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Chemical characterisation of corn plant compounds by different extraction techniques and the role of potent chemicals in the reproductive behaviour of the corn stalk borer *Sesamia nonagrioides*

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Abstract: Plant chemistry is probably the most important source of information contributing to the final decision by an insect to select a host and it is actually the balance of opposing positive and negative cues evoked by phytochemicals that determines whether a plant is accepted or rejected by a herbivore. Two different techniques, a modified steam distillation extraction and a solid phase microextraction (SPME) technique for the extraction of plant volatile chemicals from different corn hybrids were utilised and the extracts were analysed and characterised by GC-MS. For the steam distillation technique, plant material was steam distilled for 4 h in a modified apparatus with a water-cooled oil receiver, to reduce hydrodistillation overheating artifacts and the extract after appropriate treatment was analysed by GC-MS. The SPME fibre was introduced in a 40ml vial that contained plant material and left to collect analytes for 60 min. Following collection, analytes were chromatographed and analysed by GC-MS. The two techniques are compared in terms of extraction efficiency and for both qualitative and quantitative differences in the chemical profile of the extract. The oviposition preference of the corn stalk borer, *Sesamia nonagrioides*, was evaluated in bioassays using artificial substrates impregnated with the steam distillation extract. The chemical composition of the steam distillation extract of different corn hybrids obtained is correlated to the egg laying behaviour of the insect and the role of potent chemicals on this behaviour is discussed.

Key words: *Sesamia nonagrioides*, plant chemicals, oviposition preference, corn hybrids,

Introduction

Host plant recognition and selection in Lepidoptera is primarily a function of the ovipositing female and since newly emerged larvae are often limited in their dispersal abilities, oviposition is particularly crucial as it determines survival of their progeny (Renwick, 1989).

Visual factors such as shape, colour and size, in many cases studied, were significant (Renwick and Chew, 1994), but chemical cues unambiguously play the major,if not decisive, role in host selection (Udayagiri and Mason, 1995). The latter must be

especially true for insects that are active during the night. Plant chemistry is probably the most important source of information contributing to the final decision by an insect to oviposit or not and it is actually the balance of opposing positive and negative cues evoked by phytochemicals that determines whether a plant is accepted or rejected by a herbivore (Huang and Renwick, 1993, Renwick and Chew, 1994).

Chemical cues of corn stimulate oviposition in the European corn borer (Lupoli et al., 1990). Pentane leaf extracts of corn containing *n*-alkanes stimulate oviposition of *Ostrinia nubilalis* (Udayagiri and Mason, 1995, 1997). On the other hand corn methanol extracts received fewer *O. nubilalis* eggs than the methanol controls (Udayagiri and Mason, 1995). A methanol soluble oviposition deterrent chemical has been discovered in the frass of fifth instar larvae of *O. nubilalis* (Dittrick et al., 1983). Derridj et al. (1986, 1992) have reported that females of *O. nubilalis* discriminate corn cultivars based on carbohydrate content.

The corn stalk borer *Sesamia nonagrioides* (Lef) major pest of maize in the Mediterranean countries is considered as an oligophagus pest and it attacks plants of the *Graminae* family, with its major host being the corn plant. Corn hybrids that are resistant to corn stalk borer have been identified but the defensive mechanisms involved are as yet undermined (Butron et al., 1998, Velasco et al., 1999). Selection of corn hybrids that are not preferred as host plants for oviposition by the corn stalk borer is valuable for the development of insect-resistant plants.

This paper reports comparative studies on the chemical composition of corn hybrids, employ two extraction methods steam distillation and solid phase microextraction and on the effects of steam distillation extracts to the egg laying behaviour of the corn stalk borer.

Material and methods

Insects. The insects used were obtained from a laboratory colony maintained under a 16:8 h L:D regime at 25 ± 1 ^{o}C and 65 ± 5 % RH, on artificial diet (Tsitsipis et al., 1983). Aqueous sugar solution 10% was offered as food to the adults.

Plants. Eight corn (*Zea mays*) hybrids 33A14, 3211, 31B13, 3283W, 33R87, Konstanza kindly provided by Pioneer Hi-Bred International, Inc and two Greek corn hybrids (Dias and Aris) were used. Plants from seeds (F1 generation) derived from commercially available Konstanza hybrid in Greece were used as a susceptible control. Corn seeds were planted in small pots, in a greenhouse under 25 ± 1 ^{o}C and 65 ± 5 % RH. When the plants had developed 6-8 leaves they were used for the collection of chemicals.

Collection of plant chemicals - steam distillation. Corn leaves (25 g, 6-8 leaf stage) were steam distilled for 4 h in a steam distillation apparatus with a water-cooled oil receiver, to reduce hydrodistillation overheating artifacts (Roussis et. al., 1995). The chemicals carried by water vapours were condensed and trapped in a

layer of diethyl ether. The ether layer was dried over magnesium sulfate to remove residual water, concentrated to 200 µl under a gentle stream of nitrogen and stored at –20 °C until further analysis. For each hybrid, three replicates were performed. For the oviposition preference test, the concentrated extract of each hybrid was properly diluted to give an equivalent concentration of 1 mg of leaves per µl of extract.

Collection of plant chemicals - SPME. Corn leaves (1g, 6-8 leaf stage) were placed in a 22 ml headspace vial and covered with 11 ml of a saturated NaCl solution. The vial was placed on top of a hot plate, continuous stirring was applied by means of a magnetic stirrer and the content of the vial was heated to 40 °C. A 100 µm polydimethylsiloxane (PDMS) fibre (Supelco, USA) was exposed on the headspace for 90 min and was subsequently desorbed into the injection port of the GC-MS system for 5 min.

Oviposition preference tests. Oviposition preference of females was examined in a two-choice bioassay. A sheet of 20 cm x 20 cm common filter paper (65 g/m^2, Filtrak, Spezialpapier Filtrak Niederschlag, D-09484, Germany) was placed on top of a 21 cm x 21 cm A4 paper (80 g/m^2) and the two sheets were rolled together diagonally to form a tube. The paper rolls were placed in small vials containing water to keep the moisture high. In each cage (30 cm x 30 cm x 30 cm) there were two paper rolls. One was loaded with 500 µl of the steam distillation extract of a hybrid (equivalent to 500 mg of leaves) and one with 500 µl of solvent. One pair of 2-d-old insects was released and the number of eggs deposited was recorded 48 h later. Data for volatiles of each hybrid and the controls were collected from 10 replications for each hybrid.

Gas chromatography-mass spectrometry. Gas chromatography-mass spectrometry analysis was carried out on a Hewlett Packard 5890 Series II gas chromatograph interfaced to a Fisons VG Trio 1000 (Manchester M23 9BE, UK) quadrupole mass spectrometer. Electron impact ionization was used, with an electron energy of 70 eV and a trap current of 200 µA. All extracts were chromatographed on a 60 m x 0.25 mm(id) x 0.1 µm film thickness DB-5 column (J&W Scientific, Fisons). The oven temperature program was 50 °C for 2 min, then 5 °C/min to 250 °C hold for 1 min, then 2 °C/min to 280 °C and hold for 50 min. Helium was used as the carrier gas at a flow rate of 1 ml/min. Splitless injections were made (1 µl) at an injector temperature of 250 °C and a splitless period of 90 s. Prior to GC-MS analysis, 50 µg of *n*-tetradecane was added into the vial to serve as an internal standard.

Statistics. For statistical analysis, an index of oviposition, the oviposition index (OI) was calculated according to the formula (Udayagiri and Mason, 1997).

$$\mathrm{OI} = \frac{(\text{N eggs on hybrid} - \text{N eggs on control})}{(\text{N eggs on hybrid} + \text{N eggs on control})}$$

The OI takes positive or negative values indicating hybrids that are preferred by females for oviposition (positive values) and those that are not preferred (negative values). For comparison of the mean OI in the oviposition preference experiments the non-parametric Kruskal-Wallis test was used.

For statistical analysis of the chemical compounds present in the steam distillation extracts, only the peaks with a relative area (area of peak/total area) greater than 0.1% that had been detected in at least two of the three replicates for each hybrid were used as variables. To investigate any association of chemical composition and oviposition preference, data of all constituents, which met the above-mentioned criteria, were subjected to principal component analysis. Principal component analysis was performed on the correlation matrix and principal components were rotated using the varimax orthogonal rotation method. (Jolliffe 1986)

Results

Extraction techniques. Comparative GC-MS analysis of the steam distillation and the SPME extracts of corn leaves revealed both qualitative and quantitative differences for all hybrids evaluated. Approximately forty compounds were detected, characterised and quantified on the basis of retention time data and mass spectra, as constituents of each of the steam distillation and the SPME extract. The results, in terms of chemical compound classes are summarised in Figure 1.

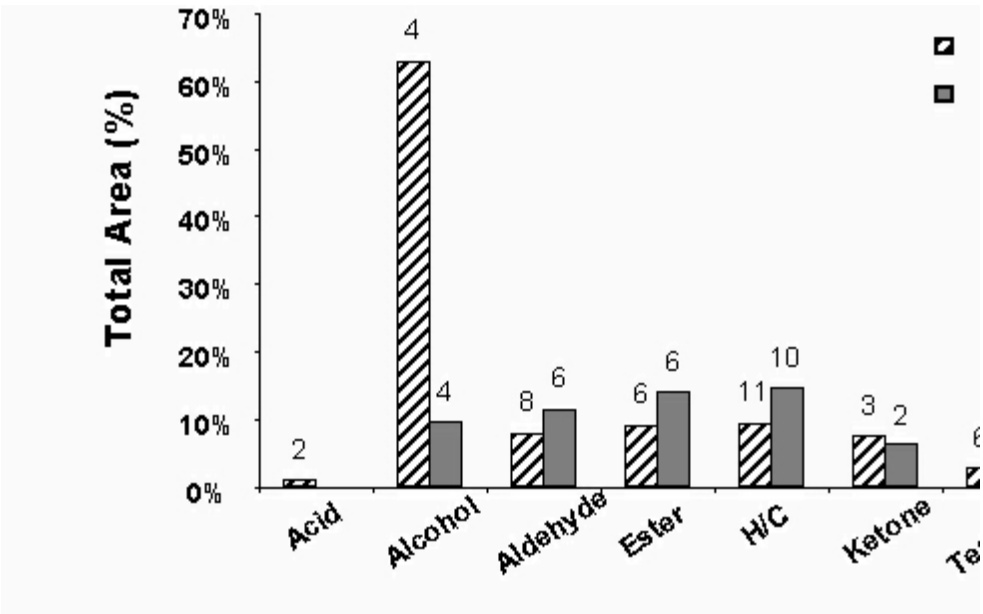

Figure 1. Distribution of chemical compound classes in corn leave extracts as determined by SPME and SD.

The two techniques compare well for compounds such as aldehydes, esters, hydrocarbons and ketones. The major differences are mainly on the alcohol and the terpenoid content of the two extracts. Although the alcohol content of the two extracts

was qualitatively the same (four compounds were detected and quantified for both techniques), steam distillation extracted a higher amount of alcohols with phytol, a hydlolysis product of chlorophyl, accounting for 53% of the total 63% of the alcohol content of the extract. For SPME, phytol accounted for only 5.7% of the total area. The efficiency of SPME to extract compounds depends, among other factors, on the affinity of the fibre coating to certain chemical characteristics, such as polarity, of the compound to be extracted. During this investigation a non-polar polydimethylsiloxane fibre was used and this can explain the lower content of polar compounds on the SPME extract. The two techniques differed both qualitatively and quantitatively on the terpenoid content. A total of fourteen compounds that accounted for the 43.5% of the total area were quantified on the SPME extract, whilst the steam distillation extract contained a total of six compounds accounting for only the 2.9% of the total area. In order to investigate possible role of corn leaves chemicals on the reproductive behaviour of *S. nonagrioides*, a physical means of performing bioassays was needed and thus the use of the steam distillation extracts in two choice bioassays was further explored.

Oviposition preference. Statistical analysis (Wilcoxon signed-ranks test, P=0.05) between solvent (diethyl ether) and steam distillation extracts of each hybrid revealed that there is a significant difference when chemicals of hybrids 3211, 31B13, 33A14 and Dias were compared to their respective controls (Table 1).

Table 1. Oviposition response of *Sesamia nonagrioides* females to hybrid volatiles in two-choice bioassays. Statistical analysis of the Oviposition Index (OI) for all hybrids and hybrid volatiles evaluated.

Hybrid	Mean OI Plants volatiles
3211	0.45 [a] (P=0.047)
Aris	-0.20 (P=0.594)
33A14	0.60 [a] (P=0.041)
33R87	-0.46 (P=0.059)
Pioneer	0.19 (P=0.646)
31B13	0.58 [a] (P=0.037)
Dias	0.59 [a] (P=0.021)

[a] Significant difference in egg laying between the control and the hybrid as determined by Wilcoxon signed-ranks test at P=0.05.

OI values (Kruskal Wallis test, X^2=15.734, df=7, P=0.028) make evident that there is a significant difference in the OI values between the eight hybrids tested: the most distinct difference occurs between the hybrids 33R87 and Dias (Fig. 2).

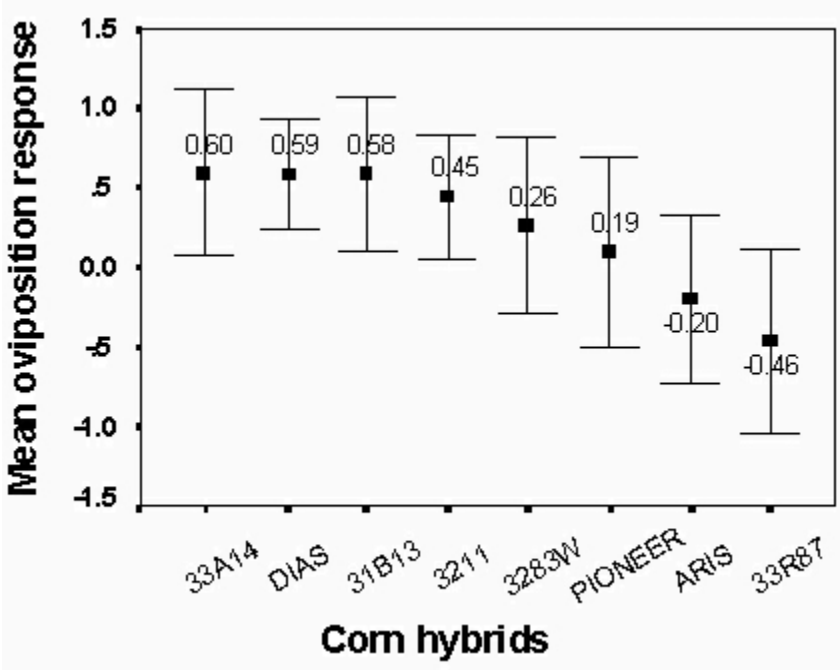

Figure 2. Oviposition response of *S. nonagrioides* females to corn hybrids volatiles [Kruskal-Wallis test, P=0.05 (Mean OI +/- SE)]

Steam distillation extracts. All peaks detected in the steam distillation extracts of the eight corn hybrids were subjected to principal component analysis resulting to the extraction of 7 principal components (PCs) that accounted for 100% of the total variation of the original data set. In order to evaluate any association of the oviposition preference of the corn stalk borer females as indicated by the bioassays and the chemical composition of the different hybrids, Pearson's correlation coefficients were computed for the OI and all 7 principal components. It was found that OI for plant chemicals was significantly correlated to Principal Component 6 (PC6) [Pearson's correlation coefficient (two-tailed) = -0.752, P = 0.031] (Figure 3) .

The coefficients presented in Table 2 indicate how these two principal components are correlated to the original variables, that is the chemical compounds detected in the steam distillation fraction of the eight hybrids. The coefficient for each compound determines how 'important' that compound is for the particular principal component. A high positive or negative value (a high loading) for the coefficient of a certain compound indicates that this compound has a strong influence on the particular principal component.

Principal component 6 separates hybrid 33R87 from the rest of the other hybrids examined. Statistical analysis of the OI values for plant chemicals (Figure 2) revealed that the two hybrids that differ most are 33R87 and Dias. These two hybrids lie on the two extreme parts of the PC6 axis [33R87 positive part of the axis and Dias negative part of the axis (Figure 3)].

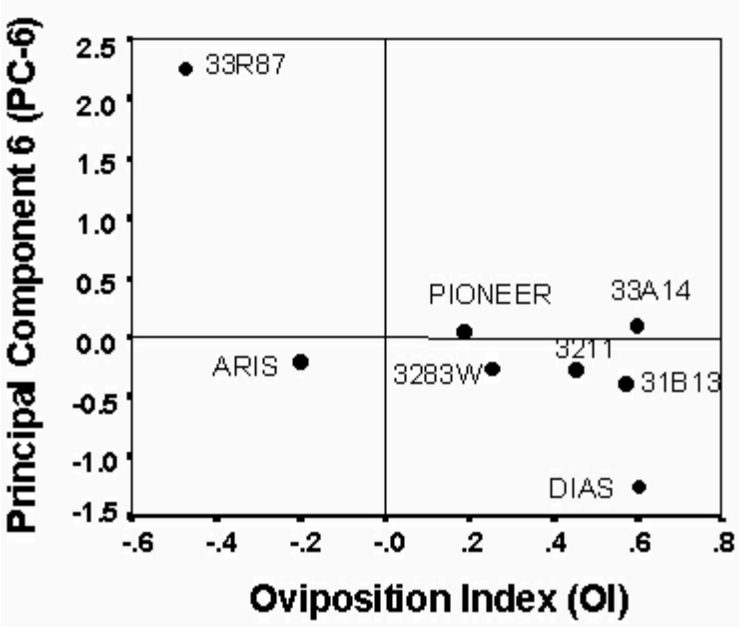

Figure 3. Scaterplot of PC-6 and Oviposition Index (OI)

Table 2. Simple correlation coefficient of chemical compounds used for statistics and Principal Component factor 6 (PC6) as extracted from principal components analysis.

Compound	PC6	Compound	PC6
(Z)-3-Hexenol	-0.118	α-Farnesene	-0.070
Terpenoid	0.031	Tetradecanal	0.295
(Z)-3-Hexenyl acetate	-0.193	Nonadecanol	0.111
Linalool	0.272	Cadinol	-0.036
Nonanal	-0.177	Unknown III (Terpenoid)	-0.156
Decanal	0.533	Pentadecanal	0.855
Propenoic Acid, Hydroxy Phenyl	-0.071	Neophytadiene	-0.042
Trans-caryophyllene	-0.083	Hexahydrofarnesyl Acetone	0.789
Ionone	-0.306	Neophytadiene (isomer)	-0.172
Uknown II(Terpenoid)	-0.157	Eicosatrienoic acid, methyl ester	0.789
Nerolidol	-0.147	Heptadecanal	-0.246
Heptadecanal	-0.246	Hexadecenol tetramethyl	-0.162
Hexadecenol tetramethyl	-0.162	Octadecenoic acid	0.531
Octadecenoic acid	0.531	Heneicosane	0.010
Heneicosane	0.010		
Phytol	-0.123		

In terms of chemical compounds, PC6 is dominated by pentadecanal, hydroxyfarnesyl acetone and eicosatrienoic acid methyl ester, all with a high positive loading. Hybrids with high positive values on that component (that is hybrids that are not preferred for oviposition; negative OI values, e.g 33R87) have large amounts of pentadecanal, hexahydrofarnesyl acetone and eicosatrienoic acid methyl ester and these could be the compounds responsible for oviposition deterrent activity. Further studies with pure synthetic compounds are in progress to verify these findings.

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