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Volatiles from *Trifolium* as feeding deterrents of redlegged earth mites

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Abstract

The volatile compounds produced by the leaves of *Trifolium glanduliferum*, *T. strictum* and *T. subterraneum* were collected by trapping on Tenax TA adsorbent and the major components were identified by GC–MS. The volatiles obtained from *T. glanduliferum* and *T. strictum* were shown to deter the redlegged earth mite (*Halotydeus destructor*) from feeding, whereas those from *T. subterraneum* did not. β-Ionone and coumarin, detected in the volatiles of *T. glanduliferum*, *T. strictum*, showed strong deterrent activity at 100 ppm. Phenylethanol, a component of the volatiles of *T. strictum*, was inactive.

Keywords: *Trifolium subterraneum*; *T. glanduliferum*; *T. strictum*; *Halotydeus destructor*; Clover; Volatiles; Insect feeding deterrents

1. Introduction

Olfactory cues play a major role in the differentiation of plants by phytophagous insects. Some insects differentiate plants based on cues perceived at a distance, whereas others do so after arrival at the plant (Viser, 1986; Visser & Piron, 1995). The identification of volatile compounds produced by plants and released into the atmosphere is an important step in the investigation of insect-plant interaction. We have been interested in the factors that determine resistance of some varieties of *Trifolium subterraneum* L. to *Halotydeus destructor* Tucker (Acarina: Penthaleidae) (redlegged earth mite), a major pest of subterranean clover (subclover) and other legumes in southern Australia.

Subclover is the most commonly used pasture legume in Australia although its productivity is declining in south-eastern Australia due, in part, to the effects of the redlegged earth mite. The adoption of new species resistant to the mite is a priority and two alternative pasture legume species, *T. glanduliferum* Boiss. and *T. strictum* L., have shown some promise. In particular, mature plants of *T. glanduliferum* have been shown to be resistant to mites under laboratory and field conditions. In previous work we have shown that the damaged-induced volatile compounds produced by cotyledons of resistant varieties of *T. subterraneum* deterred mites from feeding and contributed to the resistance of the varieties (Jiang, Ridsdill-Smith & Ghisalberti, 1996). Resistant cotyledons produced more oct-1-en-3-one than susceptible cotyledons, whereas the major volatile compound produced by susceptible varieties was (*E*)-2-hexenal, one of the components of the 'green odour' volatiles (Hatanaka, 1993; Jiang, Ghisalberti & Ridsdill-Smith, 1996). Oct-1-en-3-one deterred mites from feeding, but (*E*)-2-hexenal attracted mites in the vicinity (Jiang, Ghisalberti & Ridsdill-Smith, 1997).

The aims of the present work were to identify the volatile compounds from the mature leaves of *T. glanduliferum*, *T. strictum* and *T. subterraneum*. The volatile compounds from the three species and selected compounds produced by *T. glanduliferum* and *T. stric-*

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Table 1
Percentage composition of the volatile compounds identified from mature leaves of *T. glanduliferum*, *T. strictum* and *T. subterraneum*

Compounds	*T. glanduliferum*		*T. strictum*	*T. subterraneum*
	hydrodistillation	Tenax TA	Tenax TA	Tenax TA
Alcohols				
Pentanol	0.5	0.7	0.5	0.3
1-Penten-3-ol	5.0	1.5	0.6	1.3
2-Penten-1-ol	4.1	1.2	3.1	0.2
Hexanol	2.2	3.3	3.5	0.1
E-3-Hexen-1-ol	–	trace	0.2	–
Z-3-Hexen-1-ol	8.2	3.7	5.5	0.2
2-Hexen-1-ol	1.1	3.3	4.0	0.2
2,3-Epoxyhexanol	–	0.3	–	0.1
Octan-1-ol	0.2	–	–	–
1-Octen-3-ol	0.4	trace	1.0	5.1
Glycerol	–	–	2.4	0.2
2,6-Nonadien-1-ol	0.1	–	–	–
2-Methylcyclopentan-2-ol	–	–	2.7	–
Phenylethyl alcohol[a]	–	–	2.1	–
Aldehydes				
2-Pentenal	9.0	5.3	0.2	1.9
Hexanal	3.7	14.1	11.0	5.0
Z-2-Hexenal	3.2	1.3	1.0	1.5
E-2-Hexenal	36.9	66.2	47.0	79.0
2,4-Hexadienal	trace	0.3	0.1	0.2
Heptanal	trace	0.4	–	–
2-Octenal	–	–	0.2	–
2-Nonenal	–	–	2.0	–
2,6-Nonadienal	trace	0.2	2.0	–
Benzaldehyde	–	0.1	–	–
Salicylaldehyde	–	–	–	0.1
Ketones				
Octan-3-one	–	–	–	0.2
4-Octen-3-one	–	–	–	0.3
Cyclohexanone	–	–	0.4	–
Acids				
Propanoic acid	–	–	–	0.3
Hexanoic acid	0.2	0.1	2.5	0.1
2-Hexenoic acid	trace	0.1	1.5	0.5
Octanoic acid	–	–	0.5	–
Tetradecanoic acid	trace	–	–	2.0
Hexadecanoic acid	trace	–	–	0.3
Oxygen heterocycles				
Methylfuran	trace	–	0.4	–
2-Ethylfuran	–	0.2	2.6	–
2-Propylfuran	–	trace	1.0	–
5-Ethyl-2(3H)-furanone	–	0.2	1.2	0.3
Furfural	–	0.2	–	–
Coumarin[a]	0.4	0.4	–	–
Terpenes				
α-Ionone	0.1	–	–	–
β-Ionone[a]	0.2	–	1.2	0.1
Dehydro β-ionone	0.1	–	–	–
2,6-Dimethylocta-5,7-dien-3-ol	0.1	–	0.2	0.3
Geraniol	0.1	–	–	–
Linalool	1.0	–	–	–
p-Menth-1-en-8-ol	0.1	–	–	–

Table 1 (*continued*)

Compounds	*T. glanduliferum*		*T. strictum*	*T. subterraneum*
	hydrodistillation	Tenax TA	Tenax TA	Tenax TA
Terpenen-4-ol	0.3	–	–	–
Phytol	0.1	–	–	–

[a] Compounds tested for feeding deterrence to mites, see Table 2.

tum were assayed for feeding deterrence activity towards the redlegged earth mite.

2. Results and discussion

The volatile compounds from mature leaves of *T. glanduliferum* were obtained by two methods; hydrodistillation and by dynamic headspace analysis by trapping onto Tenax TA polymer. For *T. strictum* and *T. subterraneum*, the volatile compounds were obtained by trapping onto Tenax TA only. The composition and the identity of compounds in the volatiles were determined by GC–MS methods and by comparison of their retention time with those of authentic samples (Table 1).

In comparing the composition of the volatile components obtained by hydrodistillation and Tenax trapping, two major differences are apparent (Table 1). The proportion of aldehydes of the hydrodistillate is substantially lower (51.8%) than that obtained by trapping (87.9%). In hydrodistillation, the higher temperature involved and the presence of acids would lead to condensation of aldehydes. On the other hand, hydrodistillation affords quantities, albeit small (2.2%), of terpenes which are not detectable in the volatiles obtained by trapping. This is not due to the more forcing condition of hydrodistillation since β-ionone, 5,6-epoxy-β-ionone and 2,6-dimethyl-5,7-dien-3-ol were detectable in the volatiles of *T. subterraneum* and *T. strictum*.

A comparison of the volatiles produced by the three species reveals that the levels of aldehydes in *T. glanduliferum* and *T. subterraneum* are similar (87.9%) and higher than those of *T. strictum* (63.7%). The contributions of the 'green odour' compounds to the volatiles of all three species is significant; 91.8, 86.9 and 72.2%, respectively.

The volatiles obtained from each species were bioassayed for feeding deterrence towards the redlegged earth mite. Significant deterrence was observed for the volatiles from *T. glanduliferum* and *T. strictum*, but not for those obtained from *T. subterraneum* (var. SE014) (Table 2), which is relatively less resistant to mites. That this effect was not due to the 'green odour' compounds can be deduced from the fact that the volatiles from *T. subterraneum*, which contain similar amounts of these compounds as those from *T. glanduliferum*, were not active.

Of the components of *T. glanduliferum* and *T. strictum* that were not present in significant quantities in *T. subterraneum*, two attracted our interest. Coumarin was detected only in *T. glanduliferum* and β-ionone was present in *T. strictum* but not in *T. glanduliferum*. These compounds were also assayed for deterrent activity towards the mite. The results listed in Table 2 in-

Table 2
Mean number of mites on control and test membrane sachets containing volatile compounds from *Trifolium* species over 3 h[a]

Sample	Control (mean ± S.E.)	Treatment (mean ± S.E.)	Deterrence[b] (%)
T. glanduliferum (cv 87182)			
Volatiles (100 ppm)	7.8 ± 0.90	2.5 ± 0.28	55**
T. strictum (cv AZ2311)			
Volatiles (100 ppm)	7.2 ± 0.77	1.9 ± 0.40	58**
Volatiles (50 ppm)	4.8 ± 0.86	1.7 ± 0.26	48***
T. subterraneum (cv SE014)			
Volatiles (100 ppm)	3.1 ± 0.52	2.6 ± 0.26	9
Compounds			
Coumarin (100 ppm)	3.0 ± 0.47	0.42 ± 0.19	76**
β-Ionone (100 ppm)	5.7 ± 0.87	0.14 ± 0.04	94***
Phenylethanol (100 ppm)	2.1 ± 0.26	1.7 ± 0.17	9

[a] Both control and test membrane sachets contained 1% glucose and 5% Tween 80.
[b] $^{**}P < 0.01$; $^{***}P < 0.001$.

dicate that both compounds had significant activity when tested at 100 ppm, a level equivalent to the amount of each compound contained in the volatiles obtained from 5 g of fresh leaves (~100 μg/g). As a comparison, phenylethanol, only detected in *T. strictum*, had no activity.

A number of interesting points arise from the results described above. The volatiles from the mature leaves of the variety of *T. subterraneum* show no significant feeding deterrent activity, in agreement with previous observations with cotyledons of susceptible varieties of sub-clover (Jiang et al., 1996a,b). The 'green odour' compounds, which are dominant in the mixtures of volatiles, do not contribute to the feeding deterrence observed for the samples from *T. glanduliferum* and *T. strictum*. In fact, evidence obtained previously suggests that they can play a role as attractants (Jiang et al., 1997). However, the volatiles from these two species contain compounds, coumarin in the case of *T. glanduliferum* and β-ionone in *T. strictum*, that appear to contribute to rendering the mixture strongly deterrent to the mite. Interestingly, phenylethanol (100 ppm) has been reported as an aggregation pheromone of a *Caloglyphus* sp and a repellent to the bulb mite *Rhizoglyphus robini* (Kuwahara, 1989; Shinkaji, Okabo, Amano & Kuwahara, 1988).

There is relatively little information available on the volatile compounds produced by clover species. The essential oils of *T. repens* and *T. pratense* obtained by steam distillation have been analysed (Kami, 1978). The volatile constituents of the CH_2Cl_2 extracts from *T. pratense* have been examined. Of the 309 compounds detected, 210 have been identified including the three compounds mentioned above (Srinivas, 1988). In another study, 25 compounds in the headspace of the leaves, flowers and seed pods of *T. pratense* were identified (Buttery, Kamm & Ling, 1984) and, of these, only hexanol, (*Z*)-3-hexenol, (*E*)-2-hexenol and 1-octen-3-ol have been found in the three species examined in the present study.

3. Experimental

3.1. Plant material

T. glanduliferum (cv 87182), *T. strictum* (cv AZ2311) and *T. subterraneum* (cv SE014) were grown in experimental fields. Leaves from mature plants (3 month-old) were used.

3.2. Collection of volatiles

(a) Hydrodistillation: fresh leaves (100 g), ground in liquid nitrogen, were hydrodistilled using a Clebenger apparatus for 2 h. The volatiles recovered with ether were stored at −15°C until analysis. (b) Trapping with Tenax TA: fresh leaves (50 g), ground in liquid nitrogen, were placed in a flask into which air, passed over activated charcoal, was drawn by application of suction at the outlet and through a Tenax TA trap (200 mg; 6 × 16 mm) for 6 h at 500 ml/min. The volatile compounds were desorbed from Tenax TA with ether.

3.3. Gas chromatography–mass spectrometry

Samples of the volatiles were analysed by gas–liquid chromatography using a HP 5790A GC instrument, equipped with an Innowax column (0.24 μm film thickness, 25 m × 0.35 mm i.d.) and He as the carrier gas. The injector and FID temperatures were 250°C and the oven temperature was programmed from 40°C (isothermal for 2 min) with a ramp of 10°C/min to 230°C. For GC–MS an HP 5986 instrument was used. Tentative identifications made by GC–MS were confirmed by comparison of the mass spectral data and retention times with those of authentic samples.

3.4. Redlegged earth mites

Mites were cultured in the laboratory on vetch (*Vicia sativa* cv. Blanchefleur) (Leguminosae) in summer and collected from pasture near Perth, Western Australia, in winter. Mites were staged and young adults, starved for 2 h in a humid vial (15°C) were used for experiments.

3.5. Assay for feeding deterrence

The volatiles were bioassayed for feeding deterrence towards redlegged earth mites. The membrane sachet technique previously described was used (Jiang et al., 1996a,b). Extracts to be tested were solubilized in 5% Tween 80 containing 1% glucose, control solutions contained only 5% Tween 80 and 1% glucose. Mites were given a choice of a sachet containing a feeding stimulant (1% aqueous glucose) with or without the test sample (volatiles from each species or pure compounds). Membrane sachets were made with stretched Parafilm™. For each sachet, a membrane was stretched over a 2 cm diameter plastic ring, 35 μl of test solution added and a second membrane stretched over the top. In choice experiments, two membrane sachets were buried, nearly touching, so as to be just above the surface of the soil (4:1 sand to loam) which was moistened close to field capacity, in a three-quarters filled plastic jar. Twenty mites were released by gently tapping them from a vial onto the soil beside the membrane sachets in the plastic jar which was then sealed with stretched parafilm to contain the mites. Experiments were carried out at 15°C under fluor-

escent light in a room with no natural light. Mites were observed through the parafilm membrane and the number of mites on each membrane sachet was counted at 20 or 30 min intervals for 3 h. Deterrence in the choice test for each experiment was calculated from the average of 7 observation as follows: ((mite number on control-mite number on treatment)/total number of mites on sachets) × 100. The average of 7 observations during 3 h is presented. Difference between means were examined with paired *t*-tests.

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