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PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

Host-mediated volatile polymorphism in a parasitic plant influences its attractiveness to pollinators

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Abstract Host-plants can mediate the interactions between herbivores and their mutualists and also between parasitic plants and their mutualists. The present study reveals how a hemiparasitic plant parasitizing three host species gives rise to three distinct hemiparasite-host neighborhoods which differ in terms of volatile composition and pollinator attractiveness. The study was performed in a population of the mistletoe *Tristerix verticillatus* infecting three different species of hosts occurring in sympatry within a small area, thus exposing all individuals studied to similar abiotic conditions and pollinator diversity; we assessed the effect of hosts on the hemiparasites' visual and olfactory cues for pollinator attraction. During the study period, the hemiparasite individuals were flowering but the hosts were past their flowering stage. We collected volatile organic compounds from the hemiparasite and its hosts, measured floral display characteristics and monitored bird and insect visitors to inflorescences of *T. verticillatus*. We showed that: (1) floral patches did not differ in terms of floral display potentially involved in the attraction of pollinators, (2) hosts and hemiparasites on each host were discriminated as distinct chemical populations in terms of their volatile chemical profiles, (3) insect visitation rates differed between hemiparasites parasitizing different hosts, and (4) volatile compounds from the host and the hemiparasite influenced the visitation of hemiparasite flowers by insects. The study showed that a species regarded as "ornithophilic" by its floral morphology was actually mostly visited by insects that interacted with its sexual organs during their visits and carried its pollen, and that host-specific plant-volatile profiles within the *T. verticillatus* population were associated with differential attractiveness to pollinating insects.

Keywords Host-parasite chemical ecology · *Tristerix verticillatus* · Loranthaceae · Volatile organic compounds · Plant–plant interactions

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Introduction

Mutualistic interactions are ubiquitous in nature and have been shown to vary along spatial (Thompson 2005) and ecological gradients (Bronstein et al. 2006; Abbot et al. 2008). Most work in the field refers to the interaction of plants and their mutualists such as pollinators and dispersers. Few studies have addressed bottom-up effects in tritrophic interactions including mutualists as the third trophic level. For example, host-plants can mediate the interactions between herbivores and their mutualists (i.e., Abbot et al. 2008; Cushman 1991; Reithel and Billick 2006; Mooney and Agrawal 2008), and also between parasitic plants and their mutualists (i.e., Adler 2000; Medel et al. 2004; van Ommeren and Whitham 2002). As far as we are aware, in only two cases have the chemical

mechanisms underlying these host bottom-up effects been studied: a plant–homoptera–ant interaction (Abbot et al. 2008) and a plant–parasitic plant–pollinator interaction (Adler 2000).

Parasitic plants are physically linked to their hosts through the haustorium, an organ involved in attachment, penetration and solute transfer (Kuijt 1969; Mathiasen et al. 2008; Shen et al. 2006). Through the haustorium, parasitic plants extract water, nutrients and also a variety of primary and secondary compounds from their hosts. Primary chemicals, such as amino acids and soluble carbohydrates, can be acquired by the parasite in a host-specific manner (Pate 2001; Pate et al. 1991; Rey et al. 1991). To the best of our knowledge, no studies have evaluated how nutrient transfer through the haustorium may influence the ecological interactions between parasitic plants and mutualists. On the other hand, secondary metabolites, which can also be transferred from the host through the haustorium in a host-specific manner (e.g., Adler and Wink 2001; Cabezas et al. 2009; Lehtonen et al. 2005), have been shown to affect the interaction of the parasite with herbivores and mutualists. Thus, in the case of *Castilleja indivisa*, alkaloids from the host reduce herbivory, increase seed set and indirectly increase pollination of the hemiparasite (Adler 2000, 2003; Adler et al. 2001).

All plants produce a puzzling diversity of volatile organic compounds (Knudsen and Gershenzon 2006) whose biosynthesis, storage and emission (Ferry et al. 2004; Pichersky et al. 2006) may be affected by biotic (Dudareva et al. 2006) as well as abiotic (Peñuelas and Llusià 2003) stimuli. In turn, changes in volatile compounds may affect the plant's physiology and ecology, e.g., plant–plant communication, defense against herbivores and attraction of pollinators, predators and herbivore parasitoids. Given the intimate connectivity between host and parasite, biosynthetic precursors or regulators of the biosynthesis of volatiles may be transferred through the haustorium to the parasite leading to a host effect on composition, production and emission of parasite volatiles. The physical proximity of host and parasite may create a host-parasite bouquet neighborhood that differs between host species. These host-mediated differences in volatile chemistry can in turn affect the parasite's ecological interactions. The present study addresses this idea by examining the effect of three different host species occurring in sympatry within a small area (and hence exposing all individuals studied to similar abiotic conditions and to the same pollinator diversity) on the volatile chemical phenotype of a hemiparasitic plant and the ensuing attraction of pollinators to its flowers.

Our research was performed on the mistletoe *Tristerix verticillatus* (Loranthaceae), and demonstrates that: (1) the floral display of *T. verticillatus* does not differ with respect to the host, (2) *T. verticillatus* emits volatile compounds which depend on the host species it parasitizes, and (3) the nature and quantity of pollinators visiting flowers of *T. verticillatus* are associated with the volatile profiles of the "hemiparasite-host floral neighborhood". To the best of our knowledge, this is the first study that shows that sympatric host species affect the chemical volatile profile of a hemiparasite.

Materials and methods

Study area and species studied

The study was conducted from February to May 2005 in the Yerba Loca Sanctuary (33.31°S, 70.32°W, 70 km north east of Santiago on the road to Farellones) near Santiago, Chile. This study was performed in a small area within the sanctuary (Villa Paulina; 1,950–2,000 m elevation range), where the mistletoe *T. verticillatus* is found parasitizing three species of hosts—*Schinus montanus* (Sapindales: Anacardiaceae), *Fabiana imbricata* (Solanales: Solanaceae) and *Berberis montana* (Ranunculales: Berberidaceae). All individuals in the study population occurred within an area of ca. 15 ha, the distance between any of them and their closest neighbor was never greater than 20 m and they had similar exposure to sun light, comparable microhabitat conditions (i.e., soil, night and day mean temperature, relative humidity) and were exposed to the same diversity of pollinators.

Tristerix verticillatus is a mistletoe distributed along the Pacific rim of South America (Kuijt 1988) and collected most frequently from *Schinus* hosts. In our study area, *T. verticillatus* individuals produce several hundred flower buds that develop during the summer (December–March), and bloom from February to April. The tubular flowers are ca. 30 mm long, red and radially symmetrical, and are clustered in inflorescences of six to 20 flowers. The flowering periods of *T. verticillatus* and its hosts do not overlap (*S. montanus,* September–December; *F. imbricata*, November–January; and *B. montana*, September–November; personal observations). Hence, during the period of our study the mistletoes were flowering and the hosts were no longer bearing flowers.

Headspace volatile collection and analysis

Volatiles were collected using dynamic headspace sampling (Millar and Sims 1998). Twigs from the hemiparasite with leaves and inflorescences with ca. 80% of flowers open and twigs from the hosts with leaves and no flowers were severed from the plants. The exposed sections of the twigs were sealed with Teflon tape in order to avoid cross-contamination with volatiles released through the wounded areas. Samples were obtained from ten different infected

individuals of each of the three host species, and from one hemiparasite individual on each respective host individual. Thus, a total of 60 samples were collected (30 host samples and 30 hemiparasite samples). The amount of plant tissue collected per sample was (mean ± SD): 252 ± 3 g of *S. montanus*, 437 ± 7 g of *F. imbricata*, 201 ± 4 g of *B. montana*, and 305 ± 3 g, 296 ± 14 g, and 300 ± 6 g of *T. verticillatus* on each of its hosts, respectively.

The plant material of each sample was kept separately, brought to the laboratory and each sample introduced into a 10-l glass bell-shaped jar. Purified synthetic air (Indura, extra pure oxygen and nitrogen with undetectable organic impurities) entered the system through a flowmeter (set at 250 ml/min) and left it through a volatile-collecting glass column (60 mm long, outer diameter 6 mm, inner diameter 4 mm) containing Porapak Q® (200 mg, mesh 50/80; Supelco, Bellefonte, Pa.) held by glass wool plugs. Before use, Porapak Q® columns were washed with dichloromethane (5 ml, GC grade) and thermally desorbed under a purified nitrogen flow (200°C, 50 ml/min for 12 h). After 24 h of headspace collection, volatiles trapped in the Porapak Q® columns were eluted with 3 ml of dichloromethane (GC grade), and then concentrated to 100 µl under a slow flow of pure nitrogen (<1 ml/min). Qualitative analyses were performed by injecting 1 µl of the extract in a Hewlett-Packard 5891 gas chromatograph linked to a Hewlett-Packard 5972 mass spectrometric detector with an integrated data system (Hewlett Packard, Palo Alto, Calif.); quantitative analyses were performed by injecting 1 µl of the extract in a gas chromatograph fitted with a flame ionization detector (FID; GC-9A and FID-9, respectively; Shimadzu, Kyoto). The same capillary column (SPB-5, film thickness 0.25 µm, 30 m × 0.25 mm; Supelco, Deerfield, Ill.) was used in both instruments. The operating conditions were as follows: on-column injection; injector temperature, 250°C; detector temperature, 280°C; carrier gas, He at 1.25 ml/min; oven temperature program, 35°C for 5 min, increase to 260°C at 5°C/min, and then 260°C for 5 min. In the mass detector, ionization was by electron impact at 70 eV; scan time, 1.5 s; and acquisition mass range, 50–500 amu. Compounds in the chromatograms were identified by comparison of their mass spectra with those in the NIST98 library database, and by comparison of their retention index with those reported in the literature for the same type of column (Stein 2005) or those of commercial standards, when available. The concentration of volatile compounds in each sample of plant tissue was extrapolated from four-point calibration lines obtained by plotting peak areas in the chromatogram from the FID-fitted gas chromatograph versus concentration of commercial standards. When the abundances of a given compound differed by more than 2 orders of magnitude, two calibration curves were constructed, one for the low and one for the high concentration range. When commercial standards were not available, concentrations were extrapolated from lines generated by compounds of similar structure. These analyses yielded a total of 60 chemical profiles, i.e., all volatiles compounds identified in a sample with their respective concentration.

Assessment of visual cues for flower visitation

Floral visitors rely on visual and olfactory cues to find their resources (Dobson 1994; Proctor et al. 1996; Raguso 2008). In order to assess the effect of visual cues on flower visitors, mistletoes infecting different host species were compared in terms of their floral patch visual characteristics. The following variables were evaluated on each of the host individuals used for determining flower visitation rates (see below) and for collecting volatiles:

1. The number of hemiparasite individuals on a host individual.
2. The mistletoe to host volume ratio (total volume of all mistletoe individuals on the host individual divided by the volume of the host individual; the volume of the hemiparasite was approximated to a sphere and host volumes to hemispheres).
3. The number of open flowers per mistletoe individual on the host individual.
4. The number of mistletoe flowers per host individual.
5. The proportion of open flowers (total number of open flowers divided by the total number of flower buds per mistletoe individual on the host individual).

Flower visitation monitoring

The local population of *T. verticillatus* was subdivided in relation to the host it parasitized; thus, three hemiparasite-host systems were identified: *T. verticillatus* on *S. montanus*, *T. verticillatus* on *F. imbricata*, and *T. verticillatus* on *B. montana*. Visitation rates were recorded during the peak of the flowering season (March). One hemiparasite individual on each host individual was haphazardly selected for monitoring visitation rates. The visitation rate evaluation unit was defined as a group of ten inflorescences with more than 80% of open flowers on the first day of observations in each of the hemiparasite individuals selected. The ten infected host individuals selected for each hemiparasite-host system were the same as those used for volatile collection.

Flowers were visited by insects and birds; only visits in which contact was made with the sexual parts of the flower were taken into account. Flower visitation (number of visits and visitor species) to the evaluation unit was monitored during seven 10-min observation periods which were randomly sorted between days and time intervals during the

0900–1300 and 1400–1700 hours time periods. Sorting was performed in such a way that no focal hemiparasite was observed more than once at any given day or time of the day. All data were collected in days with similar weather conditions; observations were not performed on cloudy days. Total numbers of visits were grouped according to taxonomical families because some visitor species were very infrequent.

Statistical analysis

Floral visitation by insects and birds, and floral patch variables were compared separately across hosts and hemiparasites parasitizing each host through parametric ANOVA when all parametric assumptions were met; otherwise, the Kruskal–Wallis ANOVA on ranks was used (Siegel and Castellan 1988; Sokal and Rohlf 1995). The abundances of each volatile compound identified were also compared separately across hosts and hemiparasites parasitizing each host using the Kruskal–Wallis ANOVA on ranks. Subsequently, a factor analysis was performed separately on the abundances of the volatile compounds of hosts and of hemiparasites on each host, in order to reduce the number of variables (Härdle and Simar 2007; Manly 2005). Linear discriminant analysis was used to detect multivariate differences among groups, and canonical plots to show the dimensions that best separated the groups compared (Härdle and Simar 2007; Manly 2005). The extracted factors of the hemiparasite (P_i) and hosts (H_i) were pooled together for a general discriminant analysis (hosts and hemiparasites on the three hosts), and thereafter the factors extracted from *T. verticillatus* only (P_i) were used for a hemiparasite within-population discriminant analysis.

To test the effect of host and mistletoe volatiles (by means of their extracted factors) on the visitation rates of insect families, generalized linear models (GLM) were used; since the visitation rates were based on counts, the response variable of the model was set to have a Poisson distribution with a log link function (SAS Institute 2007). The Bonferroni correction was introduced to compensate for the number of comparisons performed (nine comparisons, $P = 0.0056$). The ANOVA and Kruskal–Wallis tests were performed with the SigmaStat 3.0 software (Systat Software 2004) and the multivariate statistics (factor analysis, linear discriminant analysis) and GLM were performed with the JMP 7 software (SAS Institute 2007).

Results

Volatile compounds of *T. verticillatus* and its hosts

A total of 53 volatile compounds were identified in the host and hemiparasite samples analyzed. *F. imbricata* was the most chemically diverse host (41 compounds), followed by *S. montanus* (23 compounds) and *B. montana* (nine compounds). The differences in the volatile profiles were not only qualitative, but also quantitative (Table 1). *T. verticillatus* produced fewer volatile compounds when compared to its hosts (except when compared to *B. montana);* only three compounds occurred in a mutually exclusive mode (sabinene was found only among volatiles collected from mistletoe individuals infecting *S. montanus*, γ-terpinene from individuals infecting *F. imbricata*, and camphene from individuals infecting *B. montana*), and (*Z*)-3-hexenyl acetate was collected only from mistletoes infecting *S. montanus* and *B. montana*. When comparing abundances of volatile compounds between host species, 46 out of the 50 compounds (92%) showed statistically significant differences [exceptions were 3-carene, hexyl acetate, borneol and (*Z*)-3-hexenyl isovalerate]; likewise, when comparing hemiparasites on different hosts, the abundances of 12 out of the 17 volatile compounds (70.6%) differed (exceptions were α–pinene, β-pinene, α–ocimene, terpinolene and α-copaene; Table 1).

Multivariate analyses of the chemical profiles of *T. verticillatus* and its hosts

The factor analysis performed on the abundances of the volatile compounds identified from hemiparasite individuals parasitizing the three host species yielded three factors (eigenvalues: $P_1 = 6.51$; $P_2 = 3.60$; and $P_3 = 1.92$) which accounted for 70.82% of the variance in the data. Each factor was highly correlated with a mixture of compounds from different structural groups. For example, loadings higher than 0.70 for the first factor (P_1) revealed that the compounds that contributed most to this factor were three alkanes (nonane, decane and undecane) and three monoterpenes (*p*-cymene, limonene and γ-terpinene). Likewise, the second factor (P_2) was highly correlated with (*Z*)-3-hexenyl acetate and three monoterpenes (camphene, α–ocimene and terpinolene); and the third factor (P_3) was highly correlated with the monoterpene α-pinene and the sesquiterpene α-copaene (a complete report of the factor loadings for all compounds is shown in Table 2).

The factor analysis on the abundances of host volatiles yielded three factors (eigenvalues: $H_1 = 21.41$, $H_2 = 6.85$ and $H_3 = 4.48$) that accounted for 65.50% of the data variation. The first factor (H_1) was highly correlated with hydrocarbons (1-dodecene, tridecane, tetradecane, 1-tetradecane and pentadecane), monoterpenes [α-thujene, linalool, terpinolene, (*Z*)-β-terpineol, thujone, (*E*)-*p*-menth-2-en-1-ol, menthone, 4-terpineol and α-terpineol] and sesquiterpenes (α-cedrene and β-cedrene); the second factor (H_2) was correlated with monoterpenes (bornylene, tricyclene, camphene and borneol) and benzaldehyde; and the

Table 1 Volatile composition[a] of *Tristerix verticillatus* and its hosts

Compound name	*Schinus montanus*	*Fabiana imbricata*	*Berberis montana*	*T. verticillatus* infecting three hosts		
				S. montanus	*F. imbricata*	*B. montana*
Methyl 2-methylbutyrate	2.15 (0.00–10.70) a	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b			
Methyl isovalerate				0.49 (0.37–0.87) b	1.61 (0.93–2.00) a	0.98 (0.80–2.12) a
(*Z*)-3-Hexen-1-ol	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b	0.08 (0.02–0.39) a			
2-Heptanone	0.00 (0.00–0.00) b	17.05 (9.65–23.68) a	0.00 (0.00–0.00) b			
Bornylene	0.00 (0.00–0.00) b	6.35 (0.00–21.39) a	0.00 (0.00–0.00) b			
Nonane	0.00 (0.00–0.00) b	8.14 (0.00–79.75) a	0.00 (0.00–0.00) b	0.46 (0.43–0.49) b	1.52 (0.97–2.00) a	1.14 (0.69–1.44) a
Tricyclene	0.00 (0.00–0.00) b	2366.02 (1360.32–4629.52) a	0.00 (0.00–0.00) b			
α-Thujene	238.76 (22.30–558.98) a	683.44 (263.21–1170.06) a	0.00 (0.00–0.00) b			
α-Pinene	103.28 (43.53–295.16) a	548.64 (408.17–606.43) a	0.00 (0.00–0.00) b	0.64 (0.41–1.10) a	1.10 (0.49–1.69) a	0.66 (0.49–1.44) a
Camphene	0.00 (0.00–0.00) b	964.23 (444.98–2085.49) a	0.01 (0.00–0.04) b	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b	0.08 (0.01–0.09) a
Benzaldehyde	0.00 (0.00–0.00) b	41.66 (30.34–97.13) a	0.00 (0.00–0.00) b			
Sabinene	57.97 (20.44–1295.50) a	428.58 (320.19–567.01) a	0.00 (0.00–0.00) b	0.03 (0.01–0.04) a	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b
β-Pinene	5.18 (2.60–8.81) a	2.21 (0.00–14.51) a	0.00 (0.00–0.00) b	0.04 (0.02–0.05) a	0.06 (0.03–0.15) a	0.07 (0.03–0.18) a
β-Myrcene	139.20 (30.83–363.79) a	58.52 (33.97–81.21) a	0.00 (0.00–0.00) b	0.07 (0.06–0.12) b	0.23 (0.16–0.34) a	0.14 (0.08–0.23) ab
Decane				0.60 (0.52–0.71) b	1.79 (1.32–3.33) a	1.47 (1.02–1.96) a
α-Phellandrene	52.23 (4.10–814.64) a	3.26 (0.00–11.48) ab	0.00 (0.00–0.00) b			
(*Z*)-3-Hexenyl acetate	0.00 (0.00–0.00) b	4.92 (0.00–30.99) a	1.19 (0.49–11.27) a	0.06 (0.05–0.07) a	0.00 (0.00–0.00) b	0.16 (0.04–0.25) a
3-Carene	0.59 (0.00–1.42) a	0.00 (0.00–0.00) a	0.00 (0.00–0.00) a			
Hexyl acetate	0.00 (0.00–0.00) a	0.00 (0.00–0.00) a	0.00 (0.00–0.04) a			
α-Terpinene	2.70 (1.24–10.58) b	61.23 (47.46–73.13) a	0.00 (0.00–0.00) b			
p-Cymene	29.84 (4.55–110.66) b	3342.80 (3209.38–4729.77) a	0.01 (0.01–0.08) b	0.02 (0.02–0.03) b	0.18 (0.10–0.31) a	0.04 (0.03–0.07) b
Limonene	551.64 (46.30–905.07) a	68.40 (5.21–99.19) ab	0.02 (0.00–0.07) b	0.36 (0.30–0.70) b	1.79 (1.00–2.96) a	0.40 (0.25–0.62) b
(*E*)-β-Ocimene	10.96 (6.78–40.39) a	0.00 (0.00–0.00) b	0.01 (0.00–0.02) b			
α-Ocimene	19.30 (0.00–143.21) ab	168.18 (58.86–182.08) a	0.38 (0.10–0.80) b	0.05 (0.03–0.06) a	0.29 (0.20–0.46) a	0.07 (0.00–0.34) a
γ-Terpinene	4.12 (2.72–46.98) a	455.66 (345.39–616.93) a	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b	0.11 (0.04–0.20) a	0.00 (0.00–0.00) b
Acetophenone	0.00 (0.00–0.00) b	5.77 (3.97–12.54) a	0.00 (0.00–0.00) b			
Linalool	0.00 (0.00–0.00) b	7.52 (4.01–14.13) a	0.00 (0.00–0.00) b			
Terpinolene	1.17 (0.75–5.68) b	103.58 (57.08–267.89) a	0.00 (0.00–0.00) b	0.01 (0.01–0.02) a	0.12 (0.08–0.15) a	0.01 (0.01–0.11) a
Methyl benzoate	1.01 (0.00–3.30) ab	11.24 (3.96–19.52) a	0.00 (0.00–0.00) b			
Undecane				0.25 (0.20–0.28) b	0.86 (0.63–1.51) a	0.44 (0.18–0.62) ab
(*Z*)-β-terpineol	0.00 (0.00–0.00) b	69.56 (45.55–125.22) a	0.00 (0.00–0.00) b			
Thujone	0.00 (0.00–0.00) b	6.71 (4.49–11.18) a	0.00 (0.00–0.00) b			
(*E*)-*p*-Menth-2-en-1-ol	0.00 (0.00–0.00) b	4.82 (4.09–10.68) a	0.00 (0.00–0.00) b			

Table 1 continued

Compound name	*Schinus montanus*	*Fabiana imbricata*	*Berberis montana*	*T. verticillatus* infecting three hosts		
				S. montanus	*F. imbricata*	*B. montana*
Allo-ocimene	2.95 (1.03–14.15) a	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b			
Camphor	0.00 (0.00–0.00) b	5.12 (1.23–9.58) a	0.00 (0.00–0.00) b			
Menthone	0.00 (0.00–0.00) b	5.61 (0.84–9.14) a	0.00 (0.00–0.00) b			
Isomenthone	0.00 (0.00–0.00) b	3.03 (0.00–5.96) a	0.00 (0.00–0.00) b			
Borneol	0.00 (0.00–0.00) a	0.44 (0.00–2.70) a	0.00 (0.00–0.00) a			
4-Terpineol*	0.02 (0.00–0.42) b	104.09 (56.45–216.35) a	0.00 (0.00–0.00) b			
1-Dodecene	0.00 (0.00–0.00) b	176.79 (113.41–517.35) a	0.00 (0.00–0.00) b			
α-Terpineol	0.00 (0.00–0.00) b	1.67 (1.14–3.77) a	0.00 (0.00–0.00) b			
Methyl salicylate	0.00 (0.00–0.00) b	18.02 (9.87–32.57) a	0.00 (0.00–0.00) b			
(*Z*)-3-Hexenyl isovalerate	0.74 (0.00–4.28) a	7.86 (0.00–13.85) a	0.04 (0.03–0.09) a			
Tridecane	0.00 (0.00–0.00) b	17.09 (9.87–33.97) a	0.00 (0.00–0.00) b			
α-Copaene	0.35 (0.13–0.50) a	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b	0.01 (0.00–0.01) a	0.01 (0.00–0.01) a	0.00 (0.00–0.00) a
1-Tetradecene	0.00 (0.00–0.00) b	24.01 (15.53–101.49) a	0.00 (0.00–0.00) b			
Tetradecane	0.00 (0.00–0.00) b	1.51 (1.12–6.74) a	0.00 (0.00–0.00) b	0.00 (0.00–0.02) b	0.04 (0.00–0.10) ab	0.08 (0.02–3.08) a
α-Cedrene	0.00 (0.00–0.00) b	0.20 (0.00–4.15) a	0.00 (0.00–0.00) b			
β-Caryophyllene	1.56 (1.24–6.63) a	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b			
β-Cedrene	0.00 (0.00–0.00) b	3.37 (0.00–10.64) a	0.00 (0.00–0.00) b			
Germacrene D	22.58 (13.63–44.30) a	0.43 (0.07–0.89) ab	0.00 (0.00–0.00) b			
Pentadecane	0.00 (0.00–0.00) b	0.42 (0.00–1.03) a	0.00 (0.00–0.00) b			
(*E,E*)-α-Farnesene	12.38 (5.13–22.62) a	0.00 (0.00–0.00) b	0.00 (0.00–0.00) b			

[a] Volatile compounds are ordered according to their retention indices. Their abundances were compared with Kruskal–Wallis ANOVA on ranks and within each row, *different letters* denote significant differences at $P < 0.05$ using the Tukey test (comparisons were separately performed between the three host species, and between the mistletoe on the three different hosts). Median value and interquartile ranges of volatile abundances (nanograms of compound per gram of fresh plant tissue) are shown for all compounds

Table 2 Factor loadings of individual volatile compounds on factors extracted from data on the concentration of compounds produced by the host plants (H_i) and the parasites on three different host (P_i). Correlation coefficients >0.70 are in *italics*

Volatile compound	Hosts			Hemiparasite		
	H_1	H_2	H_3	P_1	P_2	P_3
Methyl 2-methylbutyrate	−0.10	−0.07	0.57			
Methyl isovalerate				0.55	0.58	−0.18
(*Z*)-3-Hexen-1-ol	−0.18	−0.27	−0.39			
2-Heptanone	0.60	0.35	−0.15			
Bornylene	0.25	*0.82*	−0.12			
Nonane	0.65	−0.15	−0.07	*0.87*	0.29	−0.02
Tricyclene	0.23	*0.87*	−0.17			
α-Thujene	*0.75*	0.22	0.27			
α-Pinene	0.32	0.63	0.18	0.37	0.01	*0.76*
Camphene	0.20	*0.89*	−0.16	−0.04	*0.91*	−0.12
Benzaldehyde	0.36	*0.78*	−0.12			
Sabinene	0.25	0.21	0.49	−0.45	−0.15	0.59
β-Pinene	−0.06	0.44	0.35	0.08	−0.03	0.44
β-Myrcene	−0.03	−0.08	0.36	0.61	0.60	0.33
Decane				*0.86*	0.32	0.02
α-Phellandrene	−0.10	−0.15	0.63			
(*Z*)-3-Hexenyl acetate	−0.14	0.40	−0.26	−0.15	*0.95*	−0.11
3-Carene	−0.11	−0.16	0.61			
Hexyl acetate	−0.17	−0.26	−0.38			
α-Terpinene	0.54	0.66	0.01			
p-Cymene	0.64	0.61	−0.14	*0.86*	−0.10	−0.05
Limonene	−0.12	−0.05	*0.83*	*0.82*	−0.05	0.36
(*E*)-β-Ocimene	−0.10	−0.11	*0.79*			
α-Ocimene	0.07	0.16	0.68	0.05	*0.92*	−0.16
γ-Terpinene	*0.72*	0.55	0.07	*0.87*	−0.17	0.01
Acetophenone	0.60	0.60	−0.11			
Linalool	*0.96*	0.16	−0.09			
Terpinolene	*0.88*	0.37	−0.04	0.48	*0.79*	0.16
Methyl benzoate	0.12	0.31	0.38			
Undecane				*0.91*	0.02	0.12
(*Z*)-β-Terpineol	*0.81*	0.49	−0.10			
Thujone	*0.78*	0.52	−0.11			
(*E*)-*p*-Menth-2-en-1-ol	*0.81*	0.47	−0.10			
Allo-ocimene	−0.11	−0.10	*0.85*			
Camphor	0.49	0.55	−0.11			
Menthone	*0.71*	0.35	−0.08			
Isomenthone	0.62	0.58	−0.11			
Borneol	−0.08	*0.82*	−0.11			
4-Terpineol	*0.84*	0.44	−0.09			
1-Dodecene	*0.86*	0.36	−0.09			
α-Terpineol	*0.72*	0.33	−0.08			
Methyl salicylate	0.24	0.67	−0.10			
(*Z*)-3-Hexenyl isovalerate	0.44	0.44	0.04			
Tridecane	*0.93*	0.26	−0.09			
α-Copaene	−0.15	−0.14	*0.87*	−0.15	−0.01	*0.77*
1-Tetradecene	*0.93*	0.22	−0.08			
Tetradecane	*0.96*	0.15	−0.08	−0.06	0.12	−0.15
α-Cedrene	*0.92*	−0.13	−0.05			
β-Caryophyllene	−0.11	−0.10	0.62			
β-Cedrene	*0.92*	−0.07	−0.06			
Germacrene D	−0.10	−0.18	*0.71*			
Pentadecane	*0.77*	−0.23	−0.04			
(*E,E*)-α-Farnesene	−0.09	−0.08	*0.73*			

third factor (H_3) to monoterpenes [limonene, (*E*)-β-ocimene and allo-ocimene] and sesquiterpenes [α-copaene, germacrene D and (*E,E*)-α-farnesene; Table 2].

In the general discriminant analysis, the canonical variates significantly separated hosts, but not hemiparasites on different host plants (Hotelling-Lawley test, approximately $F = 23.77$, $df = 15$, $P < 0.0001$, 20% misclassified; Fig. 1a). The within-hemiparasite population discriminant analysis showed that the extracted factors significantly separated hemiparasite individuals parasitizing the three different hosts as three distinct chemical populations (Hotelling-Lawley test, approximately $F = 8.08$, $df = 6$, $P < 0.0001$, 13% misclassified; Fig. 1b).

Characteristics of floral patches of *T. verticillatus* on different hosts

No differences were found in terms of floral-patch visual variables between the three hemiparasite-host systems. Thus, no statistical differences were found between hosts in the number of hemiparasite individuals on each host individual, in the mistletoe to host volume ratio, in the number of open flowers per mistletoe individual on a host individual, in the number of mistletoe flowers per host individual, nor in the proportion of open flowers in each hemiparasite individual parasitizing each of the three host species (Table 3). These results indicate that the evaluation units did not differ in terms of visual attractiveness variables shown to be important for flower visitors at the flower patch level (Proctor et al. 1996).

Insect and bird visits in relation to host and the hemiparasite volatile blends

A total of 16 species of insects was found, which belong to eight families: Apidae, *Bombus dahlbomi*, *B. terrestris*, *Apis mellifera* and *Centris nigerrima*; Megachilidae, *Megachile* sp.; Vespidae, *Hypodinerus* sp. and *Vespula*

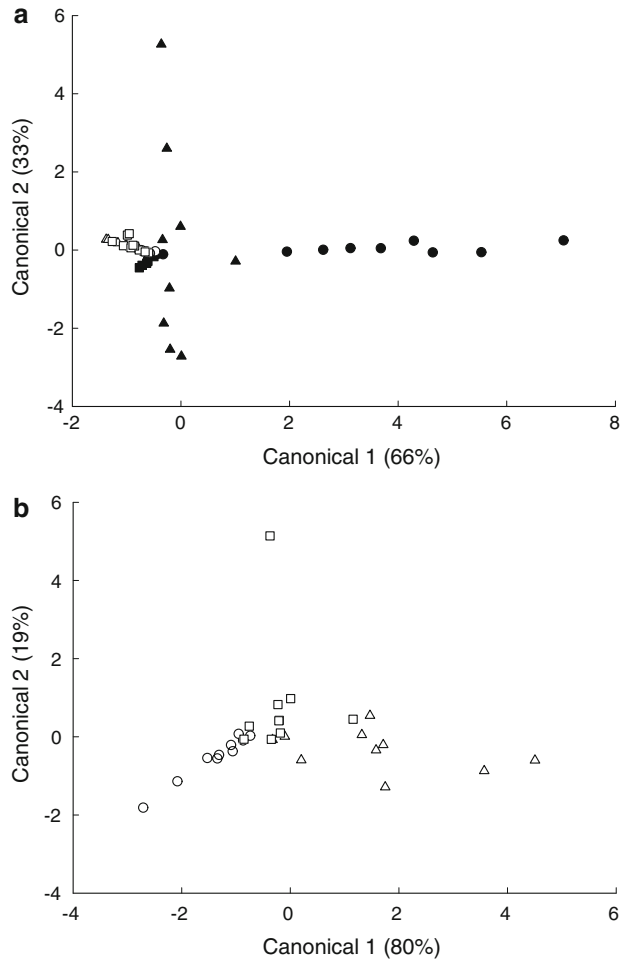

Fig. 1 **a** Differences in chemical profiles of three host plants and the parasite *Tristerix verticillatus*. **b** Differences in chemical profiles among individuals of the parasite *T. verticillatus* parasitizing different hosts. The canonical plots show that above the species level (**a**), the differences in the chemical profiles among hosts are much larger than the differences between hemiparasite individuals parasitizing different host species [mean Mahalanobis distances (MMD); $MMD_{Hosts} = 30.49$, mean $P_{Hosts} < 0.0001$; $MMD_{Hemiparasite} = 0.13$, mean $P_{Hemiparasite} = 0.83$]. Nonetheless, differences in volatile blends within the hemiparasite population (**b**) are explained by the host species parasitized by *T. verticillatus* ($MMD_{Hemiparasite} = 6.06$, mean $P_{Hemiparasite} = 0.0029$). Amount of variance explained by each canonical variate is shown *in parentheses* on the axis labels (*filled circle Schinus montanus*, *filled triangle Fabiana imbricata*, *filled square Berberis montana*, *open circle T. verticillatus* on *S. montanus*, *open triangle T. verticillatus* on *F. imbricata*, and *open square T. verticillatus* on *B. montana*)

germanica; Tachinidae and Syrphidae, one unidentified species each; Acroceridae, *Lasia rufa*; Pieridae, *Tatochila* sp., *Colias vauthieri*; Nymphalidae, *Vanessa carye, Iramea cytheris*, *Cosmosatyrus* sp. and *Danaus plexipus*. Only one species of hummingbird (Trochilidae: *Sephanoides sephanoides*) was recorded visiting the flowers of *T. verticillatus*. The total number of visits differed between hemiparasites infecting different hosts; individuals of *T. verticillatus* infecting *S. montanus* received more visits than those infecting *F. imbricata* and *B. montana* ($F = 125.18$, $df = 2$, $P < 0.0001$; Table 4). Within-family comparison of visits yielded significant differences for Acroceridae and Tachinidae families of Diptera ($F = 4.36$, $df = 2$, $P < 0.02$; and $F = 37.25$, $df = 2$, $P < 0.0001$, respectively), the Apidae, Megachilidae and Vespidae families of Hymenoptera ($F = 33.86$, $df = 2$, $P < 0.0001$; $F = 30.86$, $df = 2$, $P < 0.0001$; and $F = 30.52$, $df = 2$, $P < 0.0001$, respectively), and the Pieridae family of Lepidoptera ($F = 17.12$, $df = 2$, $P < 0.0001$; Table 4). However, flower visits by hummingbirds (*S. sephanoides*) did not differ between hemiparasite individuals on the three host species, in accordance with the lack of differences in the visual appearance of floral patches between hosts and the fact that hummingbird pollination has been shown to be largely driven by visual cues (Fenster et al. 2004). Overall, hemiparasites infecting *S. montanus* received the majority of visits by hymenopterans (Apidae, Megachilidae and Vespidae) and pierids, whereas tachinid flies showed a preference for flowers of hemiparasites infecting *F. imbricata*. It should be noted also that Apidae and Megachilidae showed 20 and 10 times as many visits as hummingbirds, respectively (Table 4).

The GLM procedure was performed on insect visitors, with the factors extracted from the volatile compounds of the hemiparasite (P_1, P_2 and P_3) and its hosts (H_1, H_2 and H_3) included as predictors in the model. The results showed a significant fit of the model for five of the eight insect families tested (Table 4). Furthermore, host volatiles (H_3 for Vespidae and Acroceridae), hemiparasite volatiles (P_2 for Pieridae), and both host and hemiparasite volatiles (different combinations of all factors for Tachinidae, Apidae, and Megachilidae) had a significant influence on flower visits by these insect families. Also, the overall number of visits by insects was influenced by both host and hemiparasite volatiles (H_3 and P_2). However, for Syrphidae and Nymphalidae, none of the model parameters were significant (Table 4).

Discussion

When the volatile profiles of hosts and mistletoes were combined for the discriminant analysis, two distinct levels of discrimination were found. There were clear differences in the volatile profiles of the three host species which noticeably segregated them as three distinct chemical groups, while all mistletoes remained clustered together (Fig. 1a). However, when only the mistletoe volatile profiles were analyzed (intra-population level), there was a clear discrimination between mistletoes infecting different hosts (Fig. 1b). This mistletoe within-population divergence in

Table 3 Floral display variables assessed for each host individual (mean ± SD)

Floral-patch variable measured	*T. verticillatus* infecting three hosts			*F*	*P*
	S. montanus	*F. imbricata*	*B. montana*		
Number of mistletoe individuals	3.3 ± 0.6	2.2 ± 0.4	2.4 ± 0.5	1.79	0.19
Mistletoe/host volume ratio	0.043 ± 0.009	0.015 ± 0.003	0.033 ± 0.013	2.32	0.12
Number of open flowers per mistletoe individual	16 ± 2.7	16 ± 5.3	14 ± 5.7	1.24	0.31
Number of mistletoe flowers	35 ± 5.5	38 ± 10	26 ± 9.0	1.73	0.20
Proportion of open to total flowers	0.46 ± 0.05	0.43 ± 0.06	0.53 ± 0.11	0.42	0.66

Comparisons were made using ANOVA

volatiles emitted was qualitative (mutually exclusive occurrence of three compounds), but most of all quantitative since more than 60% of the shared volatile compounds between mistletoe individuals on the three host species differed in their relative abundances between the three host species (Table 1).

The mechanism through which the chemical divergence of *T. verticillatus* on the three different hosts occurs is not yet resolved. The biosynthesis of volatile compounds is believed to occur in the epidermal cells of plant tissues (or secretory structures of glandular trichomes) from which they are released into the atmosphere (Dudareva et al. 2004), and volatile compounds cannot be transferred in the same way as nutrients or non-volatile secondary metabolites, i.e., through the haustorial connections established between the interacting species (Pate 2001). Still, it is possible that, through the haustorial connection, precursor metabolites involved as intermediates in volatile biosynthetic pathways in the hemiparasite or regulators involved in the activation of such biosynthetic pathways are differentially transferred from the hosts. However, we cannot discard air-borne plant-to-plant signaling between the hosts and its hemiparasite, leading to changes in the biosynthetic capabilities of the hemiparasite (Dicke et al. 2003). Chemical signals in intraplant chemical communication (as one may consider the combination of host and aerial parasite) may arrive at the receiving tissue through the vascular system (the haustorium in plant–aerial parasite interactions) or through the air (Heil and Silva Bueno 2007). The demonstrated volatile-mediated host finding and host selection behaviors by the parasitic plant, *Cuscuta pentagona* (Runyon et al. 2006) supports the plausibility of air-borne plant–plant volatile signaling in plant parasite–host interactions. Interestingly, volatiles' chemical signals may also arrive through the soil (Baldwin et al. 2006; Dicke et al. 2003; Farmer 2001; Gershenzon 2007): for instance, plants have been shown to interact with root-infesting organisms through root-emitted volatiles (Hiltpold and Turlings 2008). Hence, effects similar to those described in the present work may be expected to occur also in the interaction of plants with root parasitic plants.

Our results reveal that for most insect visitors, a combination of the volatile composition of both *T. verticillatus* and its hosts influences the nature and quantity of flower visitors. The sign of each factor within the regression models may be interpreted as an attractant (+ sign) or repellent (− sign) effect towards the visitor family involved. It should be noted that although each factor is built upon all volatile compounds, it is highly correlated only with a specific group of compounds (factor loadings shown on Table 2). Thus, for example, a significant positive effect of H_3 on Acroceridae visits may be interpreted as an attraction by one or more of the compounds most correlated to H_3, i.e., limonene, (*E*)-β-ocimene, allo-ocimene, α-copaene, germacrene D and (*E*,*E*)-α-farnesene. In order to test this interpretation of factors and factor loadings, we examined the literature for reports on the attractiveness or repellence of compounds correlated with all hemiparasite/host factors that turned out to be significant predictors of insect visits. The results of this examination (Table 5) show that the literature supports the significant effect of an ample diversity of compounds in all visiting insect families, except for the negative effect of P_2 on flower visits by the pierid *Mathania leucothea.*

It has been assumed that *T. verticillatus* (and in fact, most Loranthaceae) is bird pollinated on the basis of its floral morphology (Fenster et al. 2004; Kuijt 1988; Proctor et al. 1996). Nonetheless, our results reveal that insects are responsible for 97.9% of total flower visits compared to only 2.1% by birds. The most frequent visitors (*Megachile* sp. and *B. dahlbomi* comprise 45.9% of total flower visits) actively interacted with the sexual parts of the flowers by touching the stigma and anthers during their visits, and were found carrying *T. verticillatus* pollen on their bodies (N. J. Cabezas, unpublished data); this is sufficient evidence to consider them as pollinators although their effectiveness has yet to be evaluated. Insect pollination of ornithophilous flowers of Loranthaceae has been reported in *Peraxilla tetrapetala* (Burgess et al. 2006), *Ileostylus micranthus* and *Tupeia antarctica* (Ladley et al. 1997). This is the first report of insect pollination of a *Tristerix* species, and current additional studies have shown so far

Table 4 Flower visitation by birds and insects to *T. verticillatus*, and parameter estimates for generalized linear models (GLM) predicting visitation rates by factors extracted from data on the concentration of compounds produced by host plants (H_i) and the parasites on three different hosts (P_i)

Order	Family	Total number of visits per day to *T. verticillatus* infecting three hosts			GLM parameter estimates							Whole model goodness-of-fit test	
		S. montanus	*F. imbricata*	*B. montana*	Intercept	H_1	H_2	H_3	P_1	P_2	P_3	L–R χ^2	*P*
Trochiliformes	Trochilidae[a]	1.10 ± 0.87	0.60 ± 0.70	1.30 ± 1.25									
Diptera	Acroceridae	1.50 ± 1.90 a	0.10 ± 0.32 b	0.30 ± 0.48 ab	−1.50	0.10	−1.14	**0.68**	−0.72	−0.08	0.16	19.98	*<0.005*
	Syrphidae	9.60 ± 4.58	10.60 ± 6.35	5.60 ± 2.27	2.10	0.05	0.04	0.19	0.16	−0.18	−0.01	8.94	0.18
	Tachinidae	3.90 ± 2.02 b	14.40 ± 5.46 a	2.00 ± 1.41 b	1.62	0.12	**0.42**	0.03	**0.36**	−0.22	−0.10	82.18	*<0.0001*
Hymenoptera	Apidae	24.70 ± 6.94 a	15.40 ± 5.83 b	4.70 ± 2.54 c	2.59	**0.11**	0.02	**0.36**	0.06	**−0.23**	0.02	94.53	*<0.0001*
	Megachilidae	13.30 ± 5.31 a	5.10 ± 2.18 b	1.70 ± 1.25 c	1.59	−0.13	−0.17	**0.17**	−0.05	**−1.07**	**0.26**	72.68	*<0.0001*
	Vespidae	7.50 ± 3.17 a	1.80 ± 1.14 b	1.10 ± 0.88 b	0.98	−0.15	−0.11	**0.33**	−0.23	−0.60	0.01	21.80	*<0.005*
Lepidoptera	Nymphalidae	1.40 ± 0.84	1.80 ± 1.40	2.10 ± 1.73	0.55	−0.10	0.03	−0.01	0.15	−0.08	0.04	1.58	0.95
	Pieridae	6.30 ± 2.83 a	2.40 ± 1.78 b	1.20 ± 1.14 b	0.98	−0.14	−0.08	0.23	−0.04	**−0.79**	0.09	12.85	0.046
Total visits by insects		115.40 ± 15.97 a	59.40 ± 15.92 b	23.10 ± 3.11 c	4.06	−0.04	−0.01	**0.29**	0.02	**−0.46**	0.06	28.62	*<0.0001*

The means (±SD) of the number of visits per day are shown for all visitor groups. Within each row, *different letters* denote significant differences at $P < 0.05$ (comparisons were made using ANOVA followed by post hoc Holm-Sidak tests). Within the GLM results, significant parameter estimates are highlighted in *bold*. In the last column, *italics* indicate significant model fits after the Bonferroni correction

[a] GLM analysis was not performed on *S. sephanoides* visits since hummingbird pollination has been shown to be largely driven by visual cues (Fenster et al. 2004)

that flowers protected against hummingbird visits but exposed to insects are successfully pollinated (A. J. Troncoso, in preparation). We believe potential insect visitors may have been dismissed in previous ecological studies because floral morphology alone pointed to an ornithophilic syndrome; however, based on our findings, we propose that *T. verticillatus* has a mixed pollination syndrome (Knudsen et al. 2004).

Pollinator visits are influenced by a large number of factors, such as microhabitat conditions (i.e., temperature and humidity), lure cues (visual and volatile) and reward (nectar, pollen, mating; Proctor et al. 1996), whose relative importance is pollinator specific. In our study, all hemiparasite individuals studied coexisted within a small area under similar sun exposure; moreover, all focal hemiparasites were located on the surface of the host plants, and host plants (and hence focal hemiparasites) studied did not differ significantly in terms of height above the ground, suggesting similar temperature and humidity conditions. The small area where the study was performed guarantees the exposure of focal hemiparasites to the same pollinator diversity. However, detailed studies of nectar reward in this species are necessary to test the combined importance of volatile cues and nectar reward in this system.

The influence of host plants on hemiparasite volatiles and the subsequent influence on pollinator arrival constitute a good example of chemically mediated bottom-up effects in host plant-parasitic plant–pollinator interactions. Such effects were first reported for a root hemiparasite (Adler 2000, 2003; Adler et al. 2001); the present study is the first report for an aerial hemiparasite. Moreover, taking into account the parallels existing between host plant–parasitic plant and host plant–herbivore interactions (Atsatt 1977; Pennings and Callaway 2002), we can relate our findings to the well-documented bottom-up effects of plants on herbivores and on herbivore mutualists (Abbot et al. 2008). Cushman (1991) proposed that host plants could play a role in determining the strength of herbivore–insect mutualisms in a wide range of ways. In the case of the interaction between host plants, herbivores and mutualist ants, the mutualistic outcomes differed depending on the host species (Reithel and Billick 2006), the host-dependent nutritional state of the herbivore (Abbot et al. 2008), host fertilization presumably affecting its nutritional quality (Billick et al. 2005; but see Morales and Beal 2006), and even host genotype (Mooney and Agrawal 2008). These examples of bottom-up effects of hosts on higher trophic levels are similar to the one described in the present study.

Mistletoes are regarded as an intriguing group of plants that, through their network of interactions, can serve as sensitive indicators of overall community integrity and ecosystem health (Mathiasen et al. 2008). Even though

Table 5 Reports in the literature of the effects on insects of compounds with high loadings on factors that had significant effects on visitation rates to *T. verticillatus* (see Table 4)

Insect family	HHF[a]	Compound	Source	Relative effect
Acroceridae	$+H_3$	Limonene	Viljoen et al. (2006)	Compounds present in flowers pollinated by acrocerid flies
		(*E*)-β-Ocimene		
		Allo-ocimene		
		α-Copaene		
		Germacrene D		
Tachinidae	$+H_2$	Benzaldehyde	James (2005)	Attractants for tachinid flies
		Borneol	Roland et al. (1995)	
Apidae	$+H_1$	1-Dodecene	Blight et al. (1997)	Compounds reported as bee attractants
		Tridecane	Jakobsen et al. (1994)	
		Tetradecane		
		α-Thujene	Gerlach and Schill (1991)	
		Linalool	Blight et al. (1997), Borg-Karlson et al. (2003), Henning and Teuber (1992)	
		Terpinolene	Williams and Whitten (1983)	
		Thujone		
		Menthone		
	$+H_3$	Limonene	Fonta and Masson (1984)	Elicitation of electroantennographic responses on *Bombus terrestris*
		(*E,E*)-α-Farnesene	Blight et al. (1997)	Induction of proboscis extension in *Apis mellifera*
	$-P_2$	(*Z*)-3-Hexenyl acetate	Henning and Teuber (1992)	Increased production reduced attraction of alfalfa flowers for *A. mellifera*
Megachilidae	$+H_3$	Limonene	Eltz et al. (1999), Gerlach and Schill (1991), Whitten et al. (1986)	Compounds present in orchids that are pollinated by euglossine bees
		(*E*)-β-Ocimene		
		Germacrene D		
	$+P_3$	α-Pinene	Eltz et al. (1999)	
		α-Copaene		
Vespidae	$+H_3$	Limonene	Dani et al. (1998)	Compounds are pheromone constituents of hover wasps
		(*E*)-β-Ocimene		

The sign of each factor within the regression models is interpreted as having an attractant (+ sign) or repellent (− sign) effect towards the visitor family involved

[a] Host (*H*) and hemiparasite (*P*) factors that had a significant effect in the GLM analysis of insect visits (Table 4)

their ecological roles indicate that they qualify as keystone species for many forest ecosystems (Press and Phoenix 2005; Watson 2001), many of the ecological interactions among mistletoes, their host plants, and the many organisms that depend on them for food or habitat have not yet been investigated in depth (Mathiasen et al. 2008). Here we have shown that *T. verticillatus* exhibits a volatile chemical polymorphism when parasitizing three sympatric host species and that those differences are associated with the arrival of insect pollinators.

Variation in floral scent composition among plant populations has been mainly related to geographical distance and degree of pollinator specificity (reviewed in Knudsen et al. 2006). Although floral volatiles are clearly influenced by the environment (Dudareva et al. 2004), part of such variation is heritable and floral scent can be considered a phenotype upon which natural selection can act (Raguso 2004). If this is the case, the combined assessment of host-mediated phenotype polymorphisms and pollinating agents, more specifically chemical phenotypes and insect pollinators, in future ecological studies in *Tristerix* could significantly contribute to the understanding of how ecological speciation in sympatry driven by pollinators and hosts may have occurred within this genus, as has been hypothesized by Amico et al. (2007).

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