Effect of three volatile compounds from lucerne flowers on their attractiveness towards pollinators

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Abstract

Flower volatile compounds are involved in attractiveness towards pollinators and can therefore influence seed set in lucerne (*Medicago sativa* L.). This study aimed to verify possible effects exerted on the attractiveness towards pollinators by the application on lucerne plants of high concentrations of three volatile compounds, the aldehyde *trans*-2-hexenal, the alcohol oct-1-en-3-ol, and the terpene ocimene, naturally present in lucerne flower aroma. In each of three test days, three potted plants per odour treatment were sprayed with each compound, whereas three unsprayed plants were used as a natural control treatment. All the plants were located in the proximity of an apiary with five honey bee colonies. The number of visits by both honey bees and other pollinating hymenopterans were recorded on each plant between 14.30 h and 15.30 h every day, and between 17.00 h and 17.30 h on the second and third days. Every day at 18.00 h, the 12 plants were placed back under an isolation cage, where five other plants were kept throughout as a control, not pollinated by insects. After about 40 days, number and weight of seeds were recorded on all 41 plants. Chemical analyses on bulked flowers collected from all plants confirmed previous results (high concentrations of *trans*-2-hexenal, relatively large amounts of oct-1-en-3-ol, and limited amounts of ocimene). The effect of pollinating insects was remarkable on seed set, as indicated by the comparison between the 36 plants used in the outdoor trial and the five plants always kept in the cage. There seemed to be some attractive effect of ocimene on honey bees, whereas wild pollinating hymenopterans preferred the unsprayed control plants. The results also demonstrated the preponderant role of flower visits by wild pollinating hymenopterans on lucerne seed set, whereas honey bees confirmed to be poor pollinators of this crop.

Key words: attractiveness, lucerne (*Medicago sativa* L.), pollination, volatile compounds, seed set.

Introduction

In an entomophilous plant, the factors influencing both the reproductive physiology of the plant itself and the activity of pollinators can markedly affect seed set. Insects are attracted by flowers mainly through vision and olfaction, although gustatory and tactile hints may also be important (Dobson, 1994). The preference of visits can be guided, therefore, by flower colour, odour, pollen availability, volume and sugar concentration of nectar, or different combinations of these factors (Pinzauti and Intoppa, 1996). Floral shape and "apparent" floral shape (that is, the shape perceived by the insect) may also play an important role in attracting pollinators (Celli and Maccagnani, 1994; 1998; Dafni and Neal, 1997; Celli *et al.*, 2000).

In lucerne (*Medicago sativa* L.), seed production can be an important income in addition to that represented by the forage. The species is often self-compatible and under conducive circumstances, such as high temperatures, it can experience self-pollination (Tasei, 1984). However, a good yield of quality seed requires cross fertilisation among flowers of different plants through pollination carried out by insects that, collecting pollen and/or nectar, cause the so-called "trip" of the sexual column in the flower of this legume species (Tasei, 1984).

Information on the interrelationships among flower characters, which can be attractive towards pollinators, is scarce in lucerne, and most of the investigations on the relations between plant and insect are limited to *Apis mellifera* L. (Clement, 1965; Kauffeld *et al.*, 1969; Loper and Waller, 1970; Loper *et al.*, 1974).

Kauffeld *et al.* (1969) report of lucerne clones more attractive towards honey bees than others. In the case of nectar-collecting worker bees, the difference was attributed to different qualitative and quantitative characteristics of the secreted nectar. Those characteristics, in turn, appeared to be influenced by solar radiation, which seemed to exert different effects on flower colours. Pollen-collecting worker bees seemed less prone to preferences for different clones. Without distinguishing between nectar- and pollen-collecting honey bees, Loper *et al.* (1974) noticed that the bees were attracted by those clones characterised by a predominance of the ocimene-limonene-myrcene terpenic complex in their flower aroma. Volatile emanation can vary with photoperiod (Loper and Lapioli, 1971), season (Loper and Berdel, 1978; Pecetti and Tava, 2000), temperature, physiological factors [Hampton (1925), cited in Loper and Waller (1970)], and among different genotypes (Loper and Waller, 1970). Lack of random pollination in lucerne was also reported for *Bombus* sp. Latreille (Pedersen and Bohart, 1953) and *Megachile rotundata* (Fabricius) (Pedersen, 1967).

Volatile compounds of lucerne flowers are certainly involved in the attractiveness towards pollinators (Boren *et al.*, 1962; Pedersen, 1967; Loper *et al.*, 1974) and, therefore, they can affect seed set. The emanation of volatiles has been repeatedly examined in lucerne and studies in the USA have shown that ocimene (rather a common monoterpene in nature) is one of the main, if not the main component of flower aroma in lucerne (Loper *et al.*, 1971; Loper and Lapioli, 1971; Buttery *et* al., 1982). When conditioned to the aroma of lucerne flowers, honey bees were able to recognise the ocimene, as well as the other terpenes myrcene and limonene (Loper *et al.*, 1971; Waller *et al.*, 1974). In other studies, attractiveness towards honey bees was only observed for the terpene linalool, while repulsiveness was evidenced for the ketone octan-3-one and the methylsalicilate (Henning *et al.*, 1992). Investigations carried out in Italy on lucerne flowers indicated that ocimene was present in very limited amounts and so did, in general, all the terpenes, whereas other volatile classes, such as aldehydes and alcohols, were the main components of the aroma in the examined flowers (Tava and Pecetti, 1997; Tava *et al.*, 2000). Among the individual compounds belonging to the latter two classes, *trans*-2-hexenal and oct-1-en-3-ol, respectively, were very abundant. These two compounds are commonly present in plant materials and derive from lipid degradation (Buttery and Ling, 1993). In particular, the *trans*-2-hexenal, considered to be one of the main compounds responsible of the "green" odour, originates from the linoleic acid through enzymatic breaking by peroxidases and lipoxygenases (Hatanaka *et al.*, 1987). The *trans*-2-hexenal was perceived by various insect species belonging to different orders, such as the coleopteran *Leptinotarsa decemlineata* Say (De Jong and Visser, 1988), the lepidopteran *Manduca sexta* L. (Rybczynski *et al.*, 1989) and the dipteran *Drosophila melanogaster* Meigen (Clyne *et al.*, 1997). The oct-1-en-3-ol, with a typical mushroom scent, was attractive towards some dipteran species, such as certain mosquitoes, *Aedes* sp. Meigen (Kline, 1994), and the tsetse fly, *Glossina* sp. Robineau-Desvoidy (Spath, 1995).

The present study aimed to assess for the effect, induced by ocimene, *trans*-2-hexenal, and oct-1-en-3-ol applications on lucerne plants, on the attractiveness towards honey bees and other pollinators (and, consequently, on seed set). The possible identification of attractiveness or repulsiveness elicited by these compounds naturally present in lucerne, could guide selection towards an increase or a decrease of their concentration in plants, to improve the pollination efficiency in this crop, or towards a homogeneity of concentration in the case of clones to be inter-crossed while assuring a random mating, for the constitution of synthetic or "free-hybrid" varieties.

Materials and methods

Around 60 plants of the variety 'Equipe' were sown (March 4, 1999) and grown in pots at the Istituto Sperimentale Colture Foraggere, Lodi, northern Italy. The plants were clipped on June 6 and July 5. After the second clipping they were introduced into an insect-proof plastic cage. At full bloom of the subsequent regrowth, 41 plants, with purple flowers and with substantially similar vigour, were labelled: 36 were destined to the trial of attractiveness with the volatiles, and 5 were always kept in the cage as control without pollinator visits. Fifteen racemes with newly open florets were collected at random on each of the 41 plants. The samples were bulked and immediately brought to the laboratory for the extraction of volatile compounds by the dynamic headspace (purge and trap) method. Tenax was used as adsorbent resin, and chemical determination was carried out by gas chromatography (GC) and GC/mass spectrometry (MS) analyses, according to the methodology described in detail by Tava *et al.* (2000).

To assess for attractiveness, 9 plants out of 36 were assigned at random to each of four different treatments. Three groups of 9 plants were sprayed with volatile compounds (the aldehyde *trans*-2-hexenal, the alcohol oct-1-en-3-ol, and the terpene ocimene, respectively), whereas 9 unsprayed plants were used as control treatment.

The three pure compounds were solubilised in a 5‰ solution of Triton 100. To enhance the effects induced by the three compounds and to cope with possible losses due to spray application, each of the compounds was applied at a high concentration of 3.5 μmol/mL.

On each plant (three per day per treatment), a 5-mL solution of the respective volatile was applied by spraying the whole aerial part with 10 nebulisations. In addition, a small flock of cotton wool, imbibed with 100 μL of the pure volatile compound, was placed in the middle of the plant crown. The plants of the control treatment were sprayed with the 5‰ Triton solution and a dry cotton flock was placed in the middle of the crowns.

The trial was repeated over three days. Every day, twelve plants were assigned to the four different treatments and placed outdoors according to a randomised block design with three replicates. Each replicate included a row of four plants, one for each treatment, 1.5 m apart from each other; the rows (blocks) were 3 m apart from each other. In each day of trial, a different randomisation was used to allocate the four treatments within blocks. The blocks were placed at growing distance from 3 m to 9 m southwards of an apiary with five colonies of *A. mellifera*. Small plots of lucerne, located at about 150-200 m from the apiary, were the only surrounding crops in bloom during the trial.

The trial started on July 26, 1999 at 14.30 h summer time (all times are reported as summer time), since both volatile emanation from lucerne flowers and activity of pollinators show a peak around mid-day, in correspondence with maximum solar radiation (Pecetti and Tava, 2000). For three days, three operators, one per block (row of pots), recorded for one hour (from 14.30 h to 15.30 h) the insect visits on the respective four plants. The second and third day, insect visits were recorded also between 17.00 h and 17.30 h. In the subsequent days, each operator always observed a different block (row), to vary the operator-block combination and avoid possible systematic errors. The operator stood in the middle of the block, about 50 cm in the back of the hypothetical line adjoining the four plants, so that the farthest plant to observe was a little more than 2 m from his location, well within eye range.

Insect visits were recorded distinguishing between those of honey bees and those of other pollinating hymenopterans (e.g. bumble bees or leafcutter bees). In this latter case, no attempt was made to specifically classify the visiting insects. Visits by pollinators were recorded according to the method adopted by Clement (1965): a visit was represented by a pollinator landing on a raceme and working at least one flower; the flower "trip" was not considered a necessary condition to count the visit. When the insect flew to a new raceme and worked at least one flower, this was counted as a new visit; if the insect flew back to the previous raceme and worked at least one flower this was again counted as a new visit. Crawling of an insect from one raceme to another was not considered as a new visit. The number of flowers worked per raceme and the number of "tripped" flowers were not considered.

Every day at 18.00 h, the 12 plants were placed back under the isolation cage.

After the three days of trial, all 41 plants were kept in the isolation cage until September 7, when the formed pods appeared to be mature. The total number and weight of set seeds were then recorded on each plant. In order to verify the efficacy of insect pollination, the mean seed yield per plant (in number and weight) of the 36 plants, used in the outdoor trial, and that of the five control plants, never moved from the cage, were compared with the t-test.

The main factors "treatment" (the three volatile compound treatments and the natural control) and "day" were considered fixed. The factor "day" was considered fixed, to verify the possible temporal effect of the trial development (first, second, and third day of trial) on the pollinator responses. Also the numbers of visits, recorded during the three days between 14.30 h and 15.30 h, were compared. Differences in insect visits and seed yield among treatments and days, and the interactions between these factors, were tested by analysis of variance (ANOVA) and Duncan's multiple range test, using the error term obtained by pooling the first- and second-degree interactions of the fixed factors with the random factor "block". Simple correlation coefficients among all the recorded characters were also computed.

Results and discussion

The chemical analyses on flowers collected from the plants substantially confirmed previous quantitative determinations made at Lodi, northern Italy, on the three examined volatile compounds (Tava *et al*., 2000): there was a high concentration of *trans*-2-hexenal (16.12 µg/g, corresponding to 38.2% of total volatiles), a relatively large amount of oct-1-en-3-ol (3.52 µg/g, corresponding to 8.6% of total volatiles), and a very low presence of ocimene (0.13 µg/g of *cis*-β-ocimene and 0.16 µg/g of *trans*-β-ocimene, corresponding to 0.3% and 0.4% of total volatiles, respectively) (figure 1).

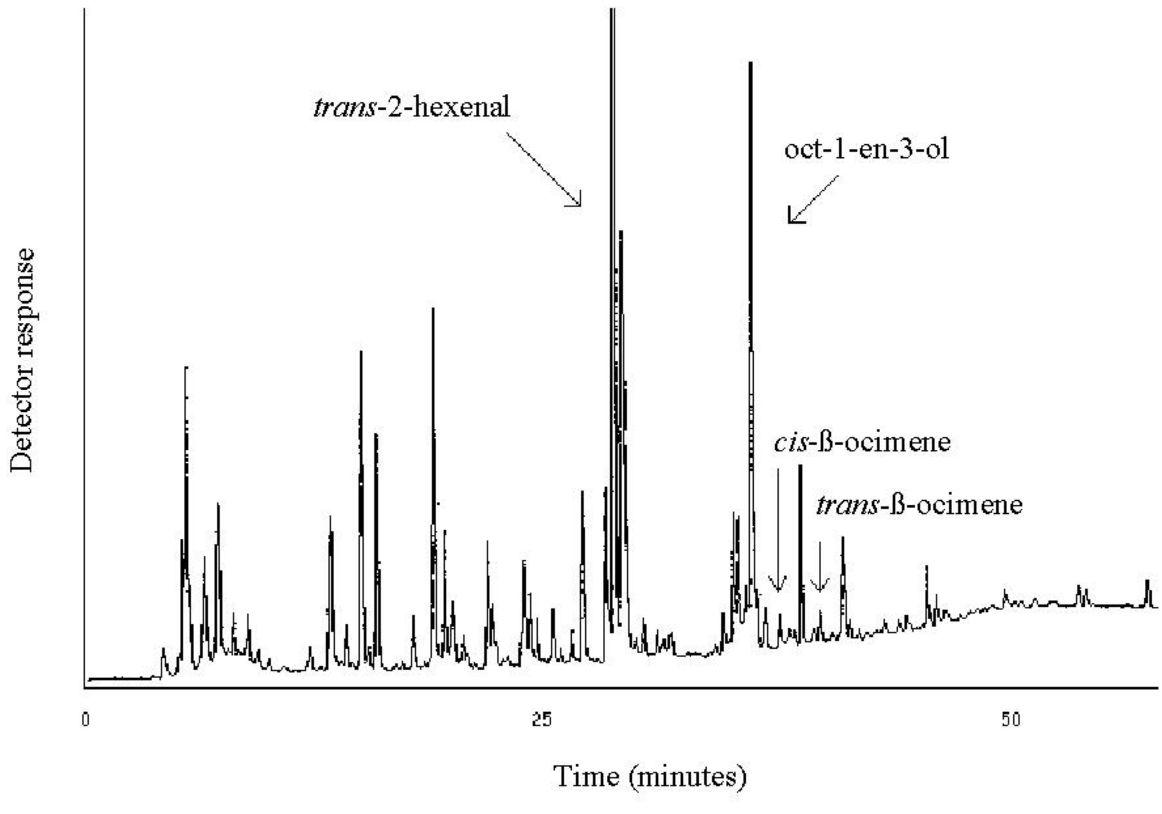

Figure 1. Gas-chromatogram of lucerne flower aroma sampled on the 41 plants used in the trial.

Table 1. Main climatic parameters recorded in each day during the trial period.

Day	Hour	Temperature (°C)	Relative humidity (%)	Solar radiation (W/m^2)	Wind speed (m/s)
July 26	14.00	29.6	45.8	821	1.5
	15.00	30.5	41.1	755	1.4
	16.00	31.1	39.1	645	1.3
	17.00	31.2	38.1	495	1.2
	18.00	30.3	40.7	327	1.3
July 27	14.00	30.6	47.3	801	1.5
	15.00	31.6	41.9	736	1.6
	16.00	31.5	43.8	556	1.5
	17.00	31.5	43.7	410	1.3
	18.00	30.7	48.0	264	1.0
July 29	14.00	26.9	55.3	653	0.8
	15.00	27.5	54.0	764	1.2
	16.00	27.2	55.5	538	1.3
	17.00	27.4	55.7	460	1.6
	18.00	26.4	61.2	317	2.0

Climatic parameters recorded in each day of testing are reported in table 1. The first day the sky was clear, temperature was high and there was weak wind from S-SE. On July 27 there was clear sky, high temperature, and weak wind from E-SE. The third day of trial was deferred to July 29 because of rainfall on July 28. In the last day, the sky was a little cloudy and the air rather sultry, with weak wind from N-NE (table 1).

Comparison of the mean seed yield of plants exposed to pollinators and that of control plants (no pollinators) indicated that there was a positive effect of insect pollination on seed set (table 2). Mechanisms, such as self-“tripping” and self-pollination may be responsible for the seed production recorded on caged plants.

Table 2. Mean seed yield of the 36 plants used outdoors in the attractiveness trial and of the 5 plants always kept in the isolation cage.

Plant group	No. seeds/ plant	Seed weight/ plant (mg)
Outdoors	90.4 **	138.7 **
In isolation cage	8.4	11.2

** t-test, $p \leq 0.01$

Significant differences among odour treatments emerged only for the number of visits of other pollinating hymenopterans ($F = 3.337$, $p \leq 0.05$), whereas the days of testing differed for the number of visits of honey bees ($F = 3.993$, $p \leq 0.05$) (table 3). The treatment × day interaction was significant only for the number of visits of other pollinating hymenopterans ($F = 2.664$, $p \leq 0.05$) (table 3). Regarding seed yield per plant (both in number and weight), no significant differences were observed (table 3).

Concerning the odour treatments, the data suggest that a certain attractive effect of ocimene towards honey bees may exist, even though no significant differences were recorded (tables 3 and 4). The number of visits by other pollinating hymenopterans was higher in the control treatment than in the applied odour treatments, and thus a certain repulsive effect on these insects may be hypothesised (table 4). The “natural” odour of lucerne flowers seems to be more attractive to wild pollinators than the scent of *trans*-2-hexenal or oct-1-en-3-ol, which represented together over 40% of the total detected volatiles. Therefore, a relationship between the relative abundance of volatile compounds in the flower aroma and the elicited responses on insects does not necessarily exist, although in this study the effect on insects may have been influenced by the adopted experimental concentration and/or the application of the volatile compounds on the whole aerial part. The quantification of volatile compounds alone could thus not be sufficient to assess for their influence on pollinator behaviour. Identifying useful tools to enhance attractiveness and pollination efficiency could therefore be difficult. The lucerne flower aroma includes over 40 volatile compounds in measurable amounts (Tava and Pecetti, 1997; Tava *et al.*, 2000) and it might be useful to verify the influence on insects of each substance by appropriate experimental techniques, such as the bioassays adopted by Loper *et al.* (1974) or the more recent ‘conditioned proboscis extension assays’ (Laloi *et al.*, 1999). If the high number of compounds to be tested represented a limit to the feasibility of this kind of tests, one could at least assess for the “mean” attractiveness of volatile classes present in the aroma, such as aldehydes, alcohols, ketones, esters, terpenes and hydrocarbons (Tava *et al.*, 2000), by appropriately mixing the main compounds belonging to each class.

Regarding both seed yield (both in number and weight) and the number of visits by other pollinating hymenopterans (non-*Apis*), an evident trend emerged for each of the four odour treatments, with the natural control being the most productive treatment (table 4).

Table 3. Analysis of variance of attractiveness towards insects and seed yield.

Source of Variation	Degrees of freedom	Mean square			
		No. visits honey bees	No. visits other pollinating hymenopterans	No. seeds/ plant	Seed weight/ plant (mg)
Treatment (T)	3	247.2 NS	264.1 *	8391.3 NS	22852.2 NS
Day (D) [a]	2	331.4 *	104.2 NS	6735.0 NS	22622.5 NS
Block	2	547.1 *	54.3 NS	3026.9 NS	7133.8 NS
T × D [a]	6	67.0 NS	107.1 *	2981.2 NS	8571.4 NS
Pooled error	22	122.9	78.2	3375.8	9035.4

[a] to test D and T × D with respect to attractiveness towards insects (first two columns), only the counts made from 14.30 h to 15.30 h were considered, and the error mean squares were 83.0 for the first column and 40.2 for the second column, respectively

NS. F-test, not significant; * F-test, $p \leq 0.05$

Table 4. Mean values (± standard error) of the different characters, recorded for the four treatments and on the three days of trial*.

	Insect attractiveness		Seed yield	
	No. visits honey bees	No. visits other pollinating hymenopterans	No. seeds/ plant	Seed weight/ plant (mg)
Treatment				
trans-2-hexenal	4.1±1.9 a	8.9±3.5 a	100.1±27.3 a	149.8±46.0 a
oct-1-en-3-ol	8.1±3.8 a	4.9±1.3 a	61.8±15.4 a	94.6±27.2 a
ocimene	16.7±7.4 a	6.0±2.1 a	70.8±12.7 a	105.2±19.7 a
control	9.2±2.9 a	16.9±5.1 b	129.1±22.8 a	205.2±36.8 a
Day				
July 26	0.9±0.5 a	3.4±1.0 a	73.4±12.7 a	106.9±20.3 a
July 27	4.4±2.0 a	7.4±3.4 a	117.5±23.9 a	188.2±42.9 a
July 29	11.2±4.3 b	9.2±1.8 a	80.4±16.2 a	121.0±20.9 a

*Different letters within the same group indicate significant statistical differences (Duncan's multiple range test $p \leq 0.05$)

Table 5. Simple correlation coefficients between seed yield and number of visits by pollinators (N = 36).

	No. visits honey bees	No. visits other pollinating hymenopterans
No. seeds/plant	-	0.47**
Seed weight/plant	-	0.50**

** $p \leq 0.01$

The number of visits of both honey bees and other pollinators increased across the three days of trial (table 4), although no significant differences were recorded. Based on the differences among days, one could hypothesise a "learning" mechanism by both honey bees and wild pollinators with respect to the new trophic source represented by the test plants. Even though the highest number of visits was recorded the third day, seed yield was 35% lower than the second day. This suggests that other external factors (e.g., relative humidity, temperature) might have interfered with the pollination process, probably causing fertilisation disorders and, thereby, lower seed set.

Simple correlation coefficients between seed yield and total numbers of insect visits recorded across the three days are reported in table 5. Seed yield (both in number and weight) seems to be significantly influenced by the number of visits by wild pollinators but not by the number of visits by honey bees. The scarce importance of visits by honey bees on seed set in lucerne confirms previous observations, reported among others by Solinas and Bin (1965a) and Tasei (1984). These bees collect pollen from lucerne only under certain circumstances (for instance, under drought). Nectar-collecting workers usually visit this crop, but they quickly learn to visit the corolla laterally, where the access to the nectaries is easier, and in this way they do not cause the flower "trip" and the subsequent pollination. Only the youngest and, therefore, unskilled workers insert their proboscis frontally into the flower, causing its "trip". According to Tasei (1984), the percentage of "tripped" flowers by honey bees in a lucerne stand in western Europe can be as low as 0.4 to 2%. Under these circumstances, the only reliable service to lucerne seed producers is provided by wild pollinating hymenopterans belonging to different genera, such as *Bombus*, *Eucera*, *Melitturga*, *Halictus*, *Melitta*, *Andrena*, *Megachile* and *Osmia* (Tasei, 1984). In Italy, Solinas and Bin (1965b) and Marletto *et al.* (1985; 1988) confirmed the limited pollinating efficacy of honey bees in lucerne and the primary activity of wild pollinators, among which most of the above-mentioned genera are reported. The present study also confirms the preponderant role of wild pollinating hymenopterans in seed set of lucerne.

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