

# Deciphering the language of plant communication: volatile chemotypes of sagebrush

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## Summary

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Received: 1 April 2014  
Accepted: 12 May 2014

New Phytologist (2014)  
doi: 10.1111/nph.12887

**Key words:** cue, eavesdropping, headspace, herbivory, heritability, signal.

- Volatile communication between sagebrush (*Artemisia tridentata*) individuals has been found previously to reduce herbivory and to be more effective between individuals that are genetically identical or related relative to between strangers. The chemical nature of the cues involved in volatile communication remains unknown for this and other systems.
- We collected headspace volatiles from sagebrush plants in the field and analyzed these using GC-MS.
- Volatile profiles were highly variable among individuals, but most individuals could be characterized as belonging to one of two chemotypes, dominated by either thujone or camphor. Analyses of parents and offspring revealed that chemotypes were highly heritable.
- The ecological significance of chemotypes and the genetic mechanisms that control them remain poorly understood. However, we found that individuals of the same chemotype communicated more effectively and experienced less herbivory than individuals of differing chemotypes. Plants may use chemotypes to distinguish relatives from strangers.

## Introduction

Although initially controversial, evidence has accumulated that many plants adjust their defenses against herbivores in response to volatile cues emitted by damaged neighbors (reviewed in Karban *et al.*, 2014). Cues that elicit responses may come from the same or a different individual. The nature of these volatile cues is poorly understood, and it is not known whether the same cues are conserved across the diverse plant species that respond to volatile cues or whether particular species use unique compounds or combinations as cues.

Sagebrush (*Artemisia tridentata*) individuals experienced less damage caused by herbivores after they were exposed to volatiles from experimentally clipped neighbors (Karban *et al.*, 2006). This effect was lost when air contact between neighbors was experimentally blocked. Responding to volatile cues was apparently beneficial for sagebrush individuals. Seedlings experienced greater rates of survival and established plants produced more new branches and inflorescences following exposure to damage-induced volatile cues (Karban *et al.*, 2012).

Volatile cues emitted by sagebrush plants following damage become public information that can be perceived and used by competing individuals. Cues are detectable over distances of up to 60 cm under field conditions (Karban *et al.*, 2006), and sagebrush populations are genetically structured such that most responding tissues will be on the same or related individuals

(Cook & Stoddart, 1960; Karban *et al.*, 2013; E. D. McArthur, pers. comm.). However, unrelated individuals, including those of other species, may also eavesdrop on these cues (Farmer & Ryan, 1990; Karban *et al.*, 2000, 2013). Interestingly, communication more effectively reduces damage experienced by receivers if emitter and receiver are genetically identical (Karban & Shiojiri, 2009) or are closely related (Karban *et al.*, 2013). These results suggest that plants may respond differently to cues from self vs non-self and kin vs strangers.

This ability to differentiate on the basis of relatedness reduces the likelihood that emitters will aid competitors, and increases the reliability and information value of cues for responders, particularly if relatives share susceptibilities. In other words, information is public, but genetic relatedness influences who receives it and how reliable it may be. Kin recognition among plants is not unique to volatile communication involving defense. Many plants have been found to recognize kin using self-incompatibility determination during pollination (Nasrallah, 2002; Charlesworth *et al.*, 2005). Plants also allocate differentially to root growth after encountering the roots of kin vs strangers (Dudley & File, 2007; Murphy & Dudley, 2009; Biedrzycki *et al.*, 2010).

The ability of plants to recognize and differentially respond to cues from different individual emitters implies a high level of specificity in these cues. The volatiles emitted by damaged sagebrush include many different compounds. The number of

candidate cues has increased as our tools for detection have improved. Personius *et al.* (1987) identified 32 volatile compounds emitted by various subspecies of *A. tridentata*. Kessler *et al.* (2006) noted that 19 volatile compounds increased by more than two times following experimental clipping. A recent review of volatiles from the *Artemisia* genus reported 40 major volatile peaks (Abad *et al.*, 2012). Many species discussed in this review exhibited volatile profiles that varied intraspecifically. Other recent studies have reported > 100 volatile components from several species of *Artemisia* (Lopes-Lutz *et al.*, 2008). Many of these volatiles have activity against microbes and other parasitic organisms. However, it has not been established that any of these specific volatile compounds are actively involved in plant communication under natural conditions.

Our objective in this study was to begin to investigate the 'language' used by sagebrush plants to communicate and to characterize the diversity of potential cues. We asked several specific questions. Do damage-induced volatile emissions vary among individual plants within a population? Can this variability be characterized by chemotypes? Are volatile chemotypes heritable? Is communication that reduces herbivory more effective among individuals of the same chemotype than among individuals of different chemotypes?

## Materials and Methods

### Characterization of the diversity of cues

We collected headspace volatiles from 59 plants in Taylor meadow at the University of California (UC) Sagehen Natural Reserve, north of Truckee, California, USA. All plants were from the same subspecies: *Artemisia tridentata* Nutt. ssp. *vaseyana* (Rybd.) Beetle. Volatiles were collected from one branch per plant. Five leaves of each branch were subjected to a 2–3-mm-long clip with scissors before collection. Each branch was enclosed in a pre-cleaned (oven baked at 120°C) polyethylene terephthalate (PET) bag (Look; Terinex Ltd, Bedford, UK), which was fastened around a woody section of the branch with insulated wire. A dynamic headspace sampling system was used, which allowed volatiles to be collected from two plants concurrently. The system consisted of pressure (Gardner Denver Thomas GmbH, Puchheim, Germany) and vacuum (Bühler Technologies GmbH, Ratingen, Germany) pumps, air filters (Wilkerson model M03-C2-X00; Wilkerson Corp., Richland, MI, USA), a rechargeable lithium ion battery (Sanyo UR18650F; Hy-line, Flurlingen, Switzerland), Teflon air inlet tubes, silicon sampling lines and a custom-built control unit, which were enclosed in a toolbox. Teflon air inlet tubes were inserted into a top corner of each bag and fastened with insulated wire. Air was filtered and pumped into each bag at a flow rate of *c.* 700 ml min<sup>-1</sup> for 10 min. The flow rate was then reduced to 220 ml min<sup>-1</sup> for the duration of sampling. The second top corner of each bag was cut and a stainless steel sample tube filled with Tenax TA and Carbopak B adsorbents (150 mg each; Markes International Ltd, Llantrisant, UK) was inserted and held in place with an insulated wire. A silicon air outlet sample line was secured

to each sample tube and air was pulled through the tubes by vacuum pumps at a rate of 200 ml min<sup>-1</sup> for 20 min. The sample tubes were closed with brass caps and stored in a freezer before delivery to the University of Eastern Finland, Kuopio for analysis. After sampling, the branches were removed, bagged, baked to dry mass and the leaves were removed and weighed.

The volatile samples were analyzed by GC-MS (Agilent 7890A GC and 5975C VL MSD; New York, NY, USA). Trapped compounds were desorbed with an automated thermal desorber (TD-100; Markes International Ltd) at 250°C for 10 min, cryofocused at -10°C and then transferred in a split mode to an HP-5 capillary column (50 m × 0.2 mm; film thickness, 0.33 µm). Helium was used as a carrier gas. The oven temperature was held at 40°C for 2 min, increased by 5°C min<sup>-1</sup> to 210°C, and then by 20°C min<sup>-1</sup> to 250°C, and held for 5 min. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 35 to 350 *m/z* with a scan rate of 5.38 scans s<sup>-1</sup>.

The compounds were identified by comparison with external standards containing 27 mono-, homo- and sesquiterpenes and 10 green leaf volatiles, and by additional comparison with compounds in the Wiley library (John Wiley & Sons Inc., Hoboken, NJ, USA). Quantification was made relative to external standards and emission rates are expressed in nmol g<sup>-1</sup> DW s<sup>-1</sup>.

We conducted two non-metric multidimensional scaling (NMDS) ordinations in the package *vegan* in R (Oksanen *et al.*, 2013; R Core Team, 2013); one used the full diversity of compounds detected and one included only four emission peaks – camphor, alpha-thujone, beta-thujone and *cis*-salvene. These four compounds were found to unambiguously separate the chemotypes. In addition, carvacrol was only detected for one of the chemotypes, but was not used in our discrimination because its detection was dependent on the chromatographic method employed. NMDS allowed us to explore the diversity and abundance of volatile compounds and to determine reproducible clusters and chemotypes. We used permutational multivariate analysis of variance to test for multivariate volatile differences between chemotypes and to estimate the percentage of variation in the chemical profiles that was explained by chemotype (Anderson, 2001).

### Characterization of the heritability of chemotypes

We collected headspace volatiles from eight *A. tridentata* ssp. *vaseyana* plants located on sagebrush knoll at UC Valentine Eastern Sierra Reserve, near Mammoth Lakes (California, USA). Seeds were collected from these maternal parents during autumn 2012. Seeds were germinated in a glasshouse in Davis in May 2013 and volatiles were collected from seven to eight seedlings per mother during February 2014. One branch (5 cm in length) from each seedling was clipped and enclosed for 20 min in a small plastic bag that was slightly larger than the cutting. A polydimethylsiloxane solid phase microextraction (SPME) fiber (Supelco, Bellefonte, PA, USA) was then inserted into the bag to collect volatiles for 10 min. This collection method was easier and less expensive and gave qualitatively similar volatile profiles.

The volatiles were injected into a GC-MS 5977 (Agilent Technologies, Santa Clara, CA, USA) with a 35 m × 0.32 mm internal diameter × 0.25 μm DB-5 column to determine their composition. The GC-MS program used a linear temperature ramp of 10°C min<sup>-1</sup> from 60 to 300°C. During the summer of 2012, we also collected the headspace volatiles from 59 plants that were haphazardly selected in the vicinity of the parent plants to estimate the relative frequencies of the chemotypes. Volatiles were collected and analyzed as described above.

We examined the relationship between offspring chemotype and parent chemotype using a binomial generalized linear mixed model (GLMM) with offspring chemotype as the response variable and parent chemotype as the predictor. To account for pseudoreplication (multiple offspring from each mother), parent was treated as a random variable in the mixed model. A likelihood ratio test was used to evaluate the effect of parent chemotype on offspring chemotypes. We estimated the broad-sense heritability ( $H^2$ ) of chemotype as twice the slope of the linear regression of the mean value for the offspring chemotype on the mother's chemotype (Falconer, 1960).

### Communication among and between chemotypes

We compared the effectiveness of communication between plants of the same and different chemotypes in the field by moving volatiles from experimentally clipped emitter plants in early spring to receiver plants and observing natural levels of herbivory throughout the season for the receivers. We initially designated 10 replicates each for communication between emitters of the camphor chemotype and receivers of camphor and thujone chemotypes and emitters of the thujone chemotype and receivers of camphor and thujone chemotypes. However, coding errors and leaf drop as a result of an unusually dry season reduced our sample size to 13 replicates of communication between pairs of the same chemotype and 21 replicates of pairs of different chemotypes. The distal half of 25% of the leaves of one branch of each emitter plant was clipped with scissors during the morning of 25 May 2013 and volatiles were collected by enclosing this branch in a plastic bag for 24 h. Volatiles were transferred from the headspace of clipped emitter plants to the headspace of a branch on receiver plants using a 1-l syringe (model S-1000; Hamilton Co., Reno, NV, USA) as described in Karban *et al.* (2013). The branch on the receiver was incubated with air from the clipped emitter for 24 h by enclosing it with a plastic bag. All receiver branches were covered by a bag for 24 h; only the source of the volatiles in the bag varied.

We measured herbivory by counting the number of leaves with any visible damage caused by herbivores on an assay branch on each receiver plant on 25 August 2013. We used herbivore damage over the season as the measure of plant resistance; we have no chemical marker of resistance for this system. Assay branches with c. 100 leaves were selected haphazardly. However, as the number of leaves on assay branches varied, the number of leaves with damage was standardized for branches with 100 leaves. This presence/absence measure of herbivory has been used in previous work in this system and correlates with the percentage of leaf area

removed. Treatment effects caused by the source of volatiles (same vs different chemotypes) were analyzed by a GLM (JMP 10.0; SAS, Cary, NC, USA) with a Poisson distribution and a log link function. This analysis allowed us to assess the relative contributions of the two chemotypes as emitters and as receivers, and whether emitter and receiver were the same or different. Poisson models are susceptible to type I error if the data are over-dispersed (Gardner *et al.*, 1995). To test our inference about the importance of chemotype similarity on damage, we also conducted a bootstrap analog of a *t*-test comparing communication between individuals of the same or different chemotypes (Efron & Tibshirani, 1993). Our a priori expectation was that communication would be more effective between individuals of the same chemotypes, and we used a one-tailed test to examine this hypothesis.

## Results

### Characterization of the diversity of cues

The chemical make-up of volatiles emitted by different individuals within the population at Taylor meadow was quite variable. For example, the largest component for some individuals was thujone, whereas other neighboring individuals emitted no detectable thujone but had camphor as their largest component. Indeed, most individuals had one or the other of these two compounds as the major constituent of their volatile emissions. NMDS ordinations from the 59 plants using the whole volatile profile, and ordinations using just thujone, camphor and *cis*-salvene, grouped plants into identical chemotype groups. Most individuals clustered around phenotypes characterized by either camphor or alpha- or beta-thujone (Fig. 1). Several plants had outlier profiles that failed to cluster into one of these two groups. Plants that had thujone as a major constituent also emitted carvacrol and *cis*- and *trans*-salvene (Fig. 2). Plants with camphor as the major peak failed to emit detectable concentrations of these

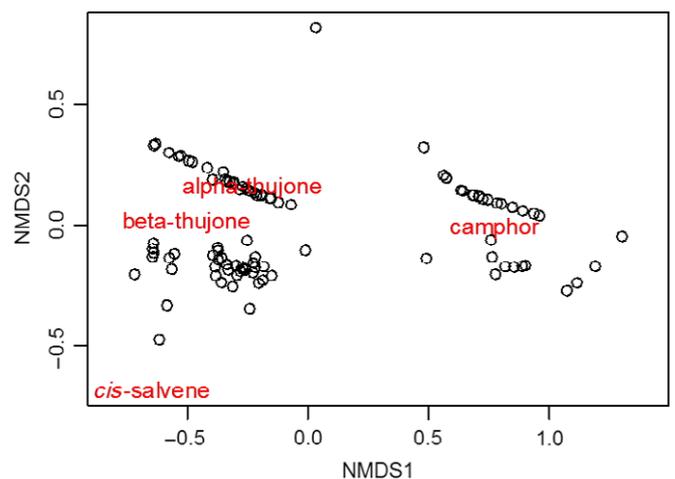
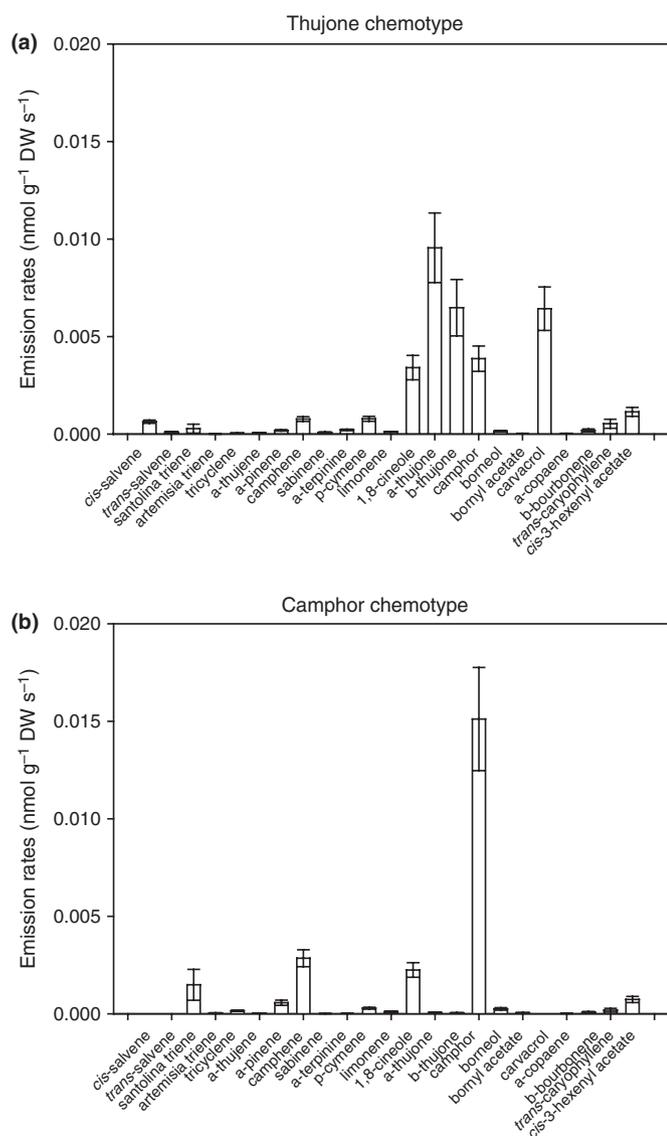


Fig. 1 Volatile emissions of the 59 *Artemisia tridentata* plants arrayed along two non-metric multidimensional scaling (NMDS) axes (stress = 0.032). The locations of the four emission peaks used to distinguish the chemotypes are also shown.



**Fig. 2** Mean ( $\pm$  1SE) volatile emission peaks for *Artemisia tridentata* plants of the two chemotypes that had either (a) thujone or (b) camphor as their major constituent.

compounds. Chemotype explained over half of the multivariate variation in thujone, camphor and *cis*-salvone abundances (permutational MANOVA pseudo- $F_{1,95} = 110.04$ ,  $P < 0.001$ ,  $R^2 = 0.54$ ).

#### Characterization of the heritability of chemotypes

Plants from the general population at our study site at the Valentine Reserve exhibited three phenotypes. Approximately 54% of the plants had thujone as the major peak, 39% had camphor as the major peak and 7% did not fit into either of these two categories.

We examined a total of 60 progeny from eight parents; five of these parents had thujone as the largest peak and three had camphor as the largest peak. A majority of progeny (48/60) had the same chemotype as their mother. Offspring from mothers of the 'thujone chemotype' were more likely to match the chemotype of

their thujone mother (34/37) than were offspring of the 'camphor chemotype' likely to match their mother (13/23) ( $\chi^2 = 8.37$ ,  $df = 1$ ,  $P = 0.004$ ). Parent chemotype was a significant predictor of offspring chemotype (likelihood ratio test  $\chi^2 = 5.67$ ,  $df = 1$ ,  $P = 0.017$ ). Broad sense heritability ( $H^2$ ) for chemotype was relatively high ( $0.98 \pm 0.36$ ).

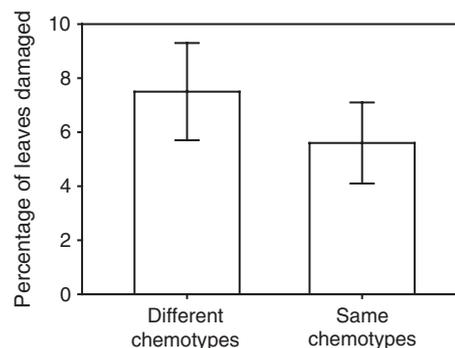
#### Communication among and between chemotypes

Damage that had accumulated over the season was used as a measure to assess the effectiveness of communication at inducing resistance to herbivory. Using this measure of host plant resistance suggested that the identity of the emitter, the identity of the receiver and whether the emitter and receiver were the same or different chemotypes all influenced the effectiveness of communication. Plants of the 'camphor chemotype' were more effective emitters ( $\chi^2 = 3.86$ ,  $df = 1$ ,  $P = 0.05$ ). Plants of the 'camphor chemotype' experienced less overall damage ( $\chi^2 = 8.81$ ,  $df = 1$ ,  $P = 0.003$ ). Of more interest, communication was more effective between plants of the same chemotype relative to plants of different chemotypes ( $\chi^2 = 9.08$ ,  $df = 1$ ,  $P = 0.003$ ; Fig. 3). As overdispersion can lead to type I error for data fitted to Poisson models (Gardner *et al.*, 1995), we used a bootstrap analog of a *t*-test to evaluate the validity of this conclusion. We found that we could reject the a priori hypothesis that communication was not more effective among plants of the same chemotypes with  $P = 0.047$ , supporting this inference.

#### Discussion

We found considerable levels of variation in the volatiles emitted by individual sagebrush bushes in a single meadow. Some volatile compounds were abundant in the profiles of some individuals and undetectable or at low concentrations in the profiles of other individuals (Fig. 2). Relatively few studies have examined intra-specific variability in damage-induced volatile emissions of plants in natural populations (Halitschke *et al.*, 2000; Staudt *et al.*, 2001; Hare, 2007; Delphia *et al.*, 2009; Schuman *et al.*, 2009; Kariyat *et al.*, 2012; Wason & Hunter, 2014). These studies generally uncovered high levels of variability among conspecifics.

The volatile emissions of some other species have been found to cluster into chemotypes, defined as 'chemically characterized



**Fig. 3** Mean ( $\pm$  1SE) percentage of leaves showing damage by herbivores for *Artemisia tridentata* plants that received cues from experimentally damaged conspecifics of the same or different chemotypes.

parts of a population of morphologically indistinguishable individuals' (Santesson, 1968, cited in Keefover-Ring *et al.*, 2008). These later authors argued for the use of multivariate statistical methods to quantitatively identify chemotypes. When we analyzed the volatile profiles of the 59 plants in our meadow using a multivariate analysis, we found that many individuals clustered into chemotypes defined by either camphor or alpha- or beta-thujone as the principal compound (Fig. 1). There were several individuals in the population that did not conform neatly to either of these categories. Interestingly, the volatile emissions of other species of *Artemisia* have also been described as chemotypes (*A. annua* – Wallaart *et al.*, 2000; Wu *et al.*, 2011; *A. judaica* – Ravid *et al.*, 1992; *A. pedemontana* – Perez-Alonso *et al.*, 2003). However, the ecological significance of chemotypic diversity has not been investigated (Linhart & Thompson, 1995; Linhart *et al.*, 2005).

We found that the chemotypes were highly heritable. Most seedlings were of the same chemotype as their mother plant. Larger samples will be required to determine the more detailed nature of inheritance of the chemotypes. In a previous study in which we did not know the relationships between individuals, we estimated the relatedness between pairs of plants using seven microsatellites that varied among these individuals (Karban *et al.*, 2013; based on Ishizaki *et al.*, 2009). That study involved the same individuals and revealed a strong correlation between genetic relatedness (estimated as Queller and Goodnight's ' $r$ ') and volatile similarity (Mantel test,  $P=0.0028$ ). As we had estimates of relatedness for the plants used in the current study, we asked whether the compounds that separated the chemotypes were correlated with relatedness. The correlation between the four chemicals that separated the chemotypes and relatedness was not statistically significant (Mantel test,  $P=0.114$ ), although a larger dataset including 12 compounds was correlated with estimates of relatedness based on microsatellites (Mantel test,  $P=0.048$ ). The result was consistent with our finding that relatives were more likely to share chemotypes. In general, the causes and consequences of chemotypic structure are not well understood, even for such well-studied examples as human blood types.

The two most common volatile compounds, camphor and thujone, are both oxygenated monoterpenes. Both are produced from the same precursor, geranyldiphosphate, but with different monoterpene syntheses catalyzing the first step in their formation (Grausgruber-Groger *et al.*, 2012; Foster *et al.*, 2013). However, these two chemically similar molecules have very different biological properties. Their signaling roles are much better understood in animal systems than in plants. Camphor binds to muscarinic acetylcholine receptors that are important in neuronal signaling. Thujone from *A. annua* is an ingredient of absinthe, although its presumed effects as a psychedelic agent acting on cannabinoid receptors now seem unlikely (Meschler & Howlett, 1999). Instead, thujone acts as a signal by binding to GABA and HT<sub>3</sub> receptors (Hold *et al.*, 2000; Deiml *et al.*, 2004). GABA signaling has been found to be important in plant responses to stresses, including herbivory, although its mode of action in plants is far less well understood than in vertebrates (Bouche &

Fromm, 2004; Mirabella *et al.*, 2008). Carvacrol is another oxygenated monoterpene that is derived from the same precursor and was only detected from the 'thujone chemotype' (Lukas *et al.*, 2010). Carvacrol has antimicrobial and antitumor properties related to alteration in phosphorylation of mitogen-activated protein kinases (Yin *et al.*, 2012), which is an early step in the development of pathogen-associated molecular pattern (PAMP)-triggered immunity in plants (Lloyd *et al.*, 2014). As these compounds are commercially available, we have performed preliminary pharmacological experiments to evaluate their effects on the levels of resistance in sagebrush. Activity is likely to be concentration dependent, and we have not measured relevant concentrations that plants experience in nature. Results from these trials were inconsistent and inconclusive.

One ecological consequence of chemotypic structure in this system is that plants communicate more effectively with individuals of the same chemotype than with individuals of other chemotypes (Fig. 3). As chemotypes are heritable, they may provide plants with one source of reliable information that can distinguish kin from strangers. This could arise by several possible mechanisms. (1) Plants may perceive cues that are most similar to their own, allowing them to coordinate their own physiological processes (Farmer, 2001; Karban *et al.*, 2006; Heil & Adame-Alvarez, 2010). (2) Plants may perceive diverse cues, but only respond to those that are likely to provide reliable information. As chemotype is a reasonable correlate of relatedness, responding to individuals of a similar chemotype may provide one mechanism to discriminate between kin and strangers.

Regardless of the mechanisms, cues that are at least partially private and heritable, rather than public, give plants the potential to provide information selectively to kin and self. This may increase the reliability of the information and decrease the likelihood that strangers may eavesdrop on the communication.

## Acknowledgements

This work was conducted at the UC Sagehen Creek Natural Reserve and the UC Valentine Eastern Sierra Reserve. We thank Jeff Brown and Dan Dawson for facilitating our fieldwork there, Kathy Eaton for field and laboratory assistance, and Cheryl Dean and Molly Hetherinton-Rauth for help with the GC. Hugh Dingle and Gideon Bradburd provided suggestions for estimating heritability. We were supported by grants from the US Department of Agriculture (NC-7), the Japanese Society for the Promotion of Science, the Academy of Finland (251898, 256050, 141053) and the European Science Foundation (EUROCORES Programme EuroVOL).

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