after FD, dark grey triangles; and TROP 24 h after FD, empty triangles. N = 10 in all cases.

3.3.2 *Volatile analysis*

After 2 h of *D. furcatus* damage, VOCs emission increased in tropical and temperate seedlings. Fourteen out of 26 volatile compounds identified were emitted exclusively by temperate seedlings after 2 h of stink bug damage, such as indole, (Z)-3-hexenyl acetate, eucalyptol, γ-terpinene, (E)-geranyl acetate, β-caryophyllene, β-sesquiphellandrene and homosalate (in all cases Kruskal Wallis Test $P < 0.05$; Supporting Information, Table S1). However, after 24 h of stink bug herbivory emission of total amounts of VOCs returned to constitutive levels in seedlings of temperate and tropical hybrids (ANOVA $P < 0.05$; Supporting Information, Table S1). PCA analysis explained 54% of the total variation in VOCs emission and only the blend from temperate seedlings emitted 2 h after herbivory separated from the rest of the blends (Fig. 3(B)). While (Z)-3-hexenyl acetate, indole, β-caryophyllene, eucalyptol, β-sesquiphellandrene, and (3E,7E)-4,8,12-trimethyl-1,3,7,11-decatetraene (TMTT) contributed to the first axis (PC1, Eigenvalue 10.6), the second axis (PC2, Eigenvalue 3.4) showed a linear relationship between β-pinene and (E)-β-ocimene (Supporting Information, Fig. S3).

3.4 Mechanically damaged seedlings

3.4.1 *Olfactometer bioassays*

Mechanical damage did not affect the preference of *D. furcatus* adults between undamaged and damaged seedlings of both hybrids. A similar proportion of stink bugs contacted both sides of the olfactometer when contrasted undamaged *vs*. mechanically damaged seedlings (ns; Fig. 4(A)).

3.4.2 *Volatile analysis*

Seedlings of both hybrids damaged mechanically emitted different blend of VOCs between 2 and 24 h (Fig. 4(B)). In addition, both hybrids emitted similar VOCs amounts and blends after damage. From the 20 identified sesquiterpenes compounds, most of them (γ-terpinene, ylangene, β-caryophyllene, β-gurjunene, α-amorphene, α-farnesene, and β-sesquiphellandrene) were released 2 h after damage and no longer after 24 h (Fig. 4(B); Supporting Information, Table S1). PCA analysis explained the 54% of the total variation, and VOCs blends were more variable 2 than 24 h after mechanical damage (Fig. 4(B)). While the first axis (PC1, Eigenvalue 7.73, 37%)

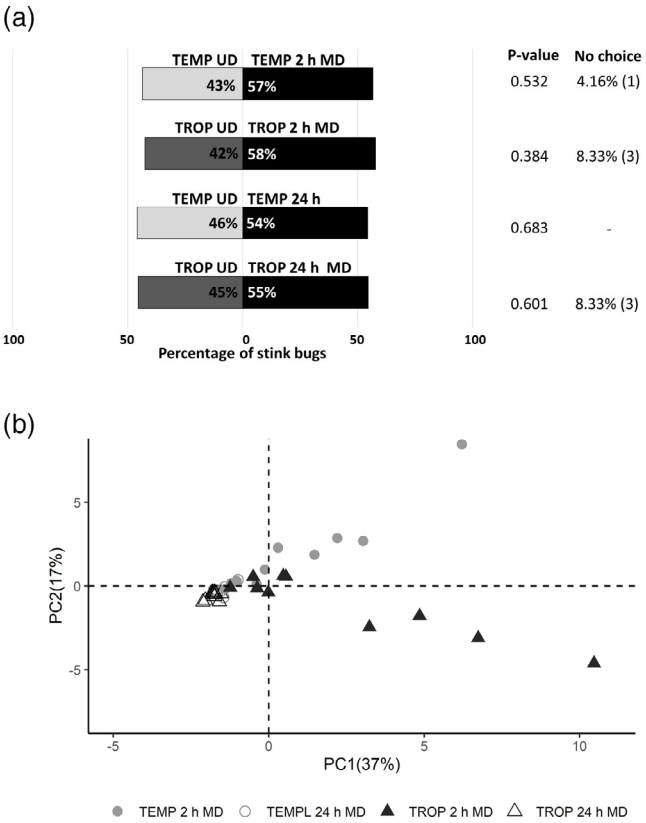

Figure 4. Dichelops furcatus olfactory orientation and volatile analysis in undamaged and mechanically damaged temperate (TEMP) and tropical (TROP) corn seedlings.(A) Percentage of *D. furcatus* adults contacting one side of the olfactometer in the following choices: TEMP seedlings undamaged (TEMP UN) *versus* mechanical damage (MD) treatments: 2 h (TEMP 2 h MD) and 24 h (TEMP 24 h MD) after damage and TROP seedlings undamaged (TROP UN) *versus* 2 h (TROP 2 h MD) and 24 h (TROP 24 h MD) after damage. Chi-square goodness-of-fit (χ^2) *$P < 0.05$ (Statistical details: Supporting Information, Table S2). (B) Principal Component Analysis (PCA) showing separation of genotypes according to mechanical damage treatment (Factors 1, PC1 and 2, PC2). Percentage of the variance explained by each axis is shown between parenthesis. The data represents the datasets of both treatments to the two hybrids: TEMP 2 h after MD, grey dots; TEMP 24 h after MD, empty dots; TROP 2 h after MD, dark grey triangles; and TROP 24 h after MD, empty triangles. N = 10 in all cases.

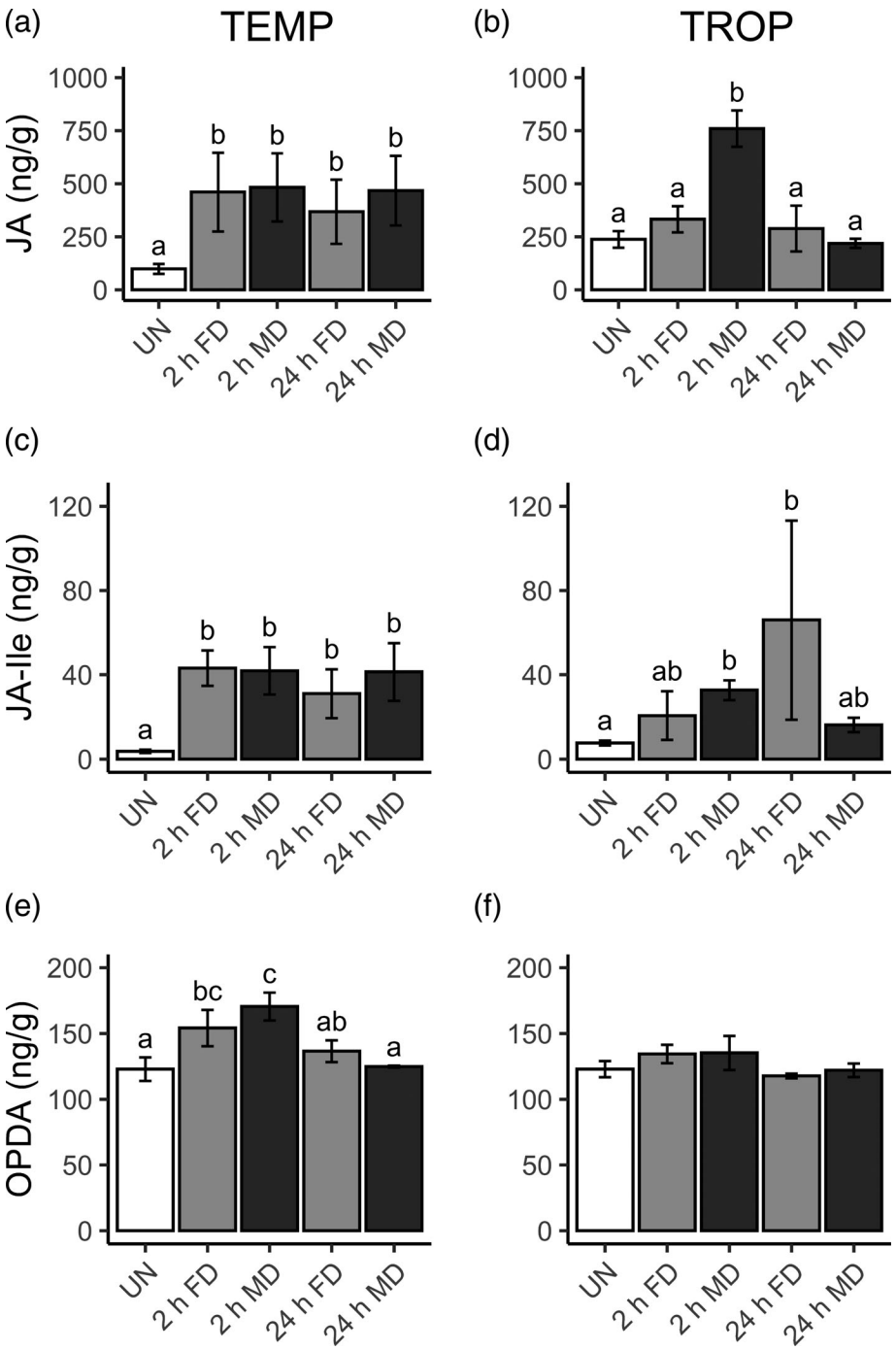

Figure 5. Hormonal determination (mean ± standard error) of jasmonic acid (JA) (A and B), jasmonic acid – isoleucine (JA-Ile) (C and D) and 12-oxo-phytodienoic acid *(OPDA)* (E and F) measured in undamaged (UN), 2 and 24 h after feeding damage (FD) and 2 and 24 h after mechanical damage (MD) of temperate (TEMP) and tropical (TROP) seedlings. Different letters denote significant differences ($P < 0.05$, ANOVA) (Statistical details: Supporting Information, Table S3).

positively correlated with ethyl benzoate, methyl salicylate, β-gurjunene, α-amorphene, α-farnesene, and TMTT, the second axis (PC2, Eigenvalue 3.61, 17%) correlated with (+) cyclosativene and calamanene (Supporting Information, Fig. S4).

3.5 Phytohormonal determination

Although 2 and 24 h after mechanical damage or herbivory JA and JA-Ile levels in temperate seedlings increased (ANOVA, $P < 0.05$; Fig. 5(A), (C)), in tropical seedlings JA and JA-Ile increased after 2 h of mechanical damage, and JA-Ile also after 24 h of herbivory (ANOVA, $P < 0.05$; Fig. 5(B), (D)). The biosynthetic jasmonate precursor OPDA was induced in temperate seedlings after 2 h of mechanical damage or herbivory but not in tropical seedlings (ANOVA, $P < 0.05$; Fig. 5(E), (F)).

SA levels were decreased in seedlings of both hybrids after 2 h of mechanical damage and herbivory (ANOVA, $P < 0.05$; Fig. 6

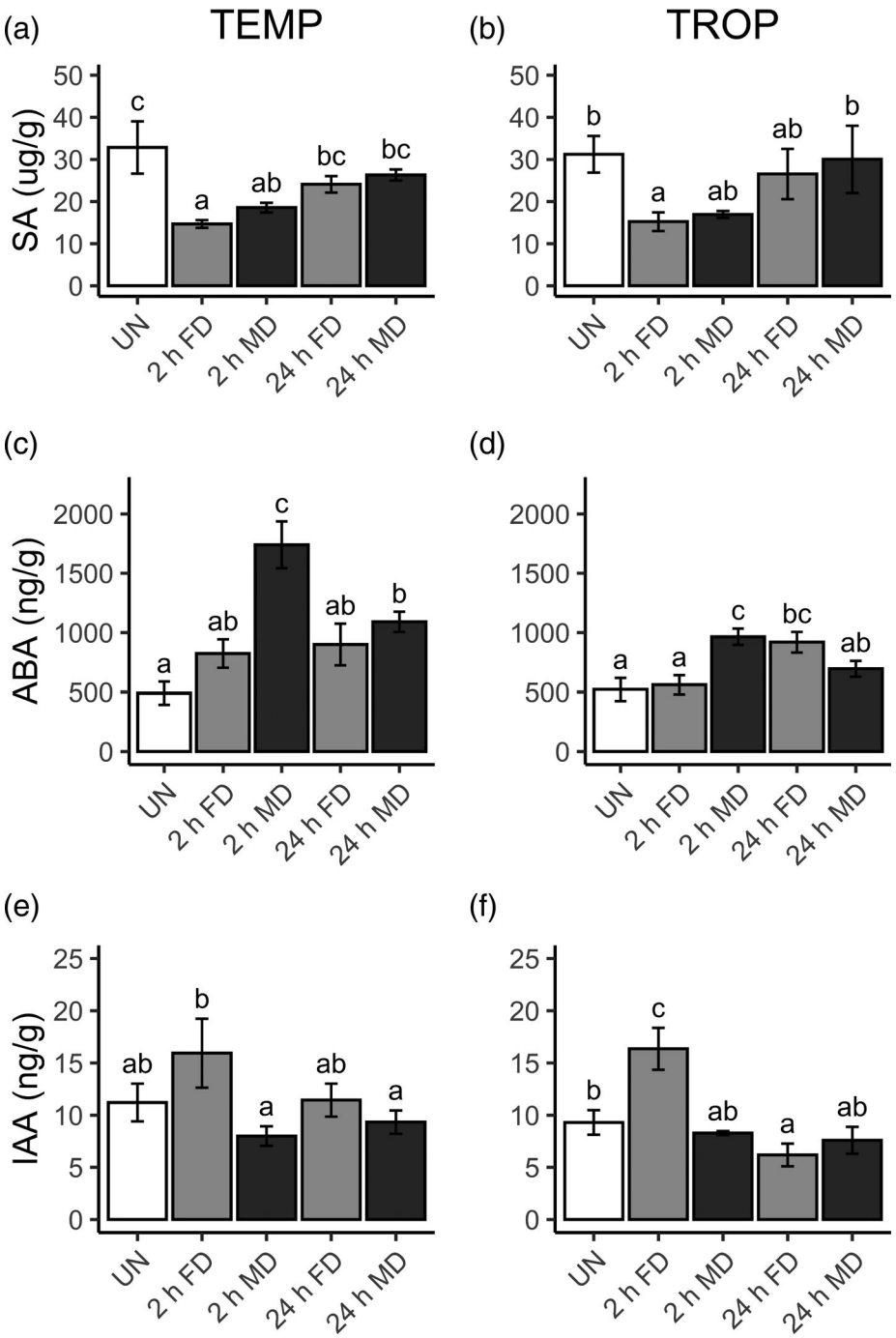

Figure 6. Hormonal determination (mean ± standard error) of salicylic acid (SA) (A and B), abscisic acid (ABA) (C and D) and indole acetic acid (IAA) (E and F) measured in undamaged (UN), 2 and 24 h after feeding damage (FD) and 2 and 24 h after mechanical damage (MD) of temperate (TEMP) and tropical (TROP) hybrids. Different letters denote significant differences ($P < 0.05$, ANOVA) (Statistical details: Supporting Information, Table S3).

(A), (B)). ABA increased in seedlings of both hybrids after 2 h of mechanical damage and in the temperate hybrid also after 24 h (ANOVA, $P < 0.05$; Fig. 6(C), (D)). IAA levels increased 2 h after herbivory in both hybrids (ANOVA, $P < 0.05$; Fig. 6(E), (F)).

3.6 Bioassay with linalool

A higher number of *D. furcatus* adults was attracted to linalool in comparison with solvent control ($P < 0.05$; Fig. 7). Nonsignificant differences were found in stink bug preferences when comparing the temperate hybrid + solvent sample *vs.* linalool ($P < 0.05$). Interestingly, the less attractive tropical hybrid was as attractive as the temperate hybrid when linalool was added ($P < 0.05$), supporting the role of linalool as an attractant (Fig. 7).

4 DISCUSSION

Understanding cues that guide *D. furcatus* adults to corn seedlings in spring can help to develop behavioral manipulation

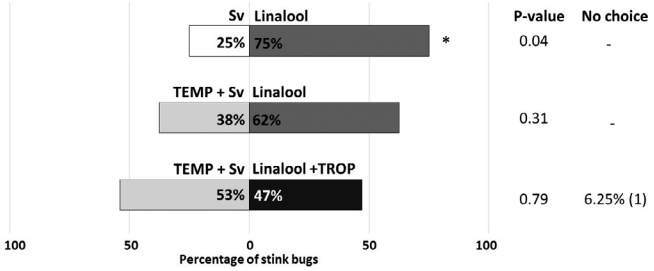

Figure 7. Dichelops furcatus olfactory orientation to Linalool: Percentage of *D. furcatus* adults contacting one side of the olfactometer for at least 10 min. Chi-square goodness-of-fit (χ^2) *$P < 0.05$. Comparisons consisted on Linalool *vs* solvent (Sv); Linalool *vs* temperate seedling (TEMP) + Sv; and Linalool + tropical seedling (TROP) *vs* TEMP + Sv (Statistical details: Supporting Information, Table S2).

strategies and divert insect damage. Detection of a particular cue as a blend of host-associated volatile organic compounds (VOCs) allows insects to identify hosts within a complex background constituted by non-host plants.[8] In this study, our experiments with the olfactometer demonstrated that linalool by itself attracted stink bugs (Fig. 6), and suggested that *D furcatus* adults orientated to corn seedlings through a blend of host-associated VOCs mainly composed by linalool. Moreover, stink bug attack increased JA, JA-Ile, ABA, and IAA, and decreased SA concentrations concomitantly with the increment of VOCs emission that induced feeding avoidance (Figs 1, 3(A), 5, and 6 and Supporting Information, Table S1). To our knowledge, no study before has shown a complete profile of defensive hormones induced by stink bugs feeding on corn and demonstrated that a blend of corn seedling-associated VOCs modulates adults of *D. furcatus* behavior and feeding preferences.

While some studies showed the importance of VOCs emitted by stink bug herbivory in tritrophic interactions,[32] little work has been carried out on the role of plant VOCs in the foraging ecology of stink bugs. Previous studies suggested that polyphagous species, such as *D. furcatus* may be attracted to GLVs that are generally expressed across a wide range of taxa.[33] However, our results suggested that mainly terpenoids are involved in the attraction of *D. furcatus* adults to corn seedlings. The PCA analysis showed that linalool, (E)-β-farnesene, ethyl benzoate, nerolidol acetate, β-pinene, (2E, 6E)-farnesyl acetate, and methyl salicylate were the main compounds in the first axis, and β-ocimene and (+) cyclosativene contributed to the second axis (Fig. 2; Supporting Information, Fig. S2). Moreover, seedlings of both hybrids emitted at least 10 different constitutive VOCs, and seedlings of temperate corn emitted three times more volatiles than the tropical ones (Supporting Information, Table S1). Quantity and quality composition of corn seedling blends are genetically determined.[34] Corn high-yielding cultivars have narrow genetic backgrounds as a result of systematic inbreeding programs.[35] Therefore, each hybrid blend depends on the alleles of the genes received across its parental lines that encode or regulate the enzymes involved in the odor compounds biosynthesis.[34] However, there are compounds as linalool commonly released by undamaged corn seedlings from a wide range of germplasms,[34] which can be used as a cue for corn location by herbivores,[36] as described for caterpillars of the generalist *Spodoptera frugiperda*[37] and the specialist leafhopper *Dalbulus maidis*.[29] In this study, the stink bug attraction and preference to feed on the undamaged temperate seedlings, in combination with olfactometer experiments suggested that linalool can be used by *D. furcatus* adults as the main cue for orientation to find and feed on corn seedlings.

Emitting VOCs as response to damage is a mechanism of plant defense that may repel herbivores and attract their natural enemies. In this study, *D. furcatus* attack increased VOCs emission of temperate seedlings and induced feeding avoidance (Figs 1–3). Although after herbivory linalool was still the most abundant compound emitted by both hybrids, other compounds with potential repellent effects were emitted by damaged seedlings (Supporting Information, Table S1). Some terpenoids induced in the temperate hybrid are bitter defense compounds against herbivores, such as caryophyllene and TMTT.[29] In addition, temperate seedlings emitted other defensive compounds, as ethyl benzoate and DMNT, and higher VOCs quantities than the tropical ones. Similarly, herbivore damage induced corn volatiles that were repellent to winged aphids (*Rhopalosiphum maidis*).[38] Moreover, damaged temperate seedlings emitted other VOCs typically released immediately after insect attack and usually related to the attraction of generalist parasitoids.[39] Attacked temperate seedlings released more mono, homo and sesquiterpenes, GLVs (*i.e.* (Z)-3-hexenyl acetate), and aromatic compounds (*i.e.* indole) than tropical seedlings. However, mechanical damage induced emission of different blend of VOCs in both hybrids than those induced by herbivory (Fig. 3). In addition, mechanical damage did not induce feeding avoidance in the temperate seedlings (Fig. 3), suggesting the participation of *D. furactus*'s saliva in the modulation of VOCs emission. Herbivore saliva can modulate VOCs emission in an intra-specific way which might depend on corn hybrids genetic background.[19] The watery saliva of Stink bugs is one of the first fluids to come in contact with the plant during herbivory, and its possible effectors on defensive hormonal pathways have been recently studied.[2,3,20]

Plant recognition of stink bug damage leads to rapid transcription and activation of MAPK signaling pathways that induce JA/ET and SA-regulated defenses.[3] Although it is well known that induction of JA increases VOCs emission,[40] the role of the other hormones in fine-tuning regulation of qualitative VOCs emission is not completely understood. As expected, we observed that both stink bug and mechanical damage increased JA and JA-Ile and decreased SA concentrations in seedlings of the tropical and temperate hybrids (Figs 5 and 6). The JA and SA pathways are often antagonistic; elicitation of the JA pathway may repress SA defense responses.[41] Whereas the induction of jasmonates and OPDA after 2 h of herbivory in temperate seedlings may explain the higher emission of VOCs (Figs 3 and 5), mechanical damage induced concomitantly JA and ABA, and a particular blend of VOCs in both hybrids (Figs 4 and 6). The interaction between JA and ABA activates defenses related to herbivory and mechanical damage.[42] Although IAA was induced in both hybrids after 2 h of herbivory (Fig. 6), indole that has been described as a precursor in the biosynthesis of IAA in *Arabidopsis thaliana*, was induced only in the temperate seedlings.[43] Our results suggest that hormonal regulations of VOCs emission are constrained by the genetic background of each corn hybrid.

Even with the importance of this new pest of corn, little is known about the ecology and interactions between adults of *D. furcatus* and corn seedlings. Previous studies have mainly focused on stink bug-soybean interactions.[20–23,44–50] The ecological behavior and versatility displayed by *D. furcatus* in host selection make this species an invasive pest that can colonize new niches. This stink bug species makes use of the plant stubble left by the no-tillage system as shelter, which allows them to feed on corn seedlings in spring.[4]

Our results suggest that olfaction plays a role in *D. furcatus* by identifying the right blend of VOCs as a cue to locate and feed on corn seedling. However, feeding avoidance induced by herbivory may be not only a consequence of new VOCs emitted by damaged seedlings, but also probably of direct defenses against herbivores induced in corn. Benzoxazinoids are typically regulated by JA and induced by herbivory in corn.[51] Identifying blends of VOCs that attract *D. furcatus* adults in other plant species can be used to manipulate insect's behavior, using trap plants or a push-pull strategy. In addition, odor orientation of *D. furcatus* could be exploited to develop management strategies, as the employment of attracting and killing traps.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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