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VOLATILES FROM *MEDICAGO SATIVA* COMPLEX FLOWERS

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Key Word Index—*Medicago sativa* complex; Leguminosae; lucerne; flowers; volatiles; colour; *trans*-2-hexenal.

Abstract—Volatiles from flowers of several lucerne (*Medicago sativa* complex) genotypes, belonging to different taxonomic groups and characterised by various flower colours, were isolated by Tenax-trapping and analysed by GC and GC-mass spectrometry. Thirty-three compounds were identified, including aldehydes, alcohols, esters, ketones, terpenes and furanoids. *Trans*-2-hexenal was the most abundant volatile in all genotypes. Ranking of individual volatile compounds was similar in all genotypes but absolute values ($\mu g\ g^{-1}$) for each compound varied widely from genotype to genotype, being largely associated with flower colour intensity. In terms of total volatile quantity, the following ranking of flower colours was observed: dark green > violet > bright or light yellow.

INTRODUCTION

Lucerne (*Medicago sativa* complex) is the oldest and most widely grown forage crop worldwide, being a source of high quality proteins for animal feeding [1]. The use of this species as a pasture or hay crop has always been paralleled by a significant trade of seed which represents, therefore, an important commodity [2]. As lucerne is a cross-fertilised species, relying on insects for pollination, plants must be visited, and their flowers 'tripped' by pronubes to produce adequate amounts of good quality seed. It has been suggested that flower volatiles can be an important factor in preferential bee visitation in lucerne clones [3]. It is well known that plant volatiles, together with some other compounds, are determinant in insect–plant interactions [4]. Some studies have been carried out on the volatiles of lucerne flowers with regard to their attraction for honeybees and to investigate flavours that mediate the behaviour of lucerne pests. Knowledge of the associated volatile constituents would provide information necessary in determining which particular volatile chemical compounds attract the pollinating insects or the pests. However, information acquired on the chemical composition of lucerne flower aroma is still scanty. Loper *et al.* [5] observed traces of 47 compounds and identified 12 of these in flowers of one variety. Buttery *et al.* [3] investigated volatiles of flowers and pods of three varieties and identified a total of 33 compounds in the flowers and 31 in the pods.

Beside olfaction, vision is usually the other main stimulus that attracts insects to plants and, in this respect, flower colour may determine differential attractiveness to pronubes [6]. The *M. sativa* complex includes several taxonomic groups, that are usually given the hierarchy of subspecies because no hybridisation barriers are present among them; genetic evidence supports common ancestry [7]. The tetraploid subspecies of the complex, which represent cultivated lucernes, are characterised by a wide range of flower colour, ranging from bright yellow in subsp. *falcata* to purple-violet of subsp. *sativa*. The subsp. × *varia* is considered to be a natural hybrid between the two above-mentioned taxa and the variability in flower colour variegation that it shows is related to the different level of relative introgression of the two parental subspecies [8].

In the present study, several lucerne genotypes have been considered, encompassing different taxa and flower colours in the complex. Their volatiles have been isolated by Tenax-trapping, identified by GC mass spectrometry and quantified by GC.

RESULTS AND DISCUSSION

Table 1 shows mean values across genotypes and lowest and highest genotype values of all identified volatile constituents of flowers. Genotype values were computed by averaging the values obtained in the two analyses made for each flower sample. A number of compounds were observed in the 13 samples, ranging from *ca* 40 to *ca* 60. Of these, the 33 most abundant constituents were identified and quantified in all genotypes and are those reported in the table, listed accord-

Table 1. Volatile constituents of lucerne (*Medicago sativa* L. complex) flowers

Compounds	Mean value* ($\mu g\ g^{-1}$ fr. wt)	Range†	
		$\mu g\ g^{-1}$ (fr. wt)	%
Aldehydes			
Hexanal	6.09	1.74 – 17.31	7.5 – 11.1
trans-2-Pentenal	0.29	0.09 – 0.73	0.3 – 0.5
trans-2-Hexenal	22.80	6.69 – 59.81	25.0 – 38.5
trans-2-Nonenal	<0.01	<0.01	<0.1
trans,trans-2,4-Hexadienal	0.15	0.04 – 0.35	0.1 – 0.3
Nonadienal	<0.01	<0.01	<0.1
2-Methyl-4-pentenal	7.31	1.44 – 21.14	6.3 – 13.6
Benzaldehyde	0.45	0.02 – 1.80	<0.1 – 1.4
Ethyl benzaldehyde	0.13	<0.01 – 0.21	0.1 – 0.5
Alcohols			
Butanol	0.13	0.08 – 0.25	0.2 – 0.7
Hexanol	1.17	0.46 – 2.43	1.6 – 2.3
Octanol	1.18	0.16 – 2.94	0.9 – 2.7
Pentan-3-ol	3.12	2.15 – 4.27	2.7 – 3.9
3-Methylbutanol	0.25	0.12 – 0.81	0.2 – 0.5
trans-2-Pentenol	0.68	0.21 – 1.26	0.7 – 1.6
trans-2-Hexenol	0.82	0.36 – 1.38	0.7 – 1.5
cis-3-Hexenol	3.71	1.32 – 7.54	4.1 – 5.8
Pent-1-en-3-ol	1.25	0.57 – 3.53	1.7 – 2.5
Oct-1-en-3-ol	4.28	1.02 – 9.20	5.5 – 12.7
Octa-1,5-dien-3-ol	5.06	0.98 – 11.54	5.9 – 11.4
Benzyl alcohol	0.18	0.02 – 0.85	<0.1 – 0.3
2-Phenylethanol	0.21	0.01 – 0.45	<0.1 – 0.2
Ketones			
Pent-1-en-3-one	1.51	0.46 – 5.34	2.0 – 3.5
Pentan-3-one	0.35	0.10 – 1.81	0.4 – 0.7
Octan-3-one	1.91	0.70 – 5.06	2.5 – 3.3
Methylphenyl ketone	<0.01	<0.01	<0.1
Esters			
cis-3-Hexenylacetate	0.81	0.42 – 2.01	0.7 – 1.3
cis-3-Hexenylbutanoate	0.32	0.03 – 0.97	0.1 – 0.5
Terpenes			
Limonene	0.48	0.01 – 3.34	0.2 – 2.1
Linalool	0.10	0.02 – 0.20	<0.1 – 0.2
trans-Ocimene	1.84	0.07 – 3.73	0.6 – 2.9
Furanoids			
Furane-2-ethyl	0.07	<0.01 – 0.21	<0.1 – 0.2
5-Ethyl-2(5H)-furanone	0.11	<0.01 – 0.49	<0.1 – 0.5

* Mean of 13 genotype values (each obtained by averaging two chemical determinations).
† Among genotype values.

ing to their chemical class. Nine aldehydes, 13 alcohols, four ketones, two esters, three terpenes and two furanoid compounds were found.

Aldehydes, which formed the most abundant class in terms of quantitative presence in the aroma, were mostly represented by saturated and unsaturated aliphatic compounds. Of these, *trans*-2-hexenal was consistently the most frequent volatile in all 13 genotypes. Despite the great differences in absolute terms ($\mu g\ g^{-1}$) among genotypes for this unsaturated aldehyde, its relative presence in the flower aroma was always comprised of between 25.0 and 38.5% of the total (Table 1). *trans*-2-Hexenal has been indicated as a compound possibly involved in mechanisms of resistance to pests and diseases [9, 10].

The group of alcohols was the most represented in terms of number of compounds identified, their quantity in the flower aroma being, however, always rather low, with the possible exceptions of *cis*-3-hexenol, the so-called 'green leaf alcohol' [11], oct-1-en-3-ol, which is a mushroom aroma compound [12], and octa-1,5-dien-3-ol (Table 1).

The only two esters detected in the samples were *cis*-3-hexenyl acetate and *cis*-3-hexenyl butanoate (Table 1), these are both formed from a C_6 alcohol.

All the C_6 compounds (aldehydes, alcohols and

Table 2. Genotype, total volatiles ($\mu g\ g^{-1}$ fr. wt), flower colour description and taxon of the different clones of lucerne (*Medicago sativa* complex) analysed

Genotype	Total volatiles	Flower colour	Taxon
283/8	149.4	Dark green with little bright yellow variegations	subsp. × *varia*
317/2	97.6	Darkgreen with violet and bright yellow variegations	subsp. *sativa* × subsp. *falcata**
283/14	73.8	Bright yellow with very little purple variegations	subsp. × *varia*
316/1	65.0	Dark green-violet with little bright yellow variegations	subsp. *sativa* × subsp. *falcata**
305/7	57.8	Dark green with bright yellow variegations	subsp. × *varia*
Equipe	55.4	Violet	subsp. *sativa*
105/9	48.9	Violet	subsp. *sativa*
119/2	47.5	Violet	subsp. *sativa*
1/16	45.7	Lemon yellow with green-lilac variegations	subsp. × *varia*
91/3	43.7	Light cream with light lilac tones	Unknown (possibly cross from different taxa)
315/2	39.0	Bright yellow	subsp. *falcata*
103/19	34.1	Bright yellow	subsp. *falcata*
61/19	20.6	Very light yellow–cream with light lilac variegations	subsp. × *varia*

* Artificial cross.

esters) are known to be present in several plant families and are considered responsible for the 'green leaf odour' of plants [3, 13–17].

Ketones and furanoid compounds were also detected in the extract (Table 1). Among the former, octan-3-one had already been found in lucerne flowers by Buttery *et al.* [3]. One the contrary, these authors did not detect any furanoids in lucerne flowers, whereas Buttery and Kamm [12] identified one furanoid compound, although different from those here reported, in intact stem and leaves samples of the same species.

Terpenes were observed in the present study, though generally to a limited extent (Table 1). These results do not agree with those of Loper *et al.* [5] and Buttery *et al.* [3] who found *trans*-ocimene as one of the most or the most important aroma component in lucerne flowers. The differences between the present findings and those previously reported are most likely due to differences in environmental conditions and, particularly, clipping period [18].

The present investigation revealed a great deal of variation among lucerne genotypes for all of the identified volatile compounds as evident by the range of absolute values (Table 1). However, a thorough examination of all the combinations of genotypes and compounds (data not reported) showed that the within-genotype rankings of the individual components tended to be very similar among genotypes. What really differed from clone to clone was the absolute quantity produced of each component. Indeed, summing up the amount found ($\mu g\ g^{-1}$) of all 33 identified compounds, the 13 genotypes under investigation showed a great differentiation (Table 2). The highest total amount of volatiles, found in the entry coded as '283/8', exceeded by seven-fold the lowest one, observed in '61/19'. Noticeably, the ranking of the genotypes for total volatiles produced did not appear to be random but followed almost strictly a decreasing order of 'darkness' of flower colour (Table 2). Variegated flowers with a predominance of dark green as background colour tended to produce more volatiles than violet flowers which, in turn, tended to produce more volatiles than flowers with a predominance of yellow or lighter in colour. Values of genotypes with dark green flower colour ranged from 57.8 to 149.4 $\mu g\ g^{-1}$, genotypes with violet colour ranged from 47.5 to 55.4 $\mu g\ g^{-1}$ and genotypes with light-coloured flowers ranged from 20.6 to 45.7 $\mu g\ g^{-1}$. The possible exception was represented by the genotype '283/14', where high volatile production (73.8 $\mu g\ g^{-1}$) was associated with a predominance of bright yellow colour in the flower. This clone derives from the same natural population of subsp. × *varia* as the genotype '283/8', the one with the highest quantity of volatiles. The population clearly has a variable level of introgression of the two parental taxa, subspp. *sativa* and *falcata*, as indicated by the different degree of flower variegation in the two genotypes. However, natural selection towards high levels of volatile production may have occurred in the whole population.

While there seemed to be a volatile production typical of genotypes from subsp. *sativa* (*ca* 50 $\mu g\ g^{-1}$) and of subsp. *falcata* (*ca* 35 $\mu g\ g^{-1}$), volatile content in genotypes from subsp. × *varia* and artificial crosses between taxa seemed to depend on the flower colour intensity as resulting from the hybridisation, be it natural or artificial.

In conclusion, this investigation has evidenced a genotypic effect on the total production of flower volatiles. Further assessments are required to confirm these findings, possibly also under different environmental conditions. As volatile production would appear to be associated with flower colour, parallel studies on pigments responsible for this colour may be important.

EXPERIMENTAL

Plant material. Twelve genotypes of exotic origin, selected from natural populations and varieties belonging to the *M. sativa* L. complex within a breeding programme aimed at developing grazing-type lucerne [19], and one genotype extracted from the local variety 'Equipe' were used in this study. The materials, cloned and field-grown under rainfed conditions at Lodi, northern Italy, encompassed subspp. *sativa, falcata* and × *varia* and artificial crosses involving to various extents these taxa (Table 2). Flower colour ranged from yellow to violet, including several variegated forms (Table 2). Flowers from each entry were collected at the full bloom stage in late August 1995. Inflorescences (20–30) per genotype were clipped just below the insertion of the bottom-most flower on the raceme. Fresh inflorescences, quickly frozen in liquid N_2, were then stored at $-80°$ until chemical evaluation.

Collection of volatiles. Flower volatiles were collected by enclosing in a glass tube 2–3 g of fresh material, to which 9.75 μg of 3-methylcyclohexanone were added as int. standard, and sampling the headspace using pure N_2 at 20 ml min^{-1}, through a 3 mm diameter freshly desorbed sampling tube packed with 100 mg of Tenax. The sampling period was optimized at 30 min at room temp. Double extraction was performed for each sample.

Analysis of volatiles. A sampling tube was inserted into a thermal desorption injector of a GC or GC–MS and, as the trap heated at 240° for 10 min in the flow of carrier gas (10 ml min^{-1}), the volatiles were desorbed and transferred into the fused silica trap cooled at $-120°$ using liquid N_2. Injection into the capillary column (Cp Sil 5 CB of 50 m length, 0.32 mm i.d. and 5 μm film thickness) was obtained by flash-heating (260° for 1 min) of the cold trap. The oven temp. programme was 50° (10 min), 50–220° (8° min^{-1}), 220° (20 min) with H_2 (1 ml min^{-1}) or He (1 ml min^{-1}) carrier gas for GC and GC-MS analyses, respectively. Detection was by FID (heated at 260°) and electron impact (EI, 70 eV, interface 230°). Compounds were identified by comparison of RR_ts and MS with published data [20–22]. The percentage composition of each component was obtained from FID data.

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