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Olfactory responses of *Lobesia botrana* females (Lepidoptera: Tortricidae) to *Tanacetum vulgare* (Asteracea) flower extracts and fractions

Bruno GÁBEL[1], Frédéric MARION-POLL[2], Václav SUCHÝ[3], Roger ROEHRICH[4], Peter HRADSKÝ[5] & Denis THIERY[6]

[1]Research Institute of Viticulture and Enology, Matuškova 25, 83311 Bratislava, Slovakia
[2]INRA, Station de Phytopharmacie, Route de Saint-Cyr, 78026 Versailles Cedex, France
[3]Pharmaceutical Faculty, Department of Pharmacognosy and Botany, 83232 Bratislava, Slovakia
[4]INRA, Station de Zoologie, BP 81, 33883 Villenave-d'Ornon Cedex, France
[5]State Forest Products Research Institute, Lamačská cesta 1, 83330 Bratislava, Slovakia
[6] Laboratoire de Neurobiologie Compareé des Invertebrés, INRA-CNRS (URA 1190), BP 23, 91440 Bures sur Yvette, France

Abstract. The European grapevine moth, *Lobesia botrana* Den. et Schiff. is a phytophagous species restricted to a narrow range of host-plants. During the summer when tansy occurs in its ecosystem, females can strongly be attracted to tansy flowers. The ecological value of such an attraction is unclear, since tansy reduces the moth's mating and oviposition and is toxic for larvae. We used electro-antennogram recordings and olfactometer bioassays to clarify the responses of *L. botrana* females to tansy flower odours. Our data demonstrate that tansy odour is well detected by females, and the resultant EAG responses are dose dependent. The largest magnitude EAG responses were elicited at each dose by the two extracts of tansy (EO and E30). When presented a dual choice of a test substance *vs* solvent control, more than 80 % of the females were attracted by either of these two extracts of tansy flowers, while singly the three fractions attracted between 66 % and 56 % of females. The rate of attraction increased on the second and third days of the experiment, when females are 4 to 5 days old. This corresponds to prior field observations. A positive correlation was found between the EAG and behavioural responses, suggesting that EAG analysis can usefully screen out biologically relevant odours. Our results yielded information concerning odour extraction and fractionation procedures. It might impact the practical use of tansy odours in grapevines protection.

Key words. *Lobesia botrana*, tansy, flower extract, attraction, plant odour, electroantennogram, olfactometer.

Introduction

Plant diversity is an important feature of the environment of phytophagous insects, even if the agroecosystem structure tends to reduce this diversity. There are numerous examples of weeds that play important roles in the biology of pest insects (Perrin, 1977; Taylor, 1977). Mixed stands of plants can affect the development of phytophagous insect populations by hindering successive patterns of host-plant selection, like searching behaviour (Uvah & Coaker, 1984; Thiery & Visser, 1986), duration of the periods of activity, and oviposition behaviour (Stanton, 1982; Latheef & Ortiz, 1984).

European grapevine moth (EGVM), *Lobesia botrana* Den. et Schiff., is a serious pest of european vineyards, despite its limitation to a narrow range of alternative host-plants (Stoeva, 1982). Tansy, *Tanacetum vulgare* L. is an Asteraceae weed species that naturally occurs in many agroecosystems, including vineyards when no herbicide is applied. Tansy flowers strongly attract females of EGVM (Gábel, 1992). This attraction

can be mimicked under controlled conditions with synthetic blends of monoterpenes, that have been identified in the odour of flowering tansy (GÁBEL et al., 1992). The ecological value of such an attraction is, however, still unclear, since it has been demonstrated that tansy is not a host plant of EGVM. Further the flower odour of tansy has recently been shown to strongly reduce oviposition and also mating activity (GÁBEL & THIERY, 1994) which may prevent offspring from having to face the toxic allochemicals of tansy (LUSTNER, 1914).

The aim of the present research was to define simple and low cost extraction/ fraction procedures leading to efficient odourant baits which could be proposed for practical using in grapevine protection, and to determine the behavioural activity of females to odourants as a function of female age. The biological activity of the various extracts and fractions has been assesed by two different methods: electroantennogram (EAG) recordings and behavioural olfactometer bioassays.

Materials and methods

Plant extracts. Tansy flowers (without their pedicels) were collected in a vineyard located in Modra (south west of Slovakia) between the end of July to mid-August. Extractions were made on fresh material, immediately following the harvest.

Essential oil of tansy: 500 g of flowers were water steam distilled during two hours using a distillation-extraction device modified after Likens and Nickerson (PHARMACOPEA BOHEMOSLOVACA, 1987). Half milliliter of essential oil (EO) was obtained (equiv. to *ca* 10 g of flowers/ml).

Fractions of tansy flowers: 1000 g of flowers were macerated during 48 hours in 2000 ml of pure ethanol and than filtered through the cellulose. Two batches of 650 ml were evaporated until the ethanol solvent was removed using a vacuum rotary evaporator. The dry extracts were then diluted each in 1000 ml of hot purified water and distilled by water steam using the device described above until obtention of: a) a volume of 30 ml (sample E30; equiv. to *ca* 10 g of flowers/ml); b) 3 consecutive fractions of 50 ml each corresponding to different volatilities (samples F50-1, F50-2 and F50-3; equiv. to *ca* 2 g of flowers/ml each).

Insect material. The insects originated from a stock culture (INRA, Bordeaux, France), that was reared on semi-synthetic diet and annually infused with wild insects. Females used for both electroantennograms and bioassays were 2 days old and mated. Behavioural experiments were conducted under 14L/8D, light period being followed by artificial sunset (constant decrease from 1200–1250 to 20–30 lux during one hour) and night period followed by sunrise (inverse). Temperature (22 °C ± 1 °C) and relative humidity (60 % ± 5 %) were held constant.

Electroantennogram set-up. Electroantennogram (EAG) measurements were conducted on head-antenna preparations. Electrodes were glass capillaries with an Ag/AgCl wire inserted and filled with physiological saline solution (KAISSLING, 1987). The recording device consisted of an input probe, a Carrack PB 181-11 preamplifier, a Tektronix 5223 differential oscilloscope and a microcomputer using custom software adapted from MARION-POLL & TOBIN (1991). Preparations presenting impedences higher than 15 MOhm were discarded. The antenna was continuously bathed with purified and humidifier air (4.67 ml/sec). Odourants were placed, without dilution, onto 350 mm^2 of filter paper inserted into a 150 mm long Pasteur pipette (see amounts beneath). During stimulation (0.82 s) the odourant air from pipette (2.19 ml) was injected into the clean air flow and then blown over the prepared EGVM antenna. Four µl and 40 µl of a solution of hexanol diluted in paraffin oil (10^{-2} vol/vol dilution) were used as standards. Responses to EO were recorded from 20 females, using the following doses: 1µl, 4 µl, 8 µl, 16 µl and 40 µl. Series of stimulations including the three different fractions have been performed on 10 females with two doses (4 µl and 40 µl). The stimulation sequence was: 2 successive standards, 2 fractions, standard, 2 fractions, standard, etc.. Each EAG response, was expressed as a percentage of the value obtained with the hexanol standard (corrected according to the decrease observed with sequential stimulation by hexanol). EAG were then expressed as a mean ± SD of the recorded percent responses.

Bioassay. Behavioural activity of odour substances was measured in an olfactometer designed specially for EGVM females. It consists in a 10 L glass barrel, with two traps (100 ml glass jar with 5 mm I. D. hole made in the lid) hung at a height of 25 mm above the bottom. Purified and humidified air was blown in from the bottom of the barrel and exitted from the top, providing a constant and vertical air flow (625 ml/min, speed: 0.5 m/sec). Traps contained 26 ml of purified water with 2 drops of wetting agent (Citowett; BASF Germany), one short glass test-tube (10 mm I. D.) filled with 100 ml of paraffin oil (used as releaser) and only in odourant trap 400 ml of tested blends. Ten females were released into the barrel

per test, between 10 and 14 test repetitions were made for each fraction.The number of females caught was recorded for each trap every morning over a 3 days span.

We calculated a daily differential trapping rate (TR, in percent) between control and odourant traps: $TR = 100 \times (O - C) / (n - (m+t))$, where O is the number of females caught in odourized traps, C the number of females caught in control traps, n the total number of females, m the number of females found dead and t the number of females trapped on previous the day. The mortality observed during the experiment was equivalent for the 5 tested blends, varing within 2–7 % of the individuals released.

Statistics. Statistical differences between the evoked EAG as well as the final behavioral responses to tansy extracts and fractions were evaluated by t-test statistics (Weir, 1960). Fourfold tables procedure was used to compare the trapping rates, while relationships between EAG and olfactometer responses were examined by a linear correlation analysis (Sachs, 1984).

Results

EAG responses to the various fractions. The mean value of the responses to the hexanol standard were 1.65 ± 0.30 mV (4 µl) and 2.06 ± 0.54 mV (40 µl). The mean EAG response to EO increased as the dose deposited on the filter paper increased, but no significant differences in EAG magnitude were found between the dose levels. The mean response to EO (relative to hexanol standard at 4 µl dose) increased from 234 ± 59.6 % at the dose 1 µl to 284.5 ± 90.9 % at the dose 16 µl (Figure 1). The dose of 40 µl provoked "hyperpolarizations" (i. e., a positive polarity EAG potential) in all recorded antenna. The highest mean EAG response to fractions were recorded with stimulants of 40 µl of E30 (4.75 ± 0.76 mV) corresponding to 273.1 % to standard at the dose of 40 µl and the lowest percent EAG response (79.6 %) with 4 µl of the fraction F50-2 (1.28 ± 0.37 mV) (Table 1). At both of the two doses used the highest EAG responses were obtained with EO and E30 (t-test, $P < 0,05$). Fraction F50-1 released a lower response as compared to E30 at the same doses (t-test, $P < 0,05$). The two other fractions (F50-2 and F50-3) released the lowest responses at both doses (different from F50-1 at $P < 0,05$, t-test). Sample F50-1 at 40 µl dose elicited EAG responses of a statistically equal magnitude to those elicited by the EO and E30 samples at 4 µl dose (Table 1).

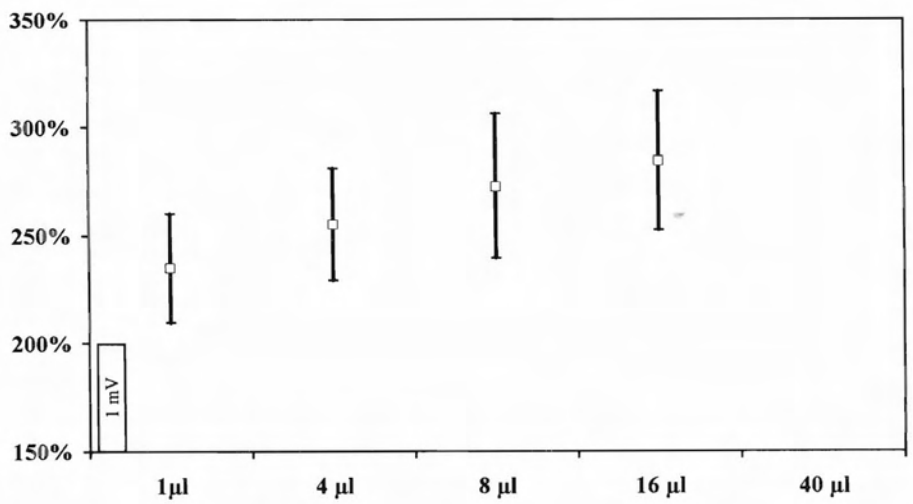

Fig. 1. Electroantennogram (EAG) responses of EGVM mated females to different doses of tansy essential oil (EO). Responses are expressed as a percentage of the response to standard (4 µl of hexanol in paraffin oil 10^{-2} v/v). Open-squares represent the mean value from 20 individuals, thin bars represent standard deviation. The 40 µl dose stimulation is not plotted because it resulted in a reversed polarity of the potential (i. e. hyperpolarization).

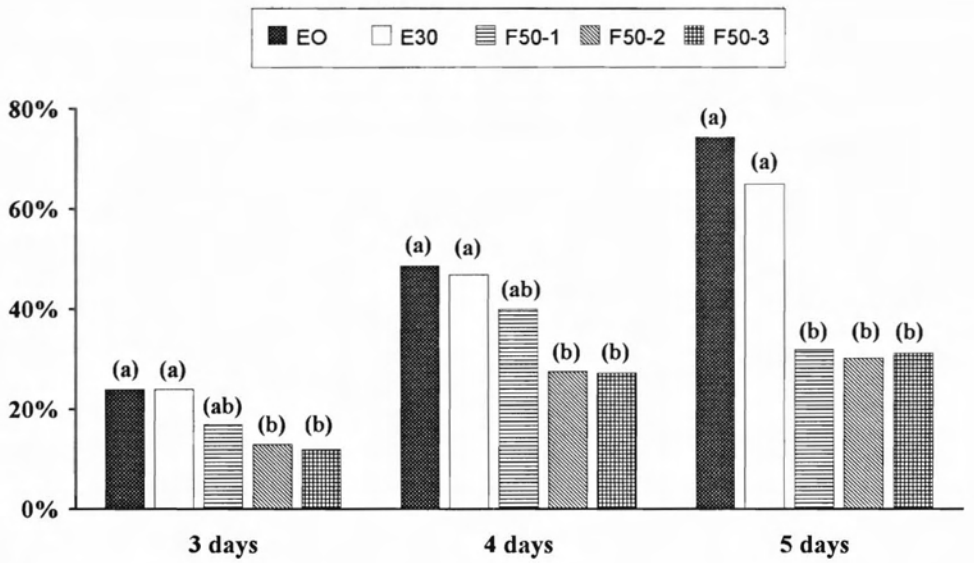

Fig. 2. Daily attractancy indexes (%) of EGVM mated females to extracts (EO, E30) and fractions (F50-1, F50-2, F50-3) of tansy flowers as a function of moths age. Index is calculated from females remaining each day in the barrels. Within an age class, different letters indicate statistical differences at $P < 0,05$ (fourfold table procedure).

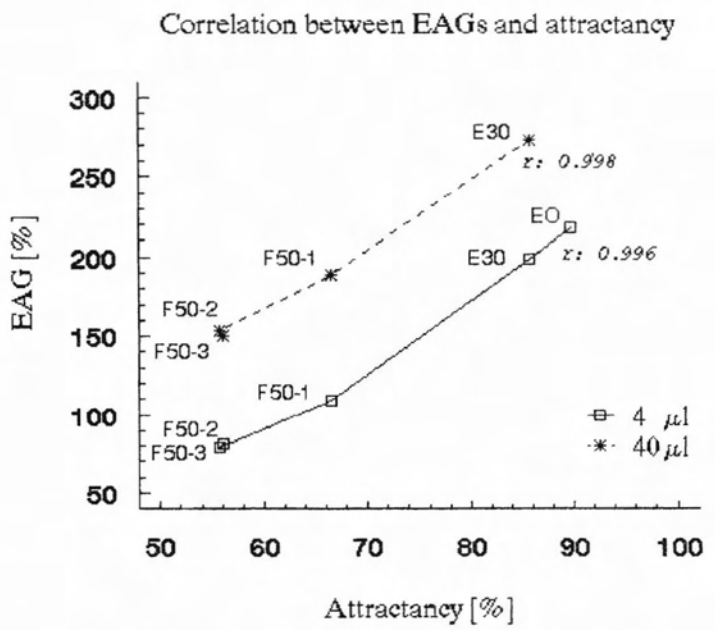

Fig. 3. Correlation between EAG relative responses and accumulative attractancy (after 72 hours) in *Lobesia botrana* for two different EAG doses of tansy odours. EO and E30 are raw extracts, others are different fractions. Data obtained with EO (40 µl) was discarded because of hyperpolarization.

Table 1. EAGs (% of standard) and percent accumulative attractancy (after 72 hours) of mated females EGVM to tansy volatiles. EAG and behavioral responses are expressed as mean values (standard deviation). EAG standards were 4µl and 40µl doses of hexanol 10^{-2}, for respective test doses. Similar letters indicate no statistical differences at $P < 0,05$ (t-test).

Product	EAG		Attractancy
	4 µl	40 µl	
EO	217.9 (12.4) b	hyperpolar.	89.6 (0.9) a'
E30	198.2 (15.2) b	273.1 (16.3) a	85.7 (1.2) a'
F50-1	109.2 (3.3) c	188.6 (5.4) b	66.4 (1.5) b'
F50-2	79.6 (4.9) e	153.8 (2.7) d	55.8 (2.7) b'
F50-3	81.6 (2.5) e	149.7 (3.9) d	56.1 (2.3) b'

Behavioural responses. The extracts EO and E30 attracted significantly more mated females (fourfold tables procedure, $P < 0,05$) than each of the three fractions of E30 sample (Table 1). The percentages of mated females caught per day were 24 %, 49 %, 74 % for EO and 24 %, 47 %, 65 % for E30.

We found an effect of age on the behavioural response. The number of females trapped by the two most attractive blends (EO and E30) significantly increased on the 2nd and 3rd day of experiment which correspond to 4 and 5 day old females (fourfold tables procedure, $P < 0,05$) (Figure 2).

Are EAG responses and attractiveness correlated? Most of EAG experiments suppose the implicit assumption that large magnitude EAG responses are released by volatiles of behavioural importance (either stimulation or inhibition) (Visser, 1979; Mayer et al., 1987). We have checked whether such an assumption is valid in *L. botrana* and which from two series of EAG responses is the best correlated with the attractancy. Significant linear correlations where found between the attractiveness and the relative EAG amplitudes for each tested blends (Figure 3). We can conclude in this first appraisal, that recording *L. botrana* EAG responses to volatiles may be used for a raw estimation of attractiveness.

Discussion

The present results show that olfactory receptors of EGVM females detect volatiles produced by tansy flowers. Significant EAG responses are observed with doses as low as 4 µl of extracts, which correspond to about 40 mg equivalent of tansy flowers (TFE). The amplitude of the EAG responses recorded with the essential oil of tansy and with the various fractions are always greater than EAG responses obtained with extracts of various parts of its grapevine host plant (B. Gábel, unpublished data). The large olfactory stimulation elicited by tansy volatiles can be explained by the high sensitivity (or high numbers of olfactory receptors) of EGVM to monoterpenes as demonstrated by coupling GC-EAD recordings (Gábel et al., 1992). High doses of essential oil reproducibly provoked positive polarity "hyperpolarizations". This is probably due to chemoreception of one of the three isomers of thujone, which combined represent the major constituents of tansy essential oil (P. Hradský, unpublished data) like in Dembitski et al. (1984). The global extract E30, obtained by a different extraction procedure, did not cause the same effect. The 3 fractions from E30 released lower EAG responses as compared to E30 and EO extracts. This is partly due to lower TFE in these fractions

(2 g/ml TFE with fractions *vs* 10 g/ml TFE with extracts). However, since fractions corresponding to 80 mg of TFE (40 µl of F50-1, F50-23, or F50-3) released lower EAG response than 40 mg of TFE for EO or E30 extracts (4 µl), we hypothezise that lower responses to each of the three fractions are the result of reduction in mixture complexity. This is supported by comparisons of GC patterns on major compounds: we found in each of the three fractions (F50-1, F50-2, F50-3) approximately two times fold less compounds than in E30 (P. HRADSKÝ, unpublished data).

The two global extracts (EO and E30) also provoke a strong attraction of females. After 3 days of experiment 89.6 % and 85.7 % of females were recovered in odourant traps baited with EO and E30, respectively. The three fractions of E30 are less attractive to females. From these fractions, F50-1 which was obtained by the first collection of the distillation (higher concentration of volatile substances measured by GC analysis), is the most attractive (as compared to F50-2 and F50-3). This fraction (F50-1) relesed also higher EAG responses as compared to F50-2 and F50-3.

Our present data also reveal significantly higher attraction with all odourants on the second and third days of experiments. This corresponds to the optimal age of females collected in the field, females about 4–5 days old (mated and having already oviposited) being the most abundant on tansy flowers (GÁBEL, 1992). We cannot physiologically explain the effect of the age on the increased responsiveness. We could not exlude sensitization (result of sustained exposure to odour) to partly interfere with the behavioural observation. Associative learning involving oviposition is probably not likely to occur since tansy odour strongly reduces the oviposition in that insect (GÁBEL & THIERY, 1994).

The aim of this work was to determine the behavioural relevance of different extraction/fractionation procedures from tansy flowers. This approach is integrated within the framework of developing natural odourant baits to trap females, and one objective was to propose low cost but efficient baits. From these results, global extracts obtained from the 2 different methods of extractions we have used can be considered as equivalent. Fractionation according to our procedure led to strong decrease of activity. Both EO and E30 elicit a good level of behavioural activity. It will serve in further experiments as standard blends in order to progressively reduce the number of constituents in different mixtures of synthetic molecules by comparing trapping capacities on EGVM females in vineyards.

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