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VARIABLE PHYTOTOXIC EFFECTS OF *THYMUS VULGARIS* (LAMIACEAE) TERPENES ON ASSOCIATED SPECIES

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Thymus vulgaris has a chemical polymorphism in which individual plants have an essential oil dominated by either nonphenolic (geraniol, α -terpineol, *cis*-sabinene hydrate, linalool, or 1,8-cineole) or phenolic (carvacrol and thymol) monoterpenes. Using the geraniol, *cis*-sabinene hydrate, and the two phenolic chemotypes, we tested three hypotheses: (1) chemotypes vary in their influences on germination and growth of associated plant species, (2) associated species respond differentially to individual chemotypes, and (3) soils under thyme canopies will influence associated species differently than do sterile soils. The study was done in Montpellier, France, in settings mimicking natural conditions. In one experiment, survival of both monocots and dicots from the seed bank was lower in soils collected from under thyme plants of the phenolic chemotypes. In a second experiment, we sowed seeds of *Daucus carota*, *Nigella damascena*, and *Bromus madritensis* into soil collected from under thyme plants and covered with canopies of fresh thyme of each chemotype or into control soils. The species showed differential responses in germination and/or growth to the four chemotypes. In a third experiment, germination and growth of *D. carota*, *N. damascena*, *Bromus erectus*, and *Picris hieracoides* on sterile soil covered with fresh thyme foliage and controls showed differential responses to the four chemotypes; however, these responses were less marked than those on soils collected under thyme plants. These results—coupled with previous demonstrations of chemotype-influenced differential herbivory and parasitism—illustrate the complex influences of genetic variability on multispecies interactions and the individuality of species responses to toxic chemicals.

Keywords: allelopathy, competition, community organization, monoterpenes, *Thymus vulgaris*, genetic polymorphism.

Online enhancements: appendix tables.

Introduction

Sessile species must deal with competition for space, nutrients, and light. Chemical interference with establishment and growth of their neighbors can help reduce the challenges posed by these neighbors in both terrestrial (McPherson and Muller 1969; Rice 1984; Williamson 1990; Crawley 1997; Inderjit et al. 1999; Lankau 2012) and marine (Jackson and Buss 1975; Thacker et al. 1998) ecosystems. Monoterpenes have been shown to be active agents of such interference in a number of intra- and interspecific interactions (Rice 1984; Williamson 1990; Denys et al. 1991; Fisher 1991; Rai et al. 2003; Ehlers and Thompson 2004; Kaur et al. 2012). One

kind of chemically mediated interaction involves the reduction of germination and growth of neighbors, usually referred to as allelopathy. Such interactions are increasingly recognized as important influences in the organization of natural communities (Weir et al. 2004; Inderjit et al. 2011; Cheema et al. 2012; Hale and Kalisz 2012; Meiners and Kong 2012).

Monoterpenes can be toxic because they disrupt various features of metabolism, and, at least in the case of phenolic ones, they influence cell membrane permeability and damage cell walls (Lambert et al. 2001; Bennis et al. 2004). They can affect a diversity of organisms, including the very plants that synthesize them (Esau 1977; Hegnauer 1989; Harborne and Tomas-Barberan 1991; Langenheim 1994; Thacker et al. 1998; Budavari et al. 2000; Zunino and Zygadlo 2004; Y. B. Linhart, J. D. Thompson, and K. Keefover-Ring, unpublished data) and their mycorrhizal associates (Hale and Kalisz 2012). In addition, some are at least partly water soluble (see below; Weidenhamer et al. 1993) and can therefore be transported away from their source toward adjacent plants, especially when they are contained in epidermal glands on leaf surfaces of species in Asteraceae, Lamiaceae, Myrtaceae, and Rutaceae.

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One of the important features of monoterpenes is that their specific chemical structure can evoke highly variable responses from organisms that interact with the plant that synthesizes them. The specificity of these responses has been particularly well documented in *Thymus* spp. (Lamiaceae). Individual thyme terpenes vary in their deterrent effects against bacteria (de Buochberg 1976; Stahl-Biskup and Saez 2002; Ehlers et al. 2012), fungi (de Buochberg 1976; Billing and Sherman 1998; Zambonella et al. 2004; Mota et al. 2012), insects (Linhart and Thompson 1999), mollusks (Gouyon et al. 1983; Linhart and Thompson 1995, 1999), and mammals (Linhart and Thompson 1999). Thyme terpenes have also been shown to reduce germination and growth of plants under experimental conditions (Vokou and Margaris 1982; Katz et al. 1987; Angelini et al. 2003; Vokou et al. 2003; Zunino and Zygadlo 2004; De Martino et al. 2010). In addition, we demonstrated that both thyme leaves and the monoterpenes extracted from those leaves—when used at concentrations comparable to those encountered in natural settings—can be autotoxic because they reduce germination of thyme as well as the grass *Brachypodium phoenicoides* (Tarayre et al. 1995). Conversely, thyme chemotypes can also vary in the patterns of insect visitation and associated pollination (Beker et al. 1989; K. Keefover-Ring, unpublished data). In addition, Ehlers et al. (2014) have shown that habitats occupied by thyme of specific chemotypes can have higher plant species diversity than nearby habitats devoid of thyme. These complex patterns have important implications for the relevance of within-species genetic variability in the context of community ecology because they imply that the chemical profiles of individual plants can generate species-specific interactions and influence the species composition of their associates.

In this study, we used the common thyme *Thymus vulgaris* L. to analyze the roles played by several monoterpenes as potential agents of allelopathy and to test whether interplant genetic variability in the synthesis of toxic chemicals can influence the specificity of responses by potential competitors. In order to make our results applicable to natural conditions, we conducted all assays in settings designed to approximate such conditions. We asked three primary questions. (1) Do different chemotypes vary in their effects on germination and growth of associated plant species? (2) Do these potential competitors respond differentially to individual chemotypes? (3) Does soil origin influence these responses?

Material and Methods

Study Organism

Thymus vulgaris is an aromatic woody perennial and a common component of plant communities in the Western Mediterranean from northern Italy to southeastern Spain. The biological complexities and genetic control of its terpene production are well understood (Vernet et al. 1986). Individual plants can be assigned a chemotype depending on the dominant monoterpene in the essential oil. Seven chemotypes are common in Mediterranean France: five are nonphenolic, geraniol, α -terpineol, *cis*-sabinene hydrate (thuyanol), linalool, and 1,8-cineole; and two are phenolic, carvacrol, and thymol. These chemotypes are constitutive, in that despite quantitative seasonal variation in the amounts of terpenes synthesized, a

given chemotype remains stable despite wounding, cloning, or growth in diverse habitats (Lamy 1985; Vernet et al. 1986; Thompson 2002; Keefover-Ring et al. 2009; J. C. Chalchat, J. D. Thompson, and Y. B. Linhart, unpublished data). Both the biosynthetic pathway involved in their synthesis and the genetic control of this synthesis of all but 1,8-cineole are well understood (Gouyon et al. 1986; Vernet et al. 1986; Passet 1971).

Because of the toxicity of monoterpenes (Hegnauer 1989; Harborne and Tomas-Barberan 1991; Budavari et al. 2000), these compounds are sequestered away from the centers of metabolic activity of plant cells (Esau 1977; Stahl-Biskup and Saez 2002). For example, in Lamiaceae such as thyme, rosemary, and mints, monoterpenes are in small glands called pellate glandular trichomes on the outside of the epidermis (Esau 1977, p. 200; Yamaura et al. 1992). These glands are thin walled, so they can burst as water falls on leaves and stems, and monoterpenes are then washed down to the soil. We have detected monoterpenes in water that flowed through fresh thyme foliage and was then analyzed by gas chromatography (see below). An extensive literature indicates that the two phenolic terpenes (carvacrol and thymol) are most toxic in both *Thymus* (Stahl-Biskup and Saez 2002) and other species (Hegnauer 1989; Budavari et al. 2000). In addition, the two phenolic terpenes are on average more deterrent to herbivores and reduce germination to a greater extent than do nonphenolic terpenes (Linhart and Thompson 1995, 1999).

In natural populations, the frequencies of the seven chemotypes vary markedly. Of more than 300 populations analyzed in southern France, about 10% are monomorphic, but most are polymorphic, with two or more chemotypes growing intermixed. There is often a strong spatial differentiation of chemotypes at both a local (Mazzoni and Gouyon 1984; Keefover-Ring et al. 2009) and a landscape (Gouyon et al. 1986; Thompson 2002; Thompson et al. 2013) scale.

Associated Species

Thyme is a low shrub, usually about 25–40 cm at maturity. In contrast, many of the annual and perennial herbs that grow intermixed with it reach 80–100 cm or more in height. Many of the species are annuals and can therefore grow quickly and, in the process, overtop and shade thyme plants for the bulk of the growing season from April through June (Y. B. Linhart and J. D. Thompson, personal observation). Under such shady conditions, flowering of thyme plants is reduced, and if succession continues, it is eliminated. Among the hundreds of species growing in these communities are Poaceae (*Bromus* spp., *Brachypodium* spp., and *Dactylis* spp.), Asteraceae (*Centaurea* spp., *Crepis* spp., *Sonchus* spp., and *Picris hieracioides*), Fabaceae (*Medicago* spp., *Melilotus* spp., and *Bituminaria bituminosa*), Apiaceae (*Daucus carota*), and Ranunculaceae (*Nigella* spp.).

Seeds of five species growing in plant communities where thyme occurs were collected for the experiments described below. The five species are *D. carota*, *Nigella damascena*, *Picris hieracioides*, *Bromus madritensis*, and *Bromus erectus*.

Experiments

Three types of experiments were conducted. In seed bank experiments, we compared the number of seedlings of all spe-

cies that emerge from native soils collected under and away from thyme canopies to determine the extent of potential competition that adult thyme plants are exposed to at the onset of the growing season. We then asked whether soil type and presence of thyme foliage could influence the rates of germination and survival of these seedlings. To do so, we compared the responses of associated species by performing tests in either native soils from thyme stands or sterile soils. In experiments with native soils, we compared the germination and growth of co-occurring species under conditions that combine use of soils from thyme stands of specific chemotypes with the presence of fresh thyme foliage. In experiments on sterile soils, we tested the influence of thyme foliage alone on germination and growth of co-occurring species in a sterile soil mix. In all three experiments, we compared the influences of two nonphenolic monoterpene chemotypes (geraniol [G] and *cis*-sabinene hydrate [U]) to the influences of the two phenolic monoterpene chemotypes (carvacrol [C] and thymol [T]) upon associated species.

Experiment 1: seed bank. To determine the possible influence of thyme litter and leached terpenes on germination and early survival of co-occurring species, we collected soil in sites where thyme occurs in dense patches and which are known to be monomorphic for a given chemotype. Within each site, soil was collected (and kept separate) from two locations: under live thyme canopy (i.e., within dense patches of the plant) and away from thyme plants, most often in open space with no plant canopy or near other species such as *Brachypodium* spp., *Bromus* spp., or *Dactylis* spp. Care was taken not to collect soil under other species that produce terpenes such as *Juniperus* spp., *Rosmarinus officinalis*, *Clinopodium nepeta*, *Lavandula angustifolia*, or *Buxus sempervirens*. The soil was collected from the top 4 cm and included, as it does in nature, leaf litter and debris. Within each stand, collections were made from a minimum of 20 locations, and the resulting collections were thoroughly mixed. Collections were made from two sites of each chemotype: geraniol (G), *cis*-sabinene hydrate (U), carvacrol (C), and thymol (T). For all samples originating under plants, the matching samples were from nearby (usually 1 m away) locations. Similar volumes of all soil samples were collected.

Soils were collected in late September at the very beginning of the rainy season, before any germination had taken place. They were kept cool and returned to the lab the same day. The next day, soils of a given origin were mixed thoroughly, and then they were spread out into small containers (10 cm × 15 cm × 5 cm) to a depth of 4 cm. Twelve replicates of each treatment were set up. All containers were set out on benches in an unheated greenhouse, and their locations were randomized. They were also moved periodically. The greenhouse doors were kept open day and night to approximate ambient temperatures. The greenhouse was located in the experimental garden of the Centre d'Ecologie Fonctionnelle et Evolutive in Montpellier, France, ~20 km from the natural populations where soils were collected.

The containers were sprayed and watered irregularly to mimic morning dew and rain, as often happens in nature. This meant that they received water one to four times per week in amounts ranging from a light fogging to about 5 mm per session. According to precipitation records, the average amount of water received in the field during the germination

season was 3.75 mm per day, and we provided the same average over the duration of the experiments.

Germination was recorded at 15 and 30 d. Seedling identity was tracked for all containers by labeling of seedlings that had germinated at 15 d with toothpicks. Those still alive at 30 d were used to compute percent early survival in soils of different origins. During this period, seedling density was never high enough to produce competition that might lead to mortality: the maximum numbers of total seedlings in a single container were 86 and 81, observed in two of the 192 containers, which works out to one seedling per 7 cm³ of soil for seedlings with a stem and root diameter of about 1 mm and a total length (shoot + root) of 2–8 cm, depending on species. New seedlings that germinated between 15 and 30 d were added to those that germinated in the previous 15 d. Very few seedlings emerged after 4 wk, and those that did were not used in analyses.

Experiment 2: performance on native soils. Seeds of three species were sown into flats filled with 4 cm of native soil of the same origins as described: either under thyme plants or away from thyme plants. The flats were then covered with either of two toppings.

1. *Live thyme.* Containers held soil from particular chemotype areas covered with live thyme twigs and foliage of the same origin; that is, soils of geraniol populations were covered with geraniol plant material, soils of thymol populations were covered with thymol plant material, and so on. The volume of plant material used was equivalent to the volume of plant material that makes up the canopy of a typical thyme plant covering an area comparable to that of a single flat. This was determined by weighing the fresh weight of field-collected material. This material was replaced with fresh material every 4–6 wk as it became dry.

Water that fell through thyme foliage and percolated to the soil was tested with gas chromatography, and we documented the presence of monoterpenes that came from the thyme foliage and branches in this water. For example, in one assay, we dripped water through fresh thyme foliage and tested the resulting rinsate by gas chromatography to determine whether monoterpenes leach from fresh foliage and into the soil. We placed about 1 g of fresh thyme branches of G, U, C, and T chemotypes into a filter apparatus consisting of a 3-cm disk of Whatman #1 filter paper clamped between a 15-mL graduated funnel and the fritted glass base (P/N XX1002514, EMD Millipore, Etobicoke, Canada). We then rinsed 15 mL of ambient-temperature distilled water through the foliage and into an Erlenmeyer flask, added 5 μL of an internal standard solution (0.1 μL mL⁻¹ *n*-propanol) to 200 μL of each rinsate, and analyzed the solution by gas chromatography with methods similar to those of Keefover-Ring et al. (2009).

2. *Control.* Containers with the same soils were covered by dry, dead thyme branches with no leaves and a thin layer of perlite. This was felt to be the best possible control available: using thyme branches meant that whatever might be in stem materials, such as tannins, could also be leached out, and using small (1–2 mm diameter) perlite was similar in structure—but not in chemistry—to the mixture of curled, dry thyme leaves that fall under thyme plants and often form a litter layer several millimeters thick.

For two species (*D. carota* and *N. damascena*), the treatments and control were applied to flats containing soils either

under thyme or away from thyme. This allowed us to compare the possible effects of soil origin alone on germination and growth. For an additional species (*B. madritensis*) for which we had fewer seeds available, treatments and control were applied only to flats containing soil from under thyme plants.

There were three replications of each treatment and control. For *N. damascena*, we sowed 30 seeds per replication. For *D. carota* and *B. madritensis*, we sowed 25 seeds per replication. Seeds were sown in late December. To obtain estimates of final plant sizes, aboveground parts and roots of plants were collected at 20–22 wk in early June and oven dried; total weights of tops plus roots for all three species are expressed as dry weights.

Experiment 3: performance on sterile soil. Seeds of four plant species were sown into containers filled with a sterile soil mix that consisted of clay loam of local origin. Soil origin was the same for all species. There were five replications of each treatment and control. The containers were then covered with either of two toppings: (1) live thyme: flats were covered with live thyme plant material of one of the four chemotypes, as described above; (2) control: flats were covered with a combination of dead thyme twigs with no leaves and perlite, as described above.

This experiment was performed with *D. carota* (40 seeds per replication), *N. damascena* (40 seeds per replication), *B. erectus* (25 seeds per replication), and *P. hieracioides* (35 seeds per replication) and was started March 23. Variation in numbers used was due to the variability of available seeds during collections.

Timing, randomization, and measurements. All experiments were carried out during the rainy period when germination and growth of associated species occur in nature, that is, between November and June. No artificial light was supplied. For all experiments, a given treatment was administered to a container of the same size (10 cm × 15 cm × 5 cm) with one species sown. These containers were randomized, and the spatial positions of individual containers were rerandomized every 2–3 wk to minimize potential influences of greenhouse location. For experiments 2 and 3, to compare plant sizes among treatments, plant densities were standardized in all containers by keeping five plants per container and randomly removing other seedlings after recording germination.

For all species except *P. hieracioides*, oven-dry weights of tops and roots of mature plants were made at the end of the growing season in late June to determine their performances. Very few *P. hieracioides* germinated, so only germination and survival were usable as indexes of performance.

Statistical Analyses

Data on plant size were analyzed by ANOVA using the PROC GLM command in SAS (SAS Institute 2003). Data on numbers of seed germinating at a given time and seedling survival are count data and were collected across different chemotype/soil origins, and they were thus analyzed using χ^2 analyses of a 2 × 2 table to test for goodness of fit for a given treatment or origin or in replicated G-tests of goodness of fit (likelihood ratio tests), following Sokal and Rohlf (2012). The

latter allow to test for heterogeneity among several (more than two) different treatments (e.g., chemotypes or soil origins). These analyses were performed by pooling replicates and soil origins within chemotypes.

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Results

Experiment 1: Seed Bank

A total of 6319 seedlings germinated, 1274 monocots, and 5045 dicots. The number of seedlings that emerged was highly variable among the 12 replicates within each category of soil origin; at 15 d, it ranged within a given replication from 0 to more than 80. Many individual replicates had fewer than five seedlings, and few had more than 50. As a result, the totals emerging at 15 d out of the 12 replications for each origin ranged from <10 per category for seven different origins to more than 100 for five origins. For this reason, results from the separate populations were combined to allow for statistical evaluation of germination rate and seedling survival between 15 and 30 d among soils of different origins. Soils from under thyme plants yielded many more seedlings: 3564 dicots and 884 monocots compared with 1481 dicots and 390 monocots in soils away from thyme plants.

Across the different comparisons, germination rates—defined as the proportion of total seedlings that had germinated after 15 d out of the total germinated after 30 d—showed little consistent variation across the different soil site origins for both monocots and dicots (fig. 1). The only significant difference was a slower germination rate of monocots in soils collected from under C plants compared with soils collected away from C plants (df = 1, $\chi^2 = 5.6$, $P < 0.05$; fig. 1). Other χ^2 values were nonsignificant ($P > 0.05$) in all comparisons.

Survival from 15–30 d was somewhat lower on soils collected from under thyme plants for soils from all four chemotype origins in both monocots and dicots (fig. 1). These differences in survival on the two types of soil were significant for dicots under both phenolic chemotypes (soils collected in the C site: df = 1, $\chi^2 = 11.3$, $P < 0.001$; soils collected in the T site, df = 1, $\chi^2 = 34.1$, $P < 0.001$). For monocots, a significantly (df = 1, $\chi^2 = 6.9$, $P < 0.01$) reduced seedling survival was observed for soils collected under the thymol chemotype compared with soils from the same site but away from thyme plants. Values for χ^2 were nonsignificant ($P > 0.05$) for the other three soil origins.

Experiment 2: Performance on Native Soils

Water-Leached Terpenes. In the experiments where we dripped water through fresh thyme foliage, we usually found

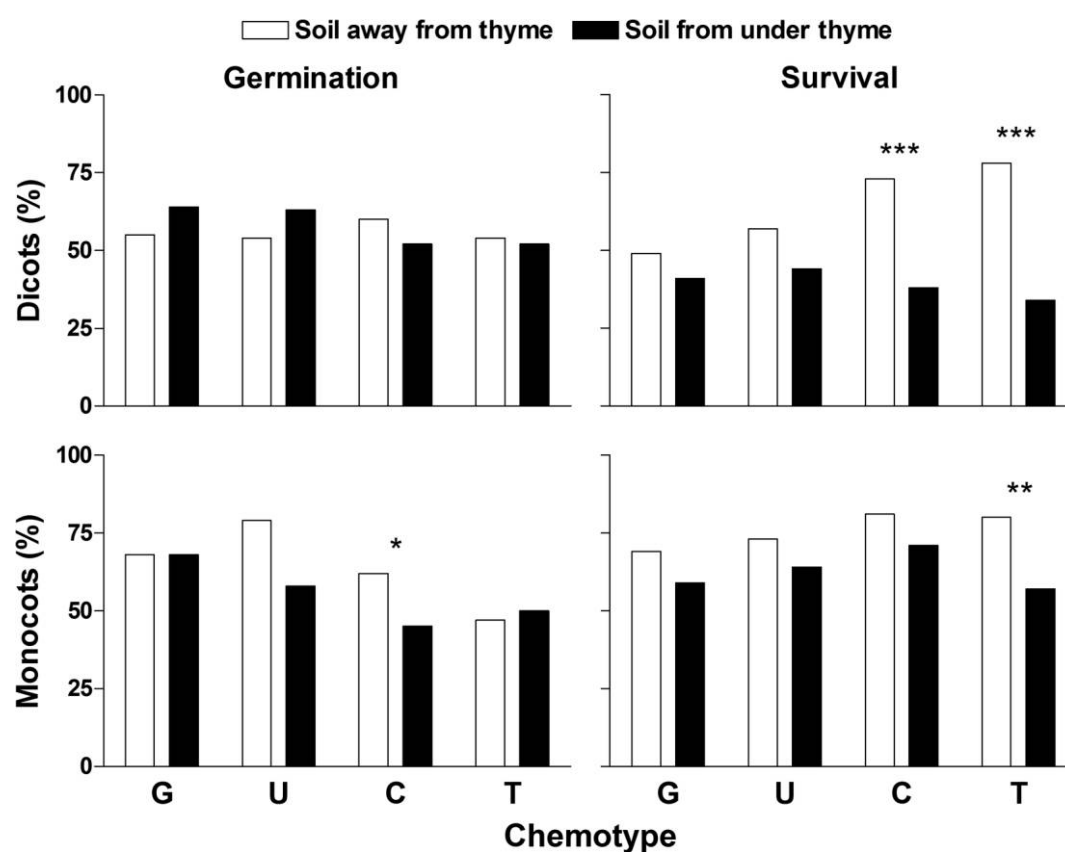


Fig. 1 Germination rate and survival for dicots and monocots that grew in soil collected either away from (open bars) or under (filled bars) thyme plants of different chemotypes. G = geraniol, U = *cis*-sabinene hydrate, C = carvacrol, T = thymol. One asterisk = $0.01 < P < 0.05$, two asterisks = $0.001 < P < 0.01$, three asterisks = $P < 0.001$.

only the most abundant monoterpene from the respective chemotypes (geraniol, *cis*-sabinene hydrate, carvacrol, and thymol) in detectable amounts. Quantities of these leached terpenes ranged from 12 to $35 \mu\text{g g}^{-1}$ fresh weight. There were no differences among chemotypes.

***Daucus carota*. Germination.** Statistical analyses of germination were done at the end of the experiment (April 9), after which no further germination was observed. Both soil origin and treatment influenced germination patterns (fig. 2a, 2b). There was a significantly reduced germination rate in soils collected from sites with the two phenolic chemotypes and covered with foliage (carvacrol: $G = 7.58$, $df = 1$, $P < 0.01$; thymol: $G = 4.26$, $df = 1$, $P < 0.05$) and an increased germination rate on soils collected from the geraniol site and covered with foliage ($G = 4.71$, $df = 1$, $P < 0.05$). The overall difference among chemotypes was significant (pooled $G = 4.89$, $df = 1$, $P < 0.05$). In addition, there was a highly significant difference among soil origins (heterogeneity $G = 14.51$, $df = 3$, $P < 0.01$). In soils collected away from thyme, germination under U, C, and T foliage was slightly decreased, but the differences were not statistically significant (pooled $G = 1.8$, $df = 1$, $P > 0.1$; heterogeneity $G = 4.92$, $df = 3$, $P > 0.1$; fig. 2a, 2b). Treatment alone, (added thyme foliage cover) produced no differences between soils collected under

and away from thyme plants on April 9 (pooled $G = 0.28$, $df = 1$, $P > 0.1$; heterogeneity $G = 1.47$, $df = 3$, $P > 0.1$).

Plant growth. Final plant biomass was reduced in the presence of *cis*-sabinene hydrate (U) (table A2; tables A1–A5 available online).

***Nigella damascena*. Germination.** Statistical analysis of germination rates were done at the end of the experiment (May 12), after which no further germination was observed. Soil origin and treatment influenced germination, and the patterns were different from those seen in *Daucus* (fig. 2c, 2d). In soils collected away from thyme plants, there was a consistently higher rate of germination in the controls compared with soils covered with thyme foliage for all chemotype origins, with C and T foliage having a statistically significant impact (pooled $G = 14.3$, $df = 1$, $P < 0.001$; heterogeneity $G = 2.26$, $df = 3$, $P > 0.1$). Conversely, on soils collected under thyme plants, foliage cover had no significant influence on germination.

In addition, there were no significant overall differences between soils collected under and away from thyme plants, whether they were covered by thyme foliage (pooled $G = 1.19$, $df = 1$, $P > 0.1$; heterogeneity $G = 1.02$, $df = 3$, $P > 0.1$) or uncovered controls (pooled $G = 0.82$, $df = 1$, $P > 0.1$; heterogeneity $G = 5.3$, $df = 3$, $P > 0.1$).

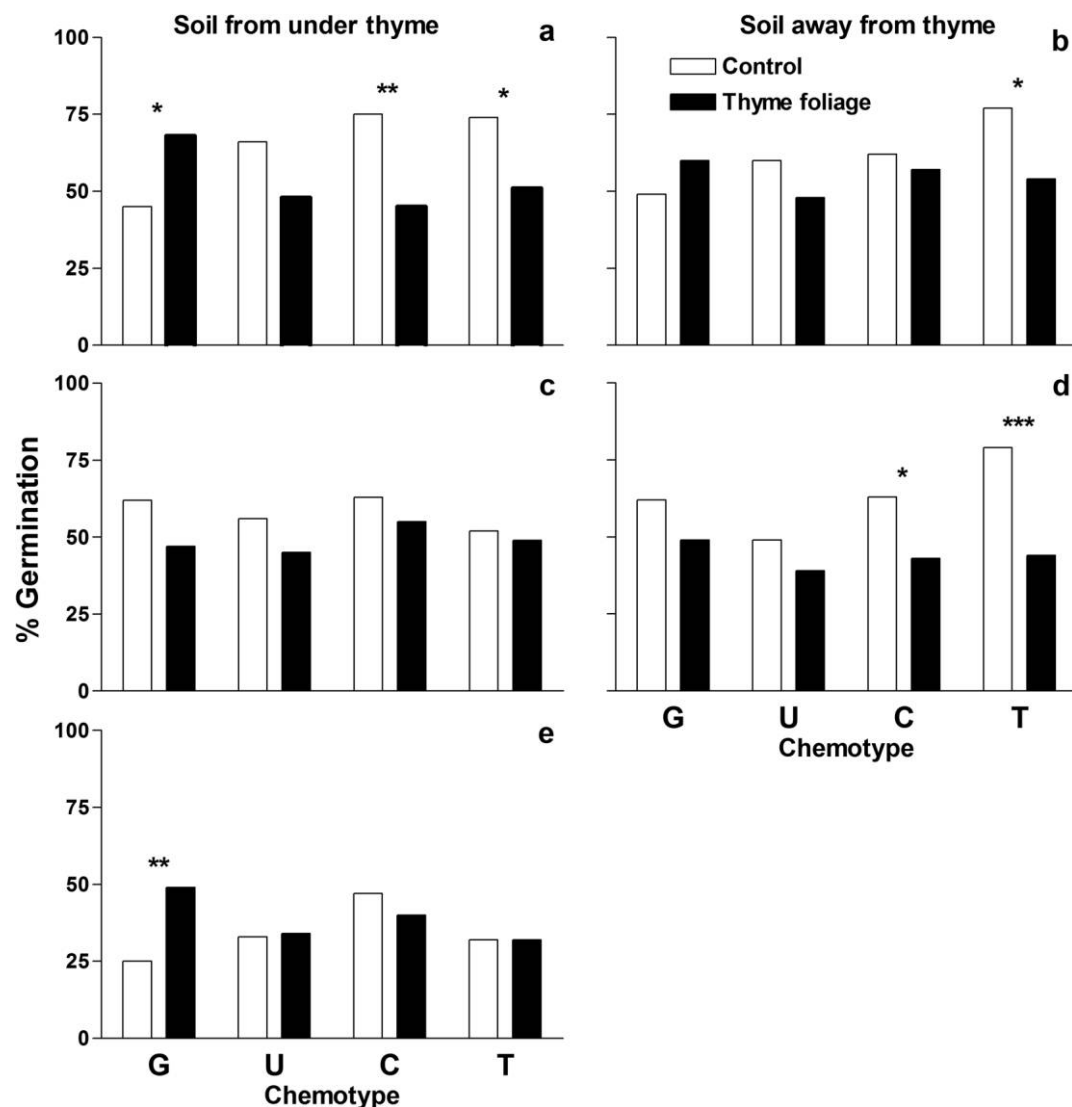


Fig. 2 Percent germination for *Daucus carota* (a, b) and *Nigella damascena* (c, d) grown in soil collected either away from or under thyme plants of different chemotypes and for *Bromus madritensis* (e) only in soil under thyme plants of different chemotypes, without (control; open bars) or with (filled bars) thyme foliage. G = geraniol, U = *cis*-sabinene hydrate, C = carvacrol, T = thymol. One asterisk = $0.01 < P < 0.05$, two asterisks = $0.001 < P < 0.01$, three asterisks = $P < 0.001$.

Plant growth. None of the treatments produced a significant reduction in final plant weight (table A3).

Bromus madritensis. Germination. At the end of the experiment (February 16), there was a significantly higher rate of germination under thyme foliage on soils from the geraniol sites compared with controls ($G = 7.93$, $df = 1$, $P < 0.01$; fig. 2e). None of the other treatments had significant effects (pooled $G = 1.11$, $df = 1$, $P > 0.1$); however, there was some variation among chemotype soil origins (heterogeneity $G = 7.39$, $df = 3$, $0.01 < P < 0.05$).

Plant growth. Final plant biomass was reduced most significantly by carvacrol and less but still significantly by thymol, and it was not affected by either geraniol or *cis*-sabinene hy-

drate (table A5). There was also a significant population by treatment interaction (table A4).

Experiment 3: Performance on Sterile Soil

Daucus carota. Germination. The presence of thyme foliage had no significant influence on germination the end of the experiment (June 1; heterogeneity $G = 8.6$, $df = 4$, $P > 0.05$), although we observed higher rates of germination on soils with geraniol foliage (90%) compared with all other soils (68%–73%).

Survival. There were no differences among treatments (fig. 3a).

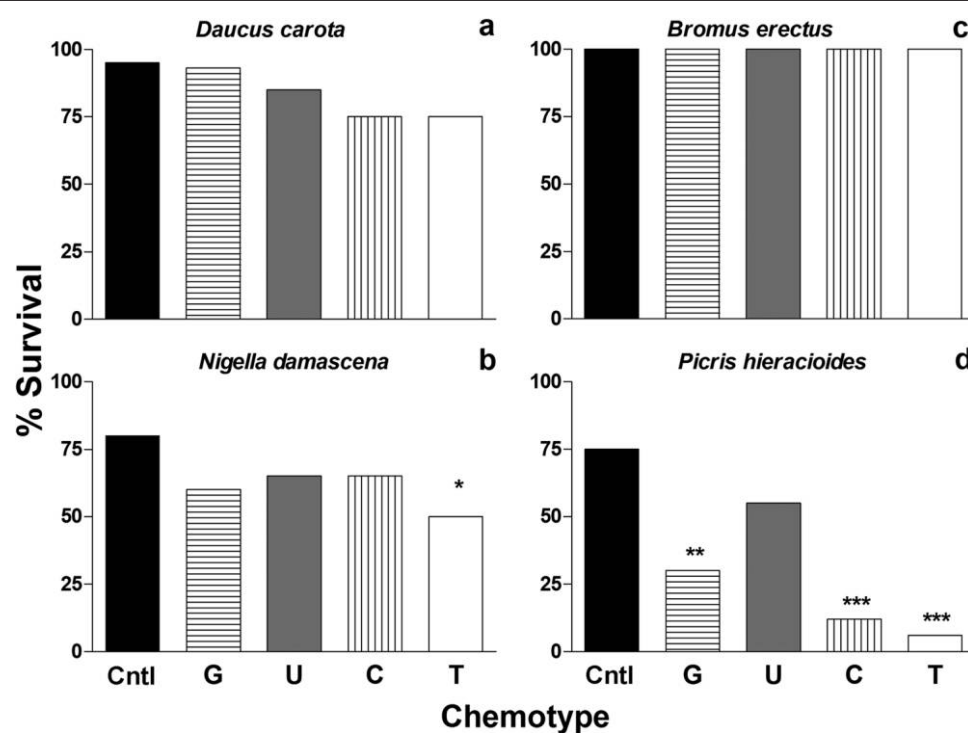


Fig. 3 Percent survival of *Daucus carota* (a), *Nigella damascena* (b), *Bromus erectus* (c), and *Picris hieracioides* (d) grown in sterile soil covered with either dead thyme twigs with no leaves and perlite (control) or live thyme plant material of four chemotypes. G = geraniol, U = *cis*-sabinene hydrate, C = carvacrol, T = thymol. One asterisk = $0.01 < P < 0.05$, two asterisks = $0.001 < P < 0.01$, three asterisks = $P < 0.001$.

Plant growth. There were significant differences among treatments: compared with the control weight of 0.355 ± 0.042 g, exposure to G (0.178 ± 0.025 g) and T (0.227 ± 0.045 g) produced the greatest reductions in biomass ($F = 3.1$, $df = 4$, $P < 0.05$).

***Nigella damascena*. Germination.** Germination showed no significant variability among treatments (heterogeneity $G = 3.8$, $df = 4$, $P > 0.1$) and varied only slightly from 45%–51% across treatments.

Survival. Seedlings exposed to T had significantly ($P < 0.025$) lower survival compared with control (fig. 3b).

Plant growth. None of the chemotypes had significant effects (data not shown).

***Bromus erectus*. Germination.** Germination varied only slightly (68%–72%) across treatments, and differences were not significant (heterogeneity $G = 0.7$, $df = 4$, $P > 0.1$).

Survival. All seedlings survived all treatments (fig. 3c).

Plant growth. There were no significant chemotype effects on growth (data not shown).

***Picris hieracioides*. Germination.** All treatments reduced germination compared with controls (39%) at the end of the experiment (geraniol = 18%, *cis*-sabinene hydrate = 9%, carvacrol = 21%, thymol = 24%; heterogeneity $G = 49.7$, $df = 4$, $P < 0.001$).

Survival. Three chemotypes reduced survival very significantly: compared with controls (68.1%), survival under G was 32.2% ($P < 0.005$), under C it was 12.9% ($P < 0.001$), and under T it was 4.8% ($P < 0.001$). Treatment U (56.2%) had no significant effect ($P > 0.20$; fig. 3d).

Plant growth. There were no significant chemotype effects on growth (data not shown).

Discussion

Effects of Terpene Leachates

The primary results of this study are that monoterpenes produced by *Thymus vulgaris* can interfere with germination, establishment, and/or growth rates of several associated species and that the responses of these species vary as a function of the individual terpenes and soil conditions to which they are exposed. These effects occurred in settings mimicking natural conditions. In experiment 1, we found that there can be residual effects of thyme litter on seedlings emerging from the seed bank in soils at the onset of the rainy season. These soils contained a large amount of dry thyme foliage that usually accumulates under and around plants at the end of the hot, dry summer. In experiments 2 and 3, we showed that performance of associated species can be influenced by exposure of their seeds and plants to terpenes present in thyme foliage, in soils from thyme stands, or both. These terpenes can be volatilized and/or carried by water running through

thyme canopies. These results complement previous experiments that documented similar patterns of reduced germination effects on a single associated species (Tarayre et al. 1995; Ehlers and Thompson 2004) and illustrate the complexity of such interactions at the community level.

The identification of monoterpenes as the direct allelopathic agents comes from the following observations. *Thymus vulgaris* produces monoterpenes in copious amounts in trichomes covering leaf and stem surfaces, and extensive extractions of its tissues indicate that monoterpenes and their precursors are the predominant components of thyme extracts (for references, see “Material and Methods”). In addition, as noted above, we have detected monoterpenes in water that has been run through thyme stems and foliage. This confirms the observation that among monoterpenes, alcohols—such as the ones produced by thyme—are the most soluble in water (Weidenhamer et al. 1993). Also, in previous experiments involving germination of seeds of *T. vulgaris* and *Brachypodium phoenicoides*, the reduction in germination produced by thyme leaves was reproduced in experiments using pure monoterpene extracts distilled from thyme leaves and placed near and in contact with seeds at concentrations comparable to or lower than those found in litter that is one leaf thick of thyme leaves (Tarayre et al. 1995).

A summary of the results of the current experiments and those of Tarayre et al. (1995) are provided in table 1. All four of the monoterpenes tested have some inhibitory effects on at least some of the associated species and under some conditions. In addition, geraniol had some positive influences on germination of *Daucus carota* and *Bromus madritensis* in experiment 2. The interspecific differences in responses to the presence of specific monoterpene-containing leachates are also notable. For example, all four terpenes reduced germination of *Thymus* and *Picris*, while three terpenes (but not geraniol) reduced germination of *D. carota* on native soils. Also, carvacrol and thymol deterred germination of *B. phoenicoides* most effectively and reduced germination of *D. carota* on native soils—but not sterile soils—while having no negative influence on germination of either *Bromus* species. Overall, it is clear that terpenes influence separate events of life history of thyme’s associated species—that is, germination, survival, and growth rates—in a highly variable manner, as detailed in table 1.

In Mediterranean ecosystems, plants face the challenge of living in environments characterized by a marked summer drought and often grow on thin, intensely weathered soils that are usually oxidized and nutrient poor, especially with regard to nitrogen and phosphorus (Thompson 2005). Under these conditions, competition for nutrients and water can be intense, and plant-plant interactions may greatly influence community structure. These ecosystems are also characterized by an abundance of aromatic plants, thereby providing ideal situations for the study of impacts of chemical interference upon plant-plant interactions. Similar species-specific effects have also been noted in allelopathy studies in other Mediterranean landscapes (Friedman et al. 1977).

The Influence of Soil Conditions

The soil within which the experiments took place also influenced certain outcomes. In the tests of germination and

survival of plants originating from the seed bank (experiment 1), on soils collected from under carvacrol plants, germination of monocots was slower. In addition, seedlings of both monocots and dicots growing in soils collected under thymol showed lower survival than did seedlings growing in soils collected under nonphenolic chemotypes (fig. 1). Although no chemical analyses were conducted on the soils, the primary difference between the two categories of soils was most likely the presence of monoterpene leachates and thyme litter in soils under thyme and the absence of such leachates or litter in soils collected away from thymes. It is also likely that the initial percolation of monoterpenes into the soil was followed by the modification of these terpenes into by-products and that the presence of the terpenes led to changes in microflora, as documented in diverse soil analyses (Inderjit et al. 1999; Ehlers et al. 2012). Mixtures of soils under thyme are known to have higher organic matter C and N than soil under *Bromus* (Ehlers and Thompson 2004). However, in our experiment, it is unlikely that there were major differences in nutrient levels, since soils away from thyme still came from under other plant species, including grasses (especially *Bromus* spp. and *Brachypodium*), *Linum* spp., *Asphodelus albus*, and *Lithodora fruticosa* (Boraginaceae).

The soils within which the associated species grew also influenced their responses. As table 1 shows, there were a total of six significant responses by *Daucus* and *Nigella*, which were used in both experiments: in experiment 2 using native soils from thyme stands compared with only two in experiment 3, which involved sterile soils.

Ecological and Evolutionary Implications

Thyme plants must cope with high densities of competition from associated species, since the soils under their canopies probably contain many more seeds than do soils in the open: as noted in experiment 1, 4448 seedlings emerged from soils under thyme compared with 1871 in comparable volumes of soil collected away from thyme canopies. In addition, individual plants must deal with disease organisms and herbivores, and those secondary compounds that are most deterrent to this multiplicity of challengers are likely to be most useful. In previous studies, we have shown that thyme terpenes can be effective deterrents and can also be species specific in their repellency to several species of herbivores. For example, linalool is simultaneously attractive to the mollusk *Helix aspersa* and repellent to the grasshopper *Leptophyes punctatissima*, while thymol shows the reverse pattern (Linhart and Thompson 1995, 1999). The overall repellency to herbivores and to bacteria and fungi (de Buochberg 1976) is greatest for the phenolic terpenes carvacrol and thymol, which are known to be fundamentally the most toxic of the six terpenes tested (Budavari et al. 2000). For this reason, we expected to see these two terpenes be the most toxic in tests of allelopathy, and there is evidence for such a pattern. Of the 33 cases where there is a significant negative effect of a terpene (table 1), 24 involve the phenolic carvacrol or thymol, while nine involve the nonphenolic geraniol or *cis*-sabinene hydrate ($\chi^2 = 6.82$, $P < 0.01$). For some species (such as *B. madritensis* and *Brachypodium*), the difference between phenolic and nonphenolic terpenes holds, while for others (*Thymus* and *Picris*), all terpenes have a deterrent effect, and

Table 1
Summary of Influences of the Four Thyme Chemotypes on Germination, Survival, and Growth of Various Plant Species

	Geraniol	<i>cis</i> -sabinene hydrate	Carvacrol	Thymol
Experiment 1 (seed bank):				
Germination:				
Dicots	ns	ns	ns	ns
Monocots	ns	ns	—*	ns
Survival:				
Dicots	ns	ns	—***	—***
Monocots	ns	ns	ns	—*
Experiment 2 (native soils + thyme canopy):				
Germination:				
<i>Daucus carota</i>	+*	ns	—**	—*
<i>Nigella damascena</i>	ns	ns	—*	—***
<i>Bromus madritensis</i>	+**	ns	ns	ns
Final plant size:				
<i>D. carota</i>	ns	—*	ns	ns
<i>N. damascena</i>	ns	ns	ns	ns
<i>B. madritensis</i>	ns	ns	—**	—*
Experiment 3 (sterile soils + thyme canopy):				
Germination:				
<i>D. carota</i>	ns	ns	ns	ns
<i>N. damascena</i>	ns	ns	ns	ns
<i>Bromus erectus</i>	ns	ns	ns	ns
<i>Picris hieracioides</i>	—**	—**	—**	—**
Survival:				
<i>D. carota</i>	ns	ns	ns	ns
<i>N. damascena</i>	ns	ns	ns	—*
<i>B. erectus</i>	ns	ns	ns	ns
<i>P. hieracioides</i>	—**	ns	—***	—***
Final plant size:				
<i>D. carota</i>	ns	—*	—*	ns
<i>N. damascena</i>	ns	ns	ns	ns
<i>B. erectus</i>	ns	ns	ns	ns
Tarayre et al. 1995:				
Germination on leaf litter:				
<i>Brachypodium phoenicoides</i>	ns	ns	—***	—***
<i>Thymus vulgaris</i>	—***	—***	—***	—***
Germination with terpenes:				
<i>B. phoenicoides</i>	ns	—***	—***	—***
<i>T. vulgaris</i>	ns	—***	—***	—***

Note. Plus sign indicates significantly higher performance of plants exposed to a specific chemotype compared with controls. Minus sign indicates significantly lower performance of plants exposed to a chemotype compared with controls. ns, not significant.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

for *Bromus erectus*, none of the terpenes influenced germination or growth. So it seems that for associated plant species, as for herbivores, the influences of individual terpenes can be species specific. In addition, herbivory and competition are only two of the contexts within which terpenes are relevant. Attraction of their principal pollinators is needed in these gynodioecious plants, and honeybees (*Apis mellifera*) are most attracted to geraniol chemotypes (K. Keefover-Ring, unpublished data). Various fungal diseases can be problematic, and geraniol is the most toxic terpene to several fungi (De Buochberg 1976). Physical conditions in which thyme grows can also be relevant: hard frosts can make the terpene-

containing glands on leaf surfaces burst, releasing the terpenes within. Under these conditions, the higher toxicity of the phenolic terpenes, carvacrol, and thymol becomes a liability: their release causes enough tissue damage to lead to higher mortality in plants with those chemotypes (Amiot et al 2005; Y. B. Linhart, unpublished data).

These results illustrate the multiple ramifications of genetic variability in an ecological context: when a target organism is variable, its diverse interactions with associated species influence the dynamics of a biotic community. In addition to its interactions with individual species, *T. vulgaris* can also influence plant species richness in microsites where it grows. Such

richness can be higher in sites with thyme than those without. Once again, these positive effects vary as a function of individual chemotypes (Ehlers et al. 2014).

In an evolutionary context, specific monoterpenes can also have strong enough physiological effects to exert selective pressures on thyme associates. For example, when monomorphic populations consisting of a single chemotype remain stable for several generations, plants of other species are exposed consistently to a single monoterpene and can adapt to such conditions. Such localized adaptation has been documented when plants are exposed repeatedly to a severe toxin (Linhart and Grant 1996). In this context, Ehlers and Thompson (2004) showed that *B. erectus* grew and reproduced better when grown in thyme and soil mixes of its home chemotype than on mixes from other chemotypes. Similar results have been obtained with other thyme species and other competitors, and they indicate that this adaptive pattern can occur under specific circumstances (Grøndahl and Ehlers 2008). In more general terms, these results document the small scale at which selective events can produce ecological and evolutionary networks and mosaics.

The multiple influences of thyme terpenes on herbivores, competitors (shown in this study), pollinators, and microor-

ganisms also suggest that the survival and fitness of any one chemotype is unlikely to be responsive to the selective pressures of any one species or functional groups of species by themselves. Instead, the differences in responses to specific chemotypes by plant associates and herbivores strongly suggest that selection by other species can be either diversifying or multidirectional. This means that genetic variability within species has diverse ramifications at the level of communities (Linhart 1991; Antonovics 1992; Agrawal 2003; Whitham et al. 2003).

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