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Effects of nitrogen application in maize (*Zea mays* L.) on host selection behaviour of the bird cherry-oat aphid (*Rhopalosiphum padi* L.)

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ABSTRACT

The nutritional status of plants affects the multitrophic plant-insect interactions, however to date, there is little evidence about such maize (*Zea mays* L.)- aphids interaction related to soil nitrogen content (N). Maize was cultivated under sufficient (15 mM) or limited (0.15 mM) nitrogen supply. We conducted the behavioural trials using a four-arm olfactometer and found that *Rhopalosiphum padi* L. preferred the maize grown under high-nitrogen conditions. The volatile organic compounds (VOCs) from maize were obtained from the plants grown under different nitrogen conditions and analyzed by gas chromatography-mass spectrometry (GC-MS). The relative amounts of (z)-2-hexenol, benzaldehyde and decane were significantly higher under high-nitrogen (15 mM) conditions than under low-nitrogen (0.15 mM) conditions (limited) nitrogen supply) and these VOCs attracted the wingless aphids. Under low-nitrogen conditions, higher contents of farnesol, cadinol, aristolene and acetic acid were found, however, the farnesol and acetic acid repelled the aphids. Furthermore, Electroantennogram (EAG) studies confirmed that aphid antennae responded to these compounds. Expressions of certain key genes associated with the synthetic pathways of (E)-2-hexenol, benzaldehyde, acetic acid and copaene were verified by Quantitative Real-time polymerase chain reaction (qRT-PCR). The chemical ecology of the interactions between maize (*Zea mays*) and aphids (*R. padi*) under different nitrogen doses was investigated to formulate the new pest management strategies.

Keywords: Bird cherry-oat aphid, Electroantennogram (EAG), GCMS, Maize, nitrogen, pest management, plant insect interactions, qRT-PCR, *Rhopalosiphum padi*, volatile organic compounds, *Zea mays* L.

INTRODUCTION

Maize (*Zea mays* L.) is most widely grown crop in the world (43). Nitrogen fertilizers are added in large amounts in modern agriculture for high crop yields (16). The nutritional status of plants affects the multitrophic plant-insect interactions. Plants on the one hand, provides necessary nutrients sugars, proteins and amino acids etc., for insect growth. Hence, this is an important factor in insect host selection. On the other hand, plants have direct and indirect defences against insects. The direct defence is a plant's defence strategy, which influences the host plant's resistance to insect damage (42). While in the indirect defence, plant releases volatile compounds to attract insect enemies to kill or drive insects away before the plants are attacked (4). Thus, the interactions between the plants and insects are complex process and are affected by many factors.

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The nutrition level and secondary metabolites are the basis of plant resistance. The plant nutrition level depends on the fertility status of growing soil. Therefore, fertilizer application is directly related to plant and insect interactions (44). For example, excessive nitrogen input reduces the C:N ratio in plants (11). A decrease in the C:N ratio affects the feeding behaviour and life cycles and may increase the number of herbivores (29,42). Overfeeding of herbivores directly affects the plant growth and development (32). Therefore, excessive fertilization is major cause of insect outbreaks in recent years.

Rhopalosiphum padi (L.) aphid is a major insect pest of cereal crops in many countries (23). Aphid feeding hinders the normal phloem transport of plant nutrients, delays the nitrogen transport to cells and disrupts the photosynthetic cell flow (39). The quality of plant nutrition can also affect the growth and reproduction of plant-eating insects, because aphids are sensitive to nitrogen levels in their host plants (20). Therefore, the differences in the nitrogen content in plant tissues is the main determinant for plant choice of herbivores (6). With increased nitrogen application, the amino acids and nitrate contents significantly increase in plants, which attracts more herbivores to plants (5). Although increased nitrogen input of plants changes the morphological characteristics of host plants, but also increases the growth and reproductive capacity of aphids (7).

Volatile organic compounds (VOCs) produced by plants are secondary metabolites with various biological characteristics and functions (24). Plant VOCs are categorized into terpenoids, amino acid derivatives, fatty acid derivatives and benzenoid compounds (14), These volatiles can be emitted from leaves into the atmosphere and then they interacts with their surroundings (25). VOCs can include monoterpenes, sesquiterpenes, shikimic acid pathway derivatives such as methyl salicylate (MeSA), as well as lipoxygenase (LOX) derivatives methyl jasmonate (MeJA) and C6-volatiles. C6-volatiles including the aldehydes trans-2-hexenal, hexanal and cis-3-hexenal and their corresponding alcohols as product of enzymatic activity of hydroperoxide lyase (HPL), a component of the lipoxygenase (LOX) pathway (21). Cinnamoyl-CoA hydratase/lyase catalyzes the hydration and cleavage the cinnamoyl-CoA to benzaldehyde and acetyl-CoA(2). Alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) play an important role in the synthesis of acetic acid (12). Copaene synthase encodes an enzyme that catalyzes the conversion of farnesyl diphosphate to a-copaene. By analyzing the expression of key genes in the signalling pathway, the effects of different nitrogen treatment conditions on the pathway can be known.

MATERIALS AND METHODS

Plant Samples

The maize cultivar B73 (obtained from the College of Plant Science, Jilin University, China) was sown in plastic pots (height : 20 cm, dia : 15 cm) containing a mixture of sterilized field soil and sand (3:1). The soil was nutrient-poor (organic matter 31.67 g/kg, total nitrogen 0.716 g/kg, total phosphorus 0.135 g/kg and available nitrogen 5.86 mg/kg). Fertilization started from the 7^{th} day of experiment with a modified Hoagland solution (5 mM CaCl2, 2mM MgSO4, 2 mg/L Fe, 0.5 mM KH2PO4, 50 mM H3BO4, 10 mM MnCl2, 1 mM ZnSO4, 0.3 mM CuSO4, and 0.5 mM Na2MoO4). There were two N treatments (i).

High Nitrogen/H^+ (Nutrient solution contained 15 mM KNO_3) and (ii). Low Nitrogen/H^- (Nutrient solution contained 0.15 mM) (33). The seedlings were grown in climate chamber (22 ± 2 ºC, 70 % relative humidity, 16 h:8 h light:dark). Three weeks after germination, 0.5 g maize leaves were used to collect the volatiles (15).

Insect rearing

Aphids were reared on maize plants for 6-months before use in experiments. A population of *R. padi* was obtained from single plants. The winged/wingless *R. padi* were reared in a rearing cabinet (22 ± 2 ºC, 70 % relative humidity, 16 h:8 h light:dark). Before the behavioural bioassays, the aphids were starved for 5 h in Petri dishes (9 mm dia) with moistened filter paper to prevent dehydration. The aphids were then kept in bioassay lab for 2 h to acclimatise prior to the experiments.

Volatile organic compounds collection from maize

Volatile organic compounds were collected from the maize leaves using a dynamic head space SPME method (3) under different nitrogen fertilizer conditions using solid-phase microextraction (SPME) fibre coated with poly-dimethyl-siloxane-divinylbenzene (PDMS-DVB, 65 mm) purchased from Supelco (Bellefonte, PA, United States). The SPME fibre was conditioned at 250 °C for 30 min in a gas chromatograph injection port according to the manufacturer's guidelines. Plants were kept in a glass container for 1 h before sampling to eliminate any impure volatiles from the system. At 9 AM, the SPME needle was inserted into the opening of the glass container and the fibre was extended to absorb the plant volatiles. After 1 h, the SPME needle was directly inserted into a gas chromatograph-mass spectrometer (GCMS-QP2010Ultra, SHIMADZU, Japan), equipped with an Rxi-5MS capillary column (30 m long, 0.32 mm i.d., 0.25 mm film thickness). The fiber was inserted into the injector port at 250 °C and desorbed for 5 min. After the fiber insertion, the column temperature was maintained at 60 °C for 1 min and then increased to 250 °C at 10 °C min -1, followed by a final stage of 4 min at 250 °C. Each treatment was repeated four times. The identification of separated compounds was done using the NIST 2008 (National Institute of Standards and Technology, Washington DC, United States) database. The Kovats retention index of each volatile component was calculated using the retention time and the data were matched to previously published data. The peak area of each component (tentatively identified) of the volatiles represented the relative quantity (18).

Olfactometer bioassays

A perspex four-arm olfactometer was used to determine the behavioural responses of winged *R. padi* to the test stimuli (41). Before each experiment, all glassware was washed with distilled water and dried in an oven overnight at 160 °C. A 20 W fluorescent light was placed 0.5 m above the olfactometer. The aphids' response to high- and low-nitrogen maize was tested, a 30 × 30 × 30 cm Plexi glass box was used to cover the high- and low-nitrogen maize in plastic boxes on each side of the olfactometer. To minimise the volatiles emission from the potting mixture, the pot was covered with aluminium foil. A single wingless/ winged aphid was introduced through a hole at the top of the olfactometer using a fine paint brush. Air was then drawn through the central hole @ 400 mL/min and subsequently exhausted from the room. Each aphid was allowed 2 min to acclimatise to the olfactometer,

after which the experiment was conducted for 20 min. Aphids were allowed to move freely within the olfactometer for the experimental duration and the time spent in each region was recorded. A positive response to the odour stimulation occurred, when an aphid spent significantly more time in the treated regions than in the control regions and no time spent was regarded a negative response. The olfactometer was rotated 90° every 20 min to control for any directional bias in the room. Six replicates of 20 aphids were used for each odour source tested (35). To test the response of the aphids to the VOCs, 1 ml of the test stimulus was placed on a piece of filter paper and the solvent was allowed to evaporate for 30 s before being placed in the treated arm. Odour sources were kept in glass arms, with each arm connected to holes on the sides of the olfactometer corresponding to the four regions within the olfactometer. Four control arms each contained a piece of filter paper with 1 mL of redistilled hexane. Responses of wingless *R. padi* to each of the single-compound solutions were prepared for each of the 16 compounds in hexane at doses of 10 ng/µl, 100 ng/µl and 1000 ng/µl. A single wingless aphid was introduced through a hole at the top of the olfactometer and then we observed which arm the aphid preferred. Six replicates of 20 aphids were used in each test (28).

The response of *R. padi* to the single VOCs was studied using the perspex four-arm olfactometer. All chemicals were purchased from Sigma Aldrich (Deisenhofen, Germany). One ml of the test chemicals was placed on a piece of filter paper and the solvent was allowed to evaporate for 30 s before kept in treated arm. The control arms contained a piece of filter paper with 1 mL of re-distilled hexane. Responses of *R. padi* to each individual compound solutions were prepared for each of the 16 compounds in hexane at doses of 10 ng/µl, 100 ng/µl, 1000 ng/µl. The experiment had 6-replications of 20 aphids each.

Electroantennogram recording

An electroantennogram (EAG) assay was done to measure the sensitivity of *R. padi* to benzaldehyde, farnesol, (z)-2-hexenol, (z)-3-hexenol, decane, acetic acid, naphthalene, caryophyllene, eicosane, hexadecane, cadinol, indole, aromadendrene, aristolene and copaene. Solutions of the single synthetic compounds were diluted in distilled paraffin oil. Antennae were carefully removed from the aphids at the base and several terminal segments at the distal end were excised before attaching them to the electrodes using Spectra 360 conductive gel (Parker, Fairfield, USA). Test compounds (20 µL, 100 µg/µL) were applied to a piece of filter paper, which was inserted into a syringe. The strip was placed in the syringe, which delivered a continuous humidified (60-70 %) air flow (500 mL/min) and added a compensatory flow. The duration of the stimulation was 0.1 s and the signal from the antenna was recorded for 4 s. Two minutes break was given between each stimulation to restore the sensitivity of EAG. At least five individuals were tested at 100 µg/µL and each individual was tested thrice. The response to the reference standard, (z)-3-hexen-1-ol, was measured at the beginning and end of each recording session to determine for the loss of sensitivity in the preparation. It was assumed that the decrease in sensitivity was linear with time. The data were then normalized to the standard as under (17):

,

$$\mathrm{rEAG} = \frac{\mathrm{EAG(A)}}{\mathrm{EAG(std1)} + \frac{\mathrm{EAG(std2)} - \mathrm{EAG(std1)}}{\mathrm{RT(std2)} - \mathrm{RT(std1)}} \times (\mathrm{RT(A)} - \mathrm{RT(std1)})}$$

Where, rEAG : Relative EAG response; EAG(A) : Amplitude (mV) of the EAG response to compound A; EAG(std1) : EAG response to the standard at the beginning of the recording, EAG(std2) : EAG response to the standard at the end of the recording; T(A) : Time elapsed before stimulation with compound A; T(std1) : Time of the first stimulation and T(std2) : Time of the final stimulation.

Quantitative RT-PCR (RT-qPCR)

Total RNA was extracted from maize roots using RNAiso Plus (Takara, Dalian, China) following the manufacturer's protocol. PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A, Takara, Dalian, China) was used for cDNA synthesis. Specific primer pairs of selected key genes in the synthesis pathways of the four odorant volatiles with reference to the pathways in the maize GDP website (19,30) for RT-qPCR were designed using Primer Premier 5.0 software (Table 1), Actin (GenBank accession number: J01238) was used as a candidate reference gene (24,26). The PCR conditions were as follows: 95 ℃ for 30 s, followed by 40 cycles of 94 °C for 5 s, 60 °C for 10 s and 72 °C for 34 s. A final volume of 20 μL reaction mixture contained 1 μl of cDNA, 20 μl of SYBR Premix Ex Taq, 0.4 μl of 10 μM of forward primer, 0.4 μL of reverse primer, 0.4 μL of ROX Reference Dye II and 7.8 μL double distilled. After RT-qPCR, melting curves were evaluated to confirm single peaks and check amplification specificity. Subsequently, the relative expression level was calculated using the $2^{-\Delta\Delta Ct}$ method (27). The reaction was performed with three biological replicates and three technical replicates.

Table 1. Primer sequences used in quantitative real-time PCR

Gene Name	Forward primer	Reverse primer
Lipoxygenase2 (LOX2)	GCATTGAGCACGGGTTCTTC	CAGTACAGTGCCGAGAGGAC
Hydroperoxide lyase (HPL)	GTATAGGATCCATCGGTGGCG	AGACGAGCATGTGGATGACG
Alcohol dehydrogenase (ADH)	CCAAGAGTGTGCGTGATGGA	CACCTGTCGTCGGACAAGAT
Penylalanine ammonia-lyse (PAL)	GCATTGAGCACGGGTTCTTC	CAGTACAGTGCCGAGAGGAC
Cinnamate: coenzyme A ligase	CTCCACTTCGGCATTCCCAT	TCCACGAACACGACCTTGG
Acetyl-CoA	GTACATGGAGATCCGCGACC	CTCTCCCCAGAAATCGTCCG
Alcohol dehydrogenase1 (ADH1)	GCGAGTACACCGTCATCCAT	GCTGGTTTCGCCACATTCAG
Aldehyde dehydrogenase (ALDH)	TGATCGGCCTTGTTTGCTCT	TTCCATTGCCCACGCATTTG
Copaene synthase	CCAAGTCTGTGGGGCGATTT	CTCGGCTCTTTCCGTCATGT
ACTIN	TACCATGTTCCCTGGGATTG	GTGGCGCAATCACTTTAACC

Statistical analysis

IBM SPSS statistics version 17 (Chicago, IL, United States) was used to conduct all statistical analyses and all the data are presented as the means ± standard errors. A one-way analysis of variance (ANOVA) was used with the test of normality and homoscedasticity of data at a significance level of 0.05 and the means were separated using Tukey's test. The Bonferroni test was used for multiple testing when comparing high/low

nitrogen maize. Principal component analysis (PCA) was used to determine whether maize under high- and low-nitrogen conditions could be separated based on the relative abundance of the volatile compounds that they produced. PCA analyses were performed using R software (R version 3.2.3).

RESULTS AND DISCUSSION

Plant growth under high-nitrogen/low-nitrogen

The maize cultivar 'B73' is of medium maturity and used for the sequencing project (34). In this study under controlled growth conditions [high-nitrogen (15 mM)/low-nitrogen (0.15 mM)], high-nitrogen condition greatly increased the root length, root weight, shoot length and shoot weight than under low-nitrogen condition (Table 2). Tukey test showed significant differences ($F=1.513$; $t=7.208$; $P<0.001$) between the aphid-infested plants under (high-nitrogen and low-nitrogen) treatments. Under high-nitrogen treatment the fresh shoot biomass of maize was reduced (0.22 g) after the aphid feeding for 48 h. However, low-nitrogen treatment, reduction was only 0.04 g. After aphid feeding for 48 h, the leaf length of plants grown under ample N increased by 0.84 g, whereas under deficient N, it increased by 2.13 g. The shoot biomass loss in the low-nitrogen stressed plants was lower than plants supplied with high-nitrogen, indicating that shoot biomass loss in maize under high-nitrogen conditions with aphid feeding stress was significantly more.

Plant volatile emissions

Twenty-one volatile organic compounds were identified in maize plants (Table 3). The relative amounts of (z)-2-hexenol ($t = 15.183$; $df = 10$; $P < 0.01$), benzaldehyde ($t = 3.049$; $df = 10$; $P < 0.05$) and decane ($t = 3.792$; $df = 10$; $P < 0.01$) were higher under high-nitrogen conditions than under low-nitrogen conditions. Contrarily, the farnesol ($t = 2.692$; $df = 10$; $P < 0.05$), cadinol ($t = 7.139$; $df = 10$; $P < 0.01$), aristolene ($t = 3.085$; $df = 10$; $P < 0.05$) and acetic acid ($t = 3.414$; $df = 10$; $P < 0.01$) were produced in higher amounts under low-nitrogen conditions than under high nitrogen conditions. While, the amounts of copaene ($t = 0.735$; $df = 10$; $P = 0.479$), eicosane ($t = 0.77$; $df = 10$; $P = 0.459$), ylangene ($t = 0.222$; $df = 10$; $P = 0.8.29$), naphthalene ($t = 0.577$; $df = 10$; $P = 0.577$), decanoic acid ($t = 0.575$; $df = 10$; $P = 0.578$) were not significantly different (Table 2).

Table 2. Influence of Nitrogen (N) supply and aphid on roots and shoots length and biomass in maize

Nitrogen doses	Root Biomass		Shoot Biomass	
	Root length (cm)	Root weight (g)	Shoot length (cm)	Shoot weight (g)
CK/HN	13.23±1.51	0.73±0.02	24.63±0.8	1.27±0.08
CK/LN	11.27±0.93	0.52±0.07	19.07±2.07	0.72±0.08
Feed24h/ HN	13.93±2.39	0.71±0.11	26.97±1.74	1.17±0.16
Feed24h/LN	11.57±0.2	0.55±0.02	17.03±2.14	0.71±0.10
Feed48h/ HN	16.80±0.81	0.72±0.14	25.47±0.67	1.05±0.14
Feed48h/LN	16.10±0.81	0.57±0.04	21.20±0.90	0.68±0.07

HN: High nitrogen (15 mM), LN: Low nitrogen (0.15 mM). Measurements represent mean from six plants ± SD, CK: Control.

Table 3. VOCs emitted from maize leaves under high and low nitrogen conditions

Compound	High nitrogen condition	Low nitrogen condition
Farnesol	0.18±0.05	0.42±0.07
Cadinol	0.09±0.03	0.42±0.03
€-2-Hexenol	0.38±0.01	0.13±0.01
Benzaldehyde	0.95±0.13	0.52±0.06
Squalane	0.33±0.05	0.32±0.05
Decane	0.34±0.06	0.11±0.03
Hexadecane	0.43±0.07	0.44±0.05
Eicosane	0.51±0.07	0.42±0.1
Aromadendrene	—	0.26±0.07
Ylangene	1.29±0.09	1.14±0.22
Naphthalene	0.94±0.18	0.81±0.13
Copaene	2.7±0.35	2.27±0.47
Caryophyllene	—	0.28±0.13
Azulene	0.59±0.13	0.72±0.11
alpha.-Cubebene	0.56±0.13	0.59±0.14
(+)-Cycloisosativene	0.5±0.17	0.65±0.2
Aristolene	0.53±0.03	0.79±0.08
Indole	0.08±0.01	0.44±0.27
Acetic acid	0.08±0.03	0.35±0.07
Decanoic acid	0.69±0.14	0.58±0.13
Hexadecanoic acid	0.25±0.06	0.32±0.03

Relative amounts (mean % ± SE) of identified compounds of SPME collected from maize plants cultivated under high (15 mM) or low (0.15 mM) nitrate supply (n = 6 plants per cultivar).

PCA analysis

Principal component analysis (PCA) of maize cultivars showed that factor 1 (maize volatile organic compounds in high nitrogen condition) and factors 2 (maize volatile organic compounds in low nitrogen condition) accounted for 60 % variability and the maize volatiles were greatly affected by the N supply (Figure 1). The first principal component (33.09 %) clearly separated the samples from the different N treatments. Maize volatiles were greatly affected by the nitrogen dose.

Aphids attraction to maize grown under conditions of high and low Nitrogen

R. padi was preferentially attracted by maize odours under high-nitrogen conditions than under low-nitrogen conditions (Student's t-test: $P < 0.05$) (Figure 2). The wingless aphids were more attracted to the maize in high-nitrogen conditions than winged aphids.

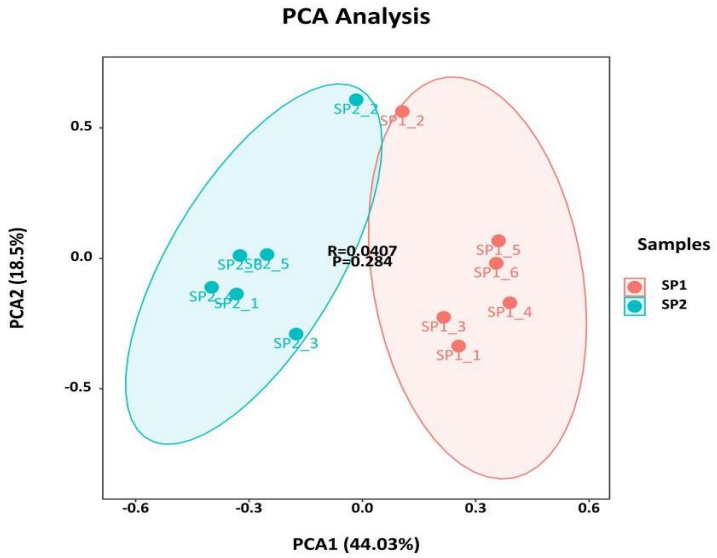

Figure 1. Principal components analysis of the composition of maize volatile organic compounds under high/low nitrogen conditions SP1(maize volatile organic compounds in high nitrogen condition) and SP2(maize volatile organic compounds in low nitrogen condition) measured as relative abundances of total peak area in individual cultivar in SPME headspace volatile collection.

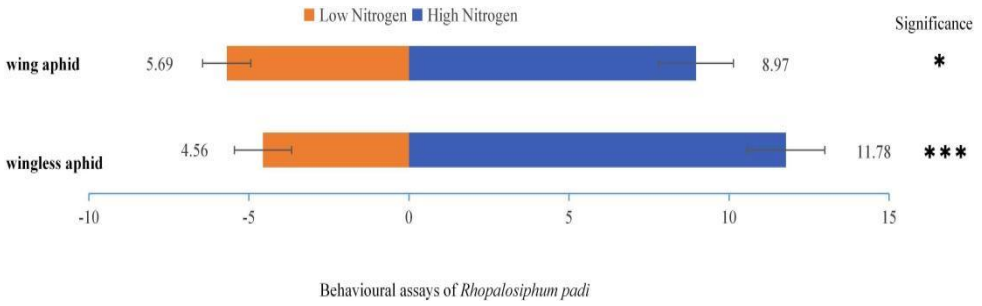

Figure 2. *R. padi* infestation tendency to maize under high and low nitrogen conditions. ∗significantly different at $P < 0.05$; ∗∗, significantly different at $P < 0.01$; ∗∗∗, significantly different at $P < 0.001$.

EAG response

The aphid antenna EAG reactions differed in benzaldehyde ($t = 27.796$, $P < 0.01$), farnesol ($t = 17.099$, $P < 0.01$), (z)-2-hexenol ($t = 22.52$, $P < 0.01$), (z)-3-hexenol ($t = 20.4$, $P < 0.01$), decane ($t = 8.388$, $P < 0.01$), acetic acid ($t = 13.119$, $P < 0.01$), naphthalene ($t =$

12.121, P < 0.01), caryophyllene (t = 7.403, P < 0.01), eicosane (t = 3.04, P < 0.05) and hexadecane (t = 32.567, p < 0.01) than CK (paraffin) (Figure 3). Cadinol (t = 6.143, P < 0.01) was higher than CK (paraffin), but the changes in indole (t = 2.058, P = 0.074), aromadendrene (t = 0.067, P = 0.948), aristolene (t = 0.175, P = 0.866) and copaene (t = 0.789, P = 0.47) were non-significant.

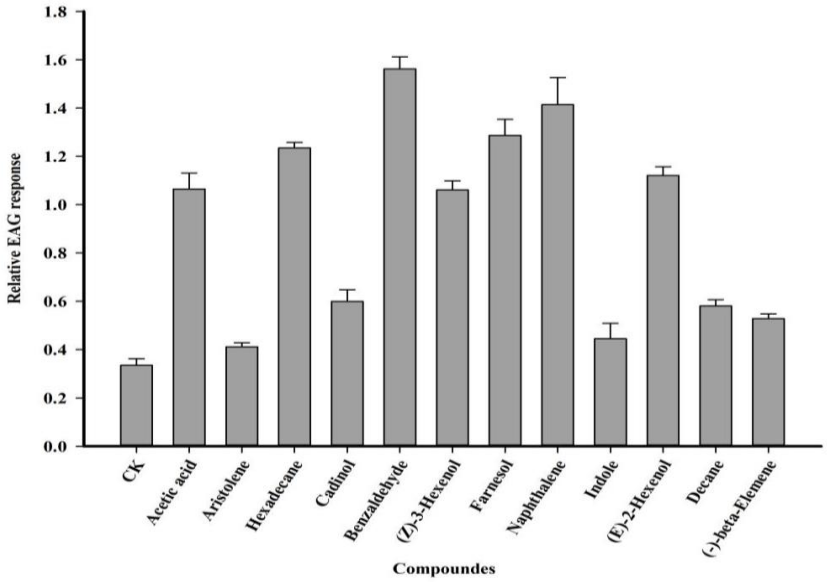

Figure 3. Relative EAG responses (mean ± SE, n = 5) of *R. padi* to different compounds.

Behavioural assays

In separate olfactometer tests, the relative attractiveness (positive values) and repellency (negative values) properties of 16-individual volatile organic compounds found in maize were studied on *R. padi* at 10 ng/µl, 100 ng/µl and 1000 ng/µl (Figure 4). Behavioural trials that compared the treatment and control odour sources at 1,000 ng/ul in the four-arm olfactometer indicated that (z)-2-hexenol, decane, benzaldehyde and naphthalene attracted the wingless aphids (Student's t-test: P < 0.05), whereas, farnesol and acetic acid repelled these at 1,000 ng/ul (Student's T-test: P < 0.05). The remaining compounds did not significantly attract or repel *R. padi* at the tested concentrations.

Quantitative real-time PCR validation

To validate the variations in the volatiles in maize under high and low nitrogen fertilizer conditions. The key genes (E-2-hexenol, benzaldehyde, acetic acid and copaene) associated with synthetic pathways of VOCs were selected for RT-qPCR assays (Figure 5). These genes included the synthetic pathways of E-2-hexenol (LOX, HPL and ADH), benzaldehyde (PAL, Cinnamate: CoA Ligase and acetyl-CoA), acetic acid (ADH1, ALDH) and copaene synthase. The trends in expression of these genes were highly consistent with those shown through the release of volatiles from maize under high and low nitrogen

fertilizer conditions. For example, the benzaldehyde, copaene and E-2-hexenol that volatilize more under high nitrogen conditions also have higher expression of key genes in their synthetic pathways under high nitrogen conditions. The key gene of Alcohol dehydrogenase was expressed less under low nitrogen condition.

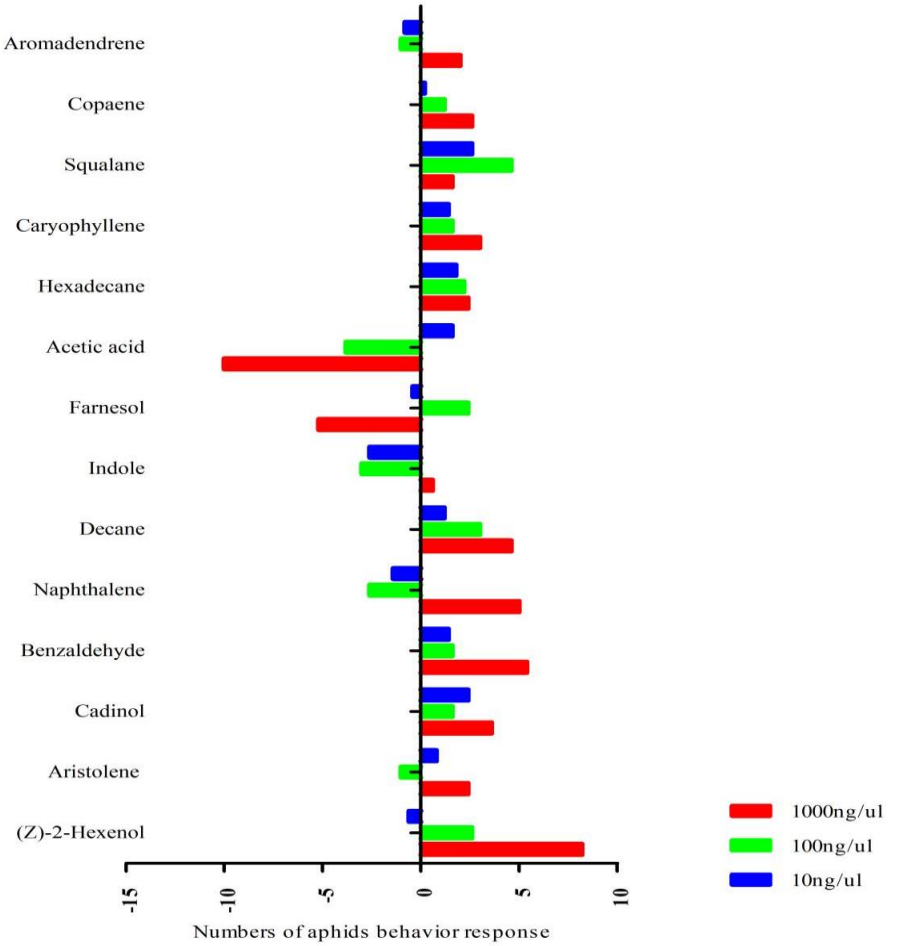

Figure 4. The relative attractiveness (positive values) and repellency (negative values) of individual volatile compounds at doses of 1000 ng, 100 ng and 10 ng found in maize cultivars to *R. padi*in in separate olfactometer tests.

Studies regarding aphid behavioural responses to volatiles under different nitrogen conditions, particularly for *R. padi*, are rare. This is the first study to evaluate the behaviour of *R. padi* in response to odours from maize under high- and low-nitrogen conditions. Although a previous study assessed the numerous volatiles of maize, in the current study,

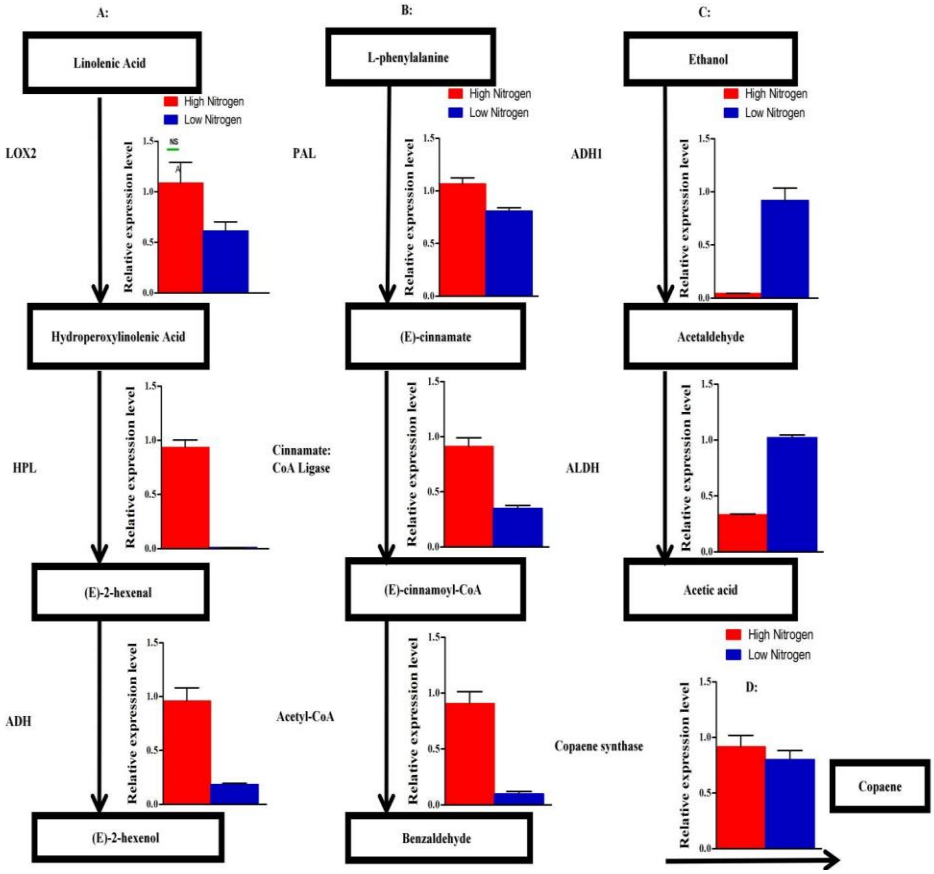

Figure 5. The gene expression of synthetase showing the pathway of biosynthesis of €-2-hexenol, benzaldehyde, acetic acid and copaene volatiles. Steps have been excluded for simplicity. LOX2: Lipoxygenase2; HPL: Hydroperoxide lyase; ADH: Alcohol dehydrogenase; PAL: Penylalanine ammonia-lyse; Cinnamate: CoA Ligase, Cinnamate: coenzyme A ligase; ADH1: Alcohol dehydrogenase1; ALDH: Aldehyde dehydrogenase

aphids varied in their behavioural responses to volatiles emitted by different fertilization of their host plants (3,28,37). In this study, *R. padi* was more attracted to volatiles emitted by maize under high-nitrogen conditions. This demonstrates that the same volatile organic compounds in different quantities can function as preferred host plant cues, depending upon the context in which they are perceived (37,41). We hypothesise that reducing nitrogen fertilizer application may reduce aphid damage. Evidence from other tritrophic interactions supports this likelihood. For example, a decrease in the carbon-to-nitrogen ratio affects the feeding behaviour and life cycle of herbivores and may increase the number of herbivores (29,42). Therefore, excessive fertilization is one of the major causes of insect outbreaks in

recent years. The winged aphid may respond weakly to the stimulation of the same plant, but it has a strong response to the smell of its winter and summer hosts (31). A wingless aphid is more sensitive to nitrogen fertilizer. This phenomenon may be because a winged aphid can search the host over a large area and a wingless aphid can discern better nutrition in the host plant.

The variation in the volatiles from maize plants is due to domestication and artificial selection (22). The herbivore-induced emissions of maize volatiles repel the corn leaf aphid, but this behaviour has not been directly linked to plant-derived €-farnesene (8). The role of plant-derived €-farnesene as an alarm pheromone in maize might decrease because of the presence of caryophyllene in the volatile blend, which inhibits the alarm response (13). This result is consistent with studies on herbivorous insects that showed that blend perception is critical for host plant recognition and behavioural responses. Behavioural evaluations of herbivorous insects using plant volatiles alone and in combination have shown that stronger responses are obtained with proper mixtures or combinations of volatiles than with single compounds (10). Aphids might prefer some volatiles and maize grown under high-nitrogen conditions releases more than that grown under low-nitrogen conditions (Figure 6).

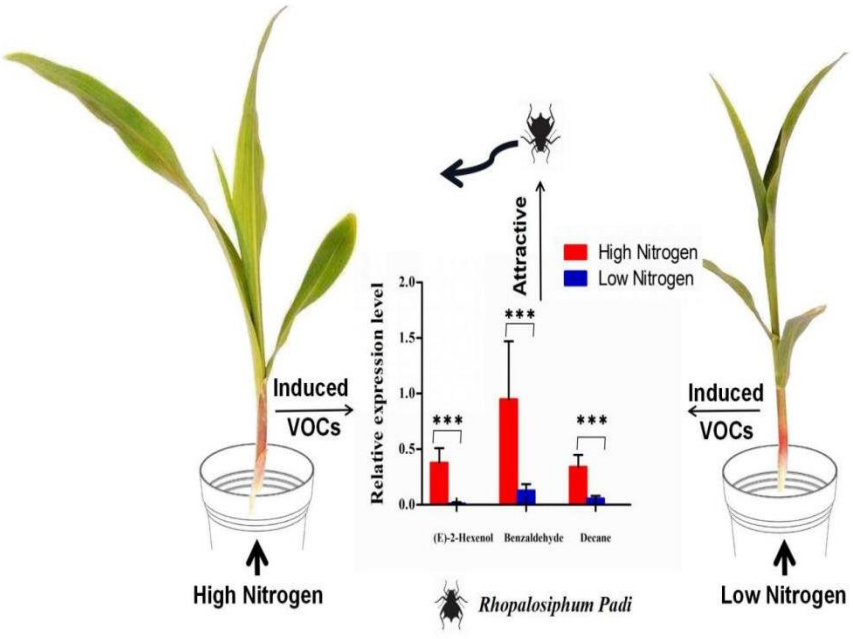

Figure 6. Pattern of *R. padi* tendency to maize were cultivated high (15 mM) or low (0.15 mM) nitrate supply.∗significantly different at $P < 0.05$; ∗∗, significantly different at $P < 0.01$; ∗∗∗, significantly different at $P < 0.001$.

Many studies have shown the species-specific VOC emissions that reflects different genetic regulations among species. The activities of different pathways biosynthesizing volatile metabolites varied among these vegetable species. It may also reflect different regulation by key enzyme(s) in a pathway, the activity of which controls the availability of metabolite precursors in the pathway or the flow through the downstream pathway (24). Hydroperoxide lyase (HPL), lipoxygenase (LOX) are key enzyme on the pathway to hexenol synthesis (21). Cinnamoyl-CoA hydratase/lyase catalyzes hydration and cleavage of cinnamoyl-CoA to benzaldehyde and acetyl-CoA (2). Alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) play an important role in the synthesis of acetic acid (12). Copaene synthase encodes an enzyme that catalyses the conversion of farnesyl diphosphate to a-copaene. So we analyzed the expression of genes of these enzymes in the signalling pathway, the effect of different nitrogen treatment conditions on the pathway can be known.

As the major part in response to allelochemicals, nitrogen play an important role in allelopathy research. Nitrogen enrichment and the presence of clethodim might lead to the excessive proliferation of *M. aeruginosa* and *R. raciborskii* and increased production of cyanotoxins in aquatic environments (9). Increased N inputs to crop plants have a positive effect on the growth and fecundity of *R. padi*. Increased nitrogen in the plant nutrition can change the plant quality and also reduce the plants resistance against aphids in cotton (5). The change in allelopathic potential seems to result from differential gene expression mediated by different N conditions, since N deprivation and other stress factors can induce gene expression associated with secondary metabolism in many crops low N nutrient conditions (36). Our assays show that the VOCs: (z)-2-hexenol, benzaldehyde, decane, which are highly influenced by nitrogen fertilization, are used by aphids to successfully locate their hosts. The mechanism of nitrogen on these compounds need further research in the future.

CONCLUSIONS

Maize volatile organic compounds significantly differed between the high (15 mMK) and low (0.15 mM) nitrogen conditions. *Rhopalosiphum padii* (*R. padi*) aphid preferred the maize grown under the high-nitrogen condition. Electroantennogram and behavioural studies confirmed that VOCs such as (z)-2-hexenol, benzaldehyde and decane were released more under high nitrogen conditions, which attract the bird cherry-oat aphid (*R. padi*) on maize. This study provided a novel perspective and strategy in maize pest resistance research and Integrated Pest Management.

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DECLARATION

We declare that all authors of this Ms. have made substantial contributions. We have not excluded any author that substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

ETHICAL APPROVAL

The authors declare that the study was carried out following scientific ethics and conduct. However, this study did not involve any use of animals, hence no ethical approval has been obtained from the concerned committee.

REFERENCES

1. Abd El-Mawla, A.M. and Beerhues, L. (2002) Benzoic acid biosynthesis in cell cultures of *Hypericum androsaemum*. Planta **214**: 727-733. https://doi.org/10.1007/s004250100657
2. Ahmed, N., Lunuwilage, H. and Darshanee, C. (2019). Host selection behavior of the green peach aphid, *Myzus persicae*, in response to volatile organic compounds and nitrogen contents of cabbage cultivars. *Frontiers in Plant Science* **79.** https://doi.org/10.3389/fpls.2019.00079.
3. Alla, S., Cherqui, A., Kaiser, L., Azzouz, H., Sangwannnorreel, B.S. and Giordanengo. P. (2010). Effects of potato plants expressing the nptii-gus fusion marker genes on physiology and exploratory behaviour of the peach-potato aphid *Myzus persicae*. *Entomologia Experimentalis Et Applicata* **106**: 95-102. https://doi.org/10.1046/j.1570-7458.2003.00013.x
4. Aqueel, M.A. and Leather, S.R. (2011). Effects of nitrogen fertilizer on the growth and survival of *Rhopalosiphum padi* (L.) and *Sitobion avenae* (F.) (Homoptera: Aphididae) on different wheat cultivars. *Crop Protection* **30**: 216-221. https://doi.org/10.1016/j.cropro.2010.09.013
5. Atanasova, E. (2008). Effects of nitrogen sources on the nitrogenous forms and accumulation of amino acids in head cabbage. *Plant Soil and Environment* **54**. https://doi.org/10.17221/438-PSE
6. Awmack, C.S. and Leather, S.R. (2002). Host plant quality and fecundity in herbivorous insects. *Annual Review of Entomology* **47**: 817-844. https://doi.org/10.1146/annurev.ento.47.091201.145300.
7. Barbour, J.D., Farrar, R.R. and Kennedy, G.G. (1991). Interactions of fertilizer regime with host-plant resistance in tomato. *Entomologia Experimentalis et Applicata* **60**: 289-300. https://doi.org/10.1007/BF00354222
8. Bernasconi, M.L., Turlings, T.C.J., Ambrosetti, L., Bassetti, P. and Dorn, S. (1998). Herbivore-induced emissions of maize volatiles repel the corn leaf aphid, *Rhopalosiphum maidis. Entomologia Experimentalis et Applicata* **87**: 133-142. https://doi.org/10.1046/j.1570-7458.1998.00315.x
9. Brêda-Alves Fernanda, de Oliveira Fernandes Valéria and Cordeiro-Araújo Micheline Kezia (2021). The combined effects of clethodim (herbicide) and nitrogen variation on allelopathic interactions between the *Microcystis aeruginosa* and *Raphidiopsis raciborskii*.[J]. *Environmental Science and Pollution Research* **28**: 11528-11539. https://doi.org/10.1007/s11356-020-11367-x
10. Bruce, T.J.A. and Pickett, J.A. (2011). Perception of plant volatile blends by herbivorous insects finding the right mix. *Phytochemistry* **72(13)**: 1605-1611. https://doi.org/10.1016/j.phytochem.2011.04.011
11. Couture, J.J., Servi, J.S. and Lindroth. R.L. (2010). Increased nitrogen availability influences the predator-prey interactions by altering host-plant quality. *Chemoecology* **20**: 277-284. https://doi.org/10.1007/s00049-010-0058-y.

12. Chugh, V., Kaur, N. and Gupta, A.K. (2011). Role of antioxidant and anaerobic metabolism enzymes in providing tolerance to maize (*Zea mays* L.) seedlings against waterlogging. *Indian Journal of Biochemistry & Biophysics* **48 (5)**: 346-352. https://doi.org/10.1016/j.plrev.2011.07.013
13. Dawson, G.W., Griffiths, D.C., Pickett, J.A., Smith, M.C. and Woodcock, C.M. (1984). Natural inhibition of the aphid alarm pheromone. *Entomologia Experimentalis et Applicata* **36(2)**: 197-199. https://doi.org/10.1111/j.1570-7458.1984.tb03427.x
14. Dudareva, N., Klempien, A., Muhlemann, J.K. and Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytologist* **198**: 16-32. https://doi.org/10.1111/nph.12145
15. Erb, M., Lenk, C., Degenhardt, J. and Turlings, T.C.J. (2009). The underestimated role of roots in defense against leaf attackers. *Trends in Plant Science* **14**: 0-659. https://doi.org/10.1016/j.tplants.2009.08.006
16. Fowler, D., Coyle, M., Skiba, U., Sutton, M.A. and Voss, M. (2013). The global nitrogen cycle in the twenty-first century. *Philosophical Transactions of The Royal Society B Biological Sciences* **368(1621)**: 20130164. https://doi.org/10.1098/rstb.2013.0164
17. Gouinguene, S., Pickett, J.A., Wadhams, L.J., Birkett, M.A. and Turlings, T.C.J. (2005). Antennal electrophysiological responses of three parasitic wasps to caterpillar-induced volatiles from maize (*Zea mays*), cotton (*Gossypium herbaceum*) and cowpea (*Vigna unguiculata*). *Journal of Chemical Ecology* **31**: 1023-1038. https://doi.org/10.1007/s10886-005-4245-1.
18. Hiltpold, I., Erb, M., Robert, C.A.M. and Turlings, T.C.J. (2011). Systemic root signalling in a belowground, volatile-mediated tritrophic interactions. *Plant Cell & Environment* **34(8)**. doi:10.1111/j.1365-3040.2011.02327.x
19. Jagtap, A.B., Vikal, Y. and Johal, G.S. (2020). Genome-wide development and validation of cost-effective kasp marker assays for genetic dissection of heat stress tolerance in maize. *International Journal of Molecular Sciences* **21(19)**: 1. https://doi.org/10.3390/ijms21197386. (Author NOT in Alphabetical order?)
20. Khan, M. and Port. G. (2008). Performance of clones and morphs of two cereal aphids on wheat plants with high and low nitrogen content. *Entomological Science* **11**: 159-165. https://doi.org/10.1111/j.1479-8298.2008.00262.x
21. Koch, T., Krumm, T., Jung, V., Engelberth, J. and Boland, W. (1999). Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. *Plant Physiology* **121(1):** 153-162. https://doi.org/10.1104/pp.121.1.153
22. Köllner, T.G., Held, M., Lenk, C., Hiltpold, I., Turlings, T.C.J., Gershenzon, J. and Degenhardt, J. (2008). A maize (E)-β-Caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. *The Plant Cell* **20**: 482-494. https://doi.org/10.1105/tpc.107.051672
23. Li, W.Q., Lu, Z.B., Li, L.L., Yu, Y., Dong, S., Men, X.Y. and Ye, B.H. (2018). Sublethal effects of imidacloprid on the performance of the bird cherry-oat aphid *Rhopalosiphum padi*. *PloS One* **13**: https://doi.org/10.1371/journal. pone.0204097.
24. Liu, Y., Zhang, H., Umashankar, S., Liang, X., Lee, H. and Swarup, S. (2018). Characterization of plant volatiles revealed distinct metabolic profiles and pathways among 12 Brassicaceae vegetables. *Metabolites* **8(4)**. https://doi.org/10.3390/metabo8040094
25. Loreto, F. and Schnitzler, J.P. (2010). Abiotic stresses and induced BVOCs. *Trends in Plant Science* **15(3)**: 154-166. https://doi.org/10.1016/j.tplants.2009.12.006
26. Manoli, A., Sturaro, A., Trevisan, S., Quaggiotti, S. and Nonis, A. (2012). Evaluation of candidate reference genes for qPCR in maize. *Journal of Plant Physiology* **169(8)**: 807-815. doi: 10.1016/j.jplph.2012.01.019.
27. Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**: e45.
28. Pingyan, W. (2009). Selectivity of branches from the various apple varieties by *Eriosoma lanigerum* with volatiles from the branches. *Scientia Silvae Sinicae* **45**: 91-95. https://doi.org/10.1007/978-1-4020-9623-5_5
29. Ponder, K.L., Pritchard, J., Harrington, R. and Bale, J.S. (2000). Difficulties in location and acceptance of phloem sap combined with reduced concentration of phloem amino acids explain lowered performance of the aphid *Rhopalosiphum padi* on nitrogen deficient barley (*Hordeum vulgare*) seedlings. *Entomologia Experimentalis et Applicata* **97(2).** pp. https://doi.org/10.1046/j.1570-7458.2000.00731.x

30. Portwood, J.L., Woodhouse, M.R., Cannon, E.K., Gardiner, J.M., Harper, L.C., Schaeffer, M.L., Walsh, J.R., Sen, T.Z., Cho, K.T., Schott, D.A., Braun, B.L., Dietze, M., Dunfee, B., Elsik, C.G., Manchanda, N., Coe, E., Sachs, M., Stinard, P., Tolbert, J., Zimmerman, S. and Andorf, C.M. (2019). Maize GDB 2018: The maize multi-genome genetics and genomics database. *Nucleic Acids Research* Nov **47(D1):**D1146-D1154. doi: 10.1093/nar/gky1046.
31. Quiroz, A., Pettersson, J., Pickett, J.A., Wadhams, L.J. and Niemeyer, H.M. (1997). Semiochemicals mediating spacing behavior of bird cherry-oat aphid, *Rhopalosiphum padi* feeding on cereals. *Journal of Chemical Ecology* **23**: 2599-2607. https://doi.org/10.1023/b:joec.0000006669.34845.0d1.
32. Rogers, A., Ainsworth, E.A. and Leakey, A.D.B. (2009). Will elevated carbon dioxide concentration amplify the benefits of nitrogen fixation in legumes? *Plant Physiology* **151**: 1009-1016. https://doi.org/10.1104/pp.109.144113
33. Schluter, U., Mascher, M., Colmsee, C., Scholz, U., Brautigam, A., Fahnenstich, H. and Sonnewald, U. (2012). Maize source leaf adaptation to nitrogen deficiency affects not only the nitrogen and carbon metabolism but also controls the phosphate homeostasis. *Plant Physiology* **160**: 1384-1406. https://doi.org/10.1104/pp.112.204420
34. Schnable, P.S., Ware, D. and Fulton, R.S. (2009). The B73 maize genome: Complexity, diversity and dynamics. *Science* **326**: 1112-1115. https://doi.org/10.1126/science.1178534.
35. Schroder, M.L., Glinwood, R., Webster, B., Ignell, R. and Kerstin, K. (2015). Olfactory responses of *Rhopalosiphum padi* to three maize, potato and wheat cultivars and the selection of prospective crop border plants. *Entomologia Experimentalis et Applicata* **157**: 241-253. https://doi.org/10.1111/eea.12359
36. Song, B.Q., Xiong, J., Fang, C., Qiu, L., Lin, R., Liang, Y.Y., Lin, W. (2008). Allelopathic enhancement and differential gene expression in rice under low nitrogen treatment. *Journal of Chemical Ecology* **34(5):** 688-95. https://doi.org/10.1007/s10886-008-9455-x.
37. Storer, J.R., Elmore, J.S. and Emden, H.F.V. (1993). Airborne volatiles from the foliage of 3-cultivars of autumn flowering chrysanthemums. *Phytochemistry* **34**: 1489-1492. https://doi.org/10.1016/S0031-9422(00)90833-1
38. Storer, J.R. and Emden, H.F.V. (1995). Antibiosis and antixenosis of chrysanthemum cultivars to the aphid *Aphis gossypii*. *Entomologia Experimentalis et Applicata* **77**: 307-314. https://doi.org/10.1007/BF02383065.
39. Turgeon, R. and Wolf, S. (2009). Phloem transport: Cellular pathways and molecular trafficking. *Annual Review of Plant Biology* **60**: 207-221. https://doi.org/10.1146/annurev.arplant.043008.0920454.
40. Turlings, T.C.J., Tumlinson, J.H. and Lewis, W.J. (1990). Exploitation of herbivore-induced plant odours by host-seeking parasitic wasps. *Science* **250**: 1251-1253. https://doi.org/10.1126/science.250.4985.1251
41. Webster, B., Bruce, T., Pickett, J. and Hardie, J. (2010). Volatiles functioning as host cues in a blend become non-host cues when presented alone to the black bean aphid. *Animal Behaviour* **79**: 451-457. https://doi.org/10.1016/j.anbehav. 2009.11.028
42. Winter, T.R. and Michael, R. (2010). Nitrogen deficiency affects bottom-up cascade without disrupting indirect plant defence. *Journal of Chemical Ecology* **36**: 642-651. https://doi.org/10.1007/s10886-010-9797-z.
43. Yang, N. and Yan, J. (2021). New genomic approaches for enhancing maize genetic improvement. *Current Opinion in Plant Biology* **60**: 101977. https://doi.org/10.1016/j.pbi.2020.11.002.
44. Yonggen, L. and Baldwin, I.T. (2004). Nitrogen supply influences herbivore-induced direct and indirect defences and transcriptional responses in *Nicotiana attenuata*. *Plant Physiology* **135**: 496-506. https://doi.org/10.1104/pp.104. 040360.

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