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Repellent Activity of Plants from the Genus *Chenopodium* to *Ostrinia nubilalis* Larvae

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Abstract

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The olfactory choices expressed by naïve neonate European corn borer (ECB) larvae were tested by recording their locomotor activities in response to odours coming from plants of the genus *Chenopodium* (producing phytoecdysteroids or not). ECB larvae were found to be repelled from phytoecdysteroid-positive species, except *C. album* and *C. polyspermum*. On the contrary, they were mildly attracted or mildly repelled by phytoecdysteroid-negative species, except *C. botrys* (which emits a rancid odour). These observations indicate that neonate ECB larvae clearly differentiate plant odours and suggest that well-defended plants may emit repellent odours.

Keywords: European corn borer; odours; larvae; orientation; behaviour

Ecdysteroids are known insect moulting hormones that promote growth, moulting and metamorphosis (Coll *et al.* 2007; Gelman *et al.* 2007). A number of plants synthesise and accumulate such hormones, which contribute to defend them against phytophagous insects (Simon *et al.* 2004) and nematodes (Soriano *et al.* 2004). Phytoecdysteroids (PEs) constitute a family of plant steroidal analogues of invertebrate steroid hormones, which include 503 molecules (Lafont *et al.* 2002). They are detectable in 5–6% of higher plant species, frequently in leaves and flowers, but less so in stems, roots and seeds (Dinan *et al.* 2001).Their levels vary enormously between species and the distribution of ecdysteroid-containing species is only partly understood (Dinan *et al.* 2001; Tarkowska & Strnad 2016).

The most common phytoecdysteroids are 20-hydroxyecdysone (Blackford *et al.* 1996; Dinan *et al.* 2002), polypodine B, makisterone A, and ponasterone A (Voigt *et al.* 2001). It has been shown that phytoecdysteroid concentrations increase in response to mechanical damage, insect herbivory, and application of methyl jasmonate (Schmelz *et al.* 1999, 2002). Phytoecdysteroids are believed to deter invertebrate predators and they play a defence role against phytophagous insects (Dinan 1998). In the tolerant insect species there is a rapid detoxification (as C-22 long-chain fatty acyl esters) and/or excretion of ingested ecdysteroids (Blackford & Dinan 1997a,b).

The question asked here is if phytoecdysteroid-producing plants would emit specific deterring or repellent odours that would deter non-tolerant phy-

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tophagous herbivores at distance. As an example of a non-tolerant phytophagous insect, European corn borer (*Ostrinia nubilalis* Hubner) was selected whose individuals are deterred from feeding by phytoecdysteroids at the larval stage (Marion-Poll & Descoins 2002) and from laying eggs at the adult stage (Calas *et al.* 2007). It is reasoned that such an insect would benefit from an early detection of plant-producing ecdysteroids, in particular through sensing odours emitted by such plants. Plants from the family *Chenopodiaceae* which include plants that produce PEs and plants which do not produce these substances were selected (Dinan 1995; Volodin *et al.* 2002).

In this study, volatiles released by phytoecdysteroid-positive *Chenopodium* plants were analysed in order to find repellent compounds. The hypothesis that odours from such plants would repel European corn borer larvae when phytoecdysteroid-negative plants would be less repellent was also tested.

MATERIAL AND METHODS

Plant culture. Experiments were performed at the Plant Growth Centre, INRA, Versailles, France. Seeds for this study were obtained from the National Botanic Garden of Belgium. *Chenopodium album*, *C. ambrosioides*, *C. glaucum*, *C. polyspermum*, *C. schraderanum*, *C. urbicum*, *C. amaranticolor*, *C. botrys*, *C. ficifolium*, *C. murale*, *C. vulvaria*, and *C. oleracea* were tested. The plants (3 per pot) were sown and grown in individual pots in a greenhouse. Daytime temperature was 22 ± 2°C, overnight temperature was 18 ± 2°C. According to Dinan (1992), there is a clear relationship between the amount of phytoecdysteroids in the vegetative parts and the levels found in the flowers and seeds, so that it is assumed that the tests performed on the seeds reflect the presence of phytoecdysteroids in the leaves (Table 1). Three green plants of each species were tested after 6 weeks of vegetation.

Insects. Behavioural experiments were performed on freshly hatched European corn borer (ECB) larvae not exposed to the food. The adult males and females (provided by INRA le Magneraud) were maintained in a cage at 25 ± 0.5°C and 70–80% relative humidity in a climatic chamber (16-h day/8-h night) and fed on sugared water (20%). Females laid eggs on wetted filter paper and put in glass vials (Ø 12 mm ID; 7.5 mm long). Each vial was sealed with a cotton plug, and put in a plastic box (25 × 15 × 10 cm). Vial plugs were lightly wetted daily with distilled water to prevent desiccation. The temperature was maintained at 25 ± 0.5°C and 70–80% relative humidity. Neonate larvae hatched from these papers were collected immediately after hatching and used for experiments. Each larva was used only once to avoid learning.

Table 1. Distribution of phytoecdysteroids in relation to the taxonomy of the plants of the genus *Chenopodium* (Dinan *et al*. 1998)

Chenopodiaceae	RIA (µg E. eg. g seed)	Against bioassay
S. oleracea L.	1185/1125/1340/2546 1033/1087/1204/1104	+++/+++/+++/+++ +++/+++/+++/+++
Ch. album L.	25/58/1370/1753/1294 428/957/790/499	+/++/+++/+++/+++ ++/+++/+++/+++
Ch. amaranticolor H.J. Coste and A. Reynie	1362	+++
Ch. ficifolium Sm.	481/975/1033	+++/+++/+++
Ch. murale L.	885/1297/169/117	+++/+++/++/++
Ch. vulvaria L.	708/1258/984	+++/+++/+++
Ch. ambrosioides L.	–	–
Ch. botrys L.	–	–
Ch. glaucum L.	–	–
Ch. polyspermum L.	1975/2/1672/0/1477/2016	+++/–/+++/–/+++/+++
Ch. schraderanum L.	–	–
Ch. urbicum L.	–	–

RIA (µg E. eg. g seed) – ecdysteroid-specific radioimmunoassay (µg ecdysone equivalents g seed); (–) below detection limit (4.3 µg 20-hydroecdysone equivalents/g; (+) 4.3–43 µg 20-hydroecdysone equivalents/g; (++) 43–430 µg 20-hydroecdysone equivalents/g; (+++) 430–4 300 µg 20-hydroecdysone equivalents/g; (++++) more than 4 300 µg 20-hydroecdysone equivalents/g

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Locomotion compensator

Locomotion compensator. The locomotion and direction of each larva were recorded by placing it onto a Syntech TrackSphere LC-100 locomotion compensator (Syntech, Löptin, Germany). The locomotion of each larva was observed during 5 min, using 3 plants of 12 species on which 10 larvae and 10 neonates for the control were tested.

Odours were obtained by pushing 40 ml/min of humidified and charcoal-filtered air through a bag of Nalophan® (Scentroid, Whitchurch-Stouffville, Canada) plastic enclosing one *Chenopodiaceae* plant, using a Stimulus Air Controller CS-55 (Syntech) which mixed it with another stream of pure air so that the total airflow directed onto the insect was 80 ml/minute. The stimulus was pulsed at 0.1 Hz (5/5 s – 5 s of continuous pure and humidified air and 5 s of pulsed air with tested plant).

Volatile collection system. Volatiles were collected from *Chenopodiaceae* plants enclosed into a Nalophan® bag. A volatile collector trap (6.35-mm OD, 76-mm long glass tube; Analytical Research Systems, Micanopy, USA) containing 30 mg of Super-Q adsorbent (Alltech Associates, Deerfield, USA) was inserted into each of 4 Tygon tubes (connection between airflow meter and collector trap). Purified, humidified air was delivered at a rate of 1.0 l/min over the plants, and a vacuum pump sucked 20% less (0.8 l/min) to avoid collecting odours from any gap of the system. Volatiles were collected from the whole plant. The volatile collection sequence (two-hour collections) was initiated after 6 weeks of growing. Six plants were taken for collection experiments plus two blanks (odours collected from empty Nalophan bags only). The results are presented in ng per 1 minute.

Chemical analyses. Volatiles were eluted from the Super-Q adsorbent with 225 µl of hexane and after this 7 ng of decane (both 95% of purity, Sigma-Aldrich, Poznań, Poland) were added as an internal standard. Volatiles were analysed by coupled gas chromatography-mass spectrometry (GC-MS). The GC-MS was a Perkin Elmer AutoSystem XL (Poland) instrument fitted with a 30-m DB-5MS capillary column (0.25-mm ID, 0.25 µm film thickness). The

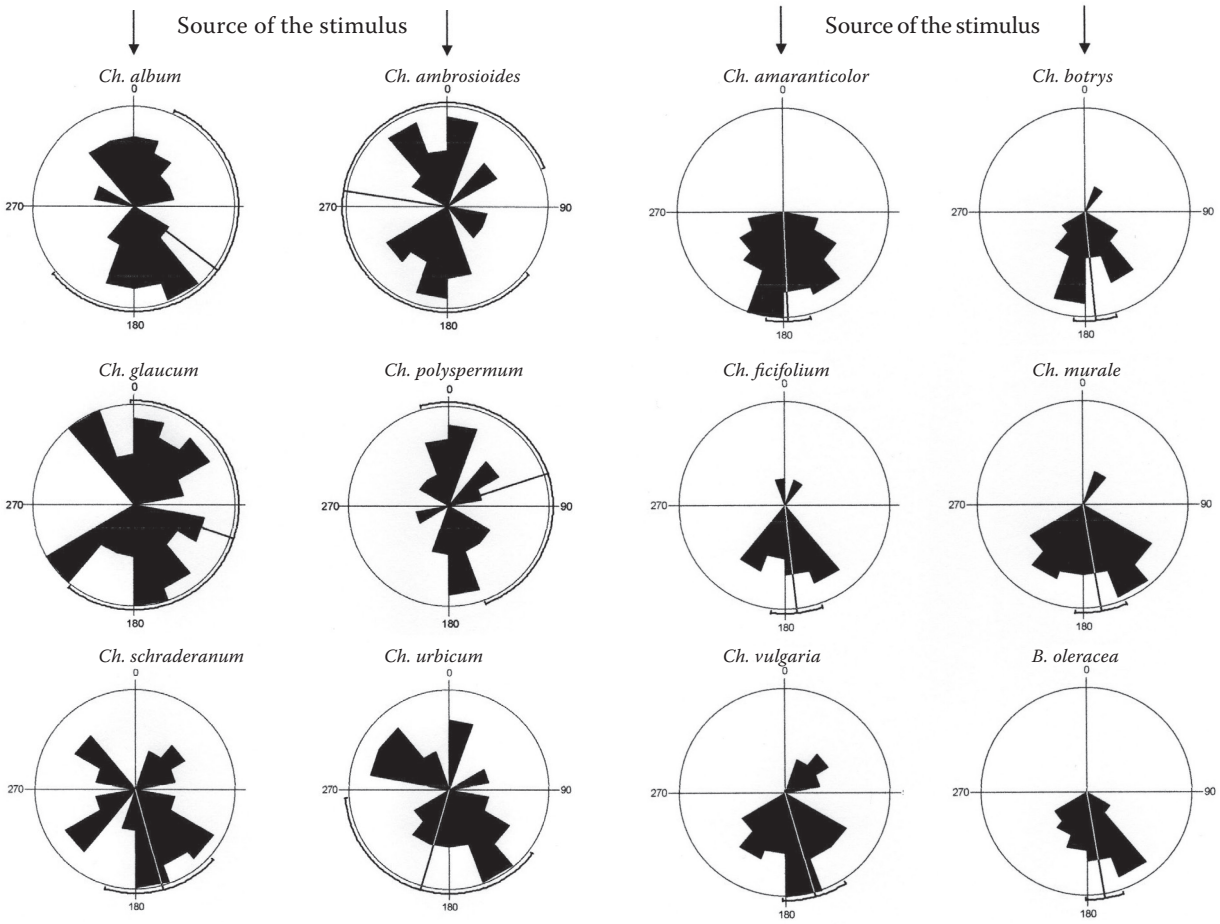

Figure 1. Orientation of neonate ECB larvae to a source of odour delivery

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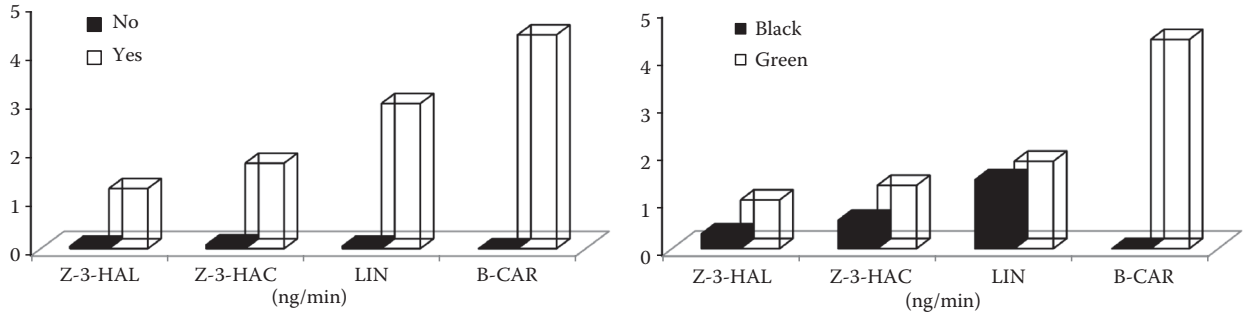

Figure 2. Mean values for (*Z*)-3-HAL, (*Z*)-3-HAC, LIN, and β-CAR

identification of volatiles was verified with authentic standards purchased from commercial sources that had the same GC retention times and mass spectra.

Track analyses. The following parameters were used to quantify the tracks: walking speed (mm/s), straightness of walking (max. 1.00, straight line), angles (*x*, *y*), and upward length (mm). For each experimental condition the mean angles by Rayleigh Z tests using the procedure described by Batschelet (1981) (U tests) were tested. Calculations and statistical tests on circular data were done using the Oriana software (Kovach Computing Services, Anglesey, UK).

Statistical analysis. All the analyses were conducted using the GenStat v17 statistical software package. Multivariate analysis of variance (MANOVA) was performed on the basis of a MANOVA procedure in GenStat v17 (Rencher 1992). Mahalanobis distance was suggested as a measure of "poly-VOCs" species similarity (Seidler-Łożykowska & Bocianowski 2012), whose significance was verified by means of critical value D_{α} called "the least significant distance" (Mahalanobis 1936).

RESULTS

The servosphere was used to observe how ECB first instar larvae orient towards or downwind of the stimulus source, depending on the tested plants and the pulsing regime. None of the tested plants of the genus *Chenopodium* attracted the larvae. Attraction/repulsion was observed in response to odours from *Chenopodium album*, *C. ambrosioides*, *C. glaucum*, *C. polyspermum*, *C. schraderanum*, and *C. urbicum* (Figure 1). A clear repellency was recorded in response to odours from *C. amaranticolor*, *C. botrys*, *C. ficifolium*, *C. murale*, *C. vulvaria*, and *C. oleracea* (Figure 1) – Track Sphere Locomotion Compensator software.

Results of MANOVA (Wilk's $\lambda = 0.0001798$; $F_{10.55} = 43.39$; $P < 0.0001$) indicate that 4 volatile organic compounds (VOCs) were released in large amounts. Results of the analysis of variance for all VOCs confirm the variability of tested species at the significance level $\alpha = 0.01$ (except for LIN, $P = 0.489$).

Mean values for observed VOCs for both factors are presented in Figure 2 [repellent on the space (Yes) or

Tab. 2. Mahalanobis distance between species

Species	No	1	2	3	4	5	6	7	8	9	10	11
Spinacia oleracea L.	1	0	7.81	12.85	3.18	1.54	6.14	8.79	9.79	8.82	8.50	8.44
Ch. album L.	2		0	14.72	8.69	8.49	9.13	1.54	13.62	1.30	1.01	0.93
Ch. amaranticolor	3			0	11.12	11.83	11.73	15.55	13.89	15.46	15.28	15.23
Ch. ficifolium Sm.	4				0	1.80	6.42	9.76	10.80	9.75	9.41	9.34
Ch. murale L.	5					0	6.09	9.52	9.96	9.54	9.21	9.15
Ch. vulvaria L.	6						0	10.59	5.52	10.39	10.12	10.04
Ch. ambrosioides L.	7							0	14.92	0.41	0.56	0.65
Ch. botrys L.	8								0	14.76	14.53	14.47
Ch. glaucum L.	9									0	0.35	0.43
Ch. polyspermum L.	10										0	0.09
Ch. schraderanum L.	11											0

$D_{0.05} = 7.73$

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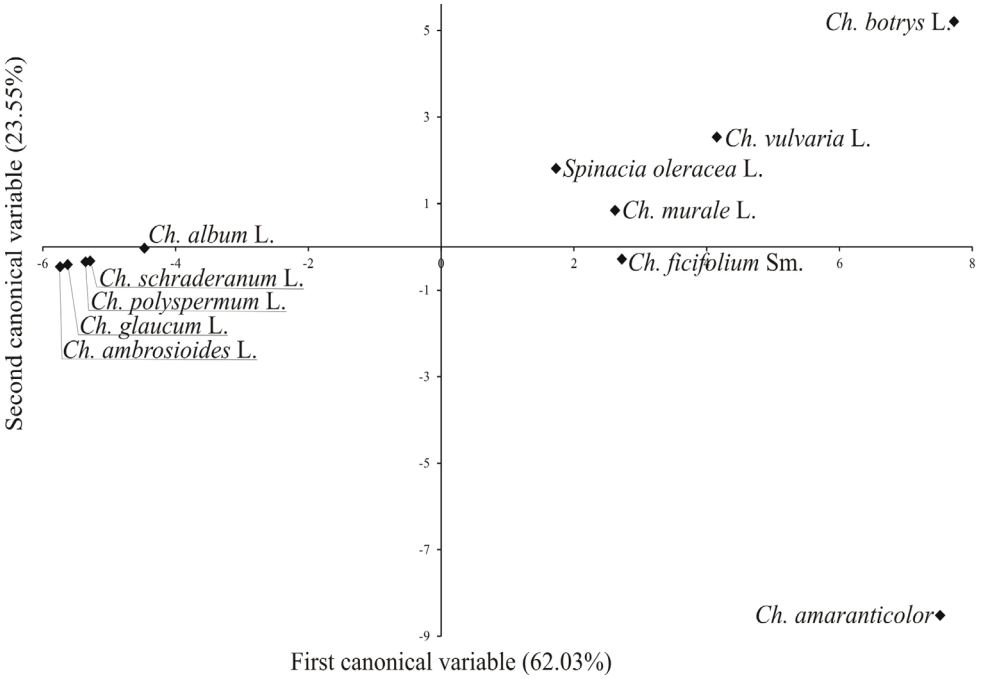

Figure 3. Distribution of ten *Chenopodiaceae* species and *Spinacia oleracea* L. in the space of the first two canonical variables

not active on the space (No) (left figure) and plants with ecdysteroids by Dinan (Green) or ecdysteroid-free plants by Dinan (Black) (right figure)]. The high mean values were observed in case of the repellent presence on the space for all four VOCs. Plants with ecdysteroids characterised higher mean values of all four VOCs than ecdysteroid-free plants (Figure 2).

The greatest variability in terms of all the analysed VOCs expressed jointly with the greatest Mahalanobis distance was recorded for *Ch. amaranticolor* and *Ch. ambrosioides* L. (the Mahalanobis distance was equal to 15.55). In turn, the greatest similarity was observed for *Ch. polyspermum* L. and *Ch. schraderanum* L. (the Mahalanobis distance for this pair was 0.09) (Table 2).

Multivariate analysis made it possible to compare tested species in terms of all four VOCs (Figure 3). The total variation explained by the first canonical variable was 62.03%, while for the second variable it was 23.55%.

DISCUSSION

The orientation of neonate ECB larvae to undamaged *Chenopodiaceae* plants which showed that these larvae clearly detect odours from such plants was recorded. Witzgall *et al*. (2012), Bengtsson *et al*. (2014), and Gonzalez *et al*. (2015) claim that many plants constitutively release VOCs, but quantities are often affected by stress, where volatiles have various functions in plant development including defence against pathogens.

While our previous work showed that larvae of the same age were unconditionally attracted to maize odours (their host plants) or to green leaf volatiles (Piesik *et al.* 2009, 2013), here, it was observed that ECB larvae were strongly repelled by odours from ecdysteroid-positive plants (Figure 2) and to a lesser extent by ecdysteroid-negative plants (Figure 1). *C. album* and *C. polyspermum* stand out in the group of plant odours being attractive/repellent. According to Dinan *et al.* (1998), these two species show important variations between samples: in *C. album,* the amounts varied between no detection to 1 to 3 levels, while for *C. polyspermum*, two of six samples were below the detection limit. In the group of repellent plants, all of them are ecdysteroid-positive except *C. botrys*.

These chemicals may be generally occurring compounds. In this work (*Z*)-3-hexenal, (*Z*)-3-hexen-1-yl acetate as well as linalool and β-caryophyllene were released in greater amounts in ecdysteroid-positive plants than in ecdysteroid-negative ones. These chemicals have been reported as repellent in different plants. Koschier *et al.* (2002) found linalool and eugenol (main compounds of *O. majorana* and *O. gratissimum*) inhibiting feeding of onion thrips (*Thrips tabaci*). Zheng *et al.* (2005) found (*Z*)-3-hexenal as a key component contributing to the aroma of omija (*Schizandra chinensis*) leaves. Moreover, (*Z*)-3-hexenal has been identified as the most abundant volatile compound in tomato (Buttery *et al.* 1987) and in orange juice

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(Buettner & Schieberle 2001). Fuhrmann and Grosch (2002) found (*Z*)-3-hexenal as one of the main odours of the apple cultivar Elstar. Fukushima *et al.* (2002) reported that (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, β-myrcene, and linalool could be released not only from infested or artificially damaged plants, but also from undamaged ones. Peacock *et al.* (2001) found (*Z*)-3-hexen-1-yl acetate and (*Z*)-3-hexenol emitted from undamaged *S. dasyclados* (Wimm) leaves.

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