

**SOIL FUNGAL EFFECTS ON FLORAL SIGNALS, REWARDS, AND
 ABOVEGROUND INTERACTIONS IN AN ALPINE POLLINATION WEB¹**

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- *Premise of the study:* Plants interact with above- and belowground organisms; the combined effects of these interactions determine plant fitness and trait evolution. To better understand the ecological and evolutionary implications of multispecies interactions, we explored linkages between soil fungi, pollinators, and floral larcenists in *Polemonium viscosum* (Polemoniaceae).
- *Methods:* Using a fungicide, we experimentally reduced fungal colonization of krummholz and tundra *P. viscosum* in 2008–2009. We monitored floral signals and rewards, interactions with pollinators and larcenists, and seed set for fungicide-treated and control plants.
- *Key results:* Fungicide effects varied among traits, between interactions, and with environmental context. Treatment effects were negligible in 2008, but stronger in 2009, especially in the less-fertile krummholz habitat. There, fungicide increased nectar sugar content and damage by larcenist ants, but did not affect pollination. Surprisingly, fungicide also enhanced seed set, suggesting that direct resource costs of soil fungi exceed indirect benefits from reduced larceny. In the tundra, fungicide effects were negligible in both years. However, pooled across treatments, colonization by mycorrhizal fungi in 2009 correlated negatively with the intensity and diversity of floral volatile organic compounds, suggesting integrated above- and belowground signaling pathways.
- *Conclusions:* Fungicide effects on floral rewards in *P. viscosum* link soil fungi to ecological costs of pollinator attraction. Trait-specific linkages to soil fungi should decouple expression of sensitive and buffered floral phenotypes in *P. viscosum*. Overall, this study demonstrates how multitrophic linkages may lead to shifting selection pressures on interaction traits, restricting the evolution of specialization.

Key words: floral evolution; fungicide; mycorrhizae; nectar larcenists; Polemoniaceae; *Polemonium viscosum*; pollination.

Plants interact with a diverse community both above- and belowground, and in many cases, interactions in one sphere alter those in the other (Morris et al., 2007). Numerous studies have examined the independent and pairwise effects of pollinators, herbivores, and soil fungi on plant traits and reproduction (e.g., Irwin et al., 2001; Wolfe et al., 2005; Gehring and Bennett, 2009). However, because plants interact with all three guilds of organisms simultaneously, any one partner or antagonist may influence opportunities for selection by others (Strauss and Irwin, 2004). Simultaneously examining above- and belowground relationships can provide insights into selection on interaction traits in complex natural systems missing from more narrowly focused studies. In this study, we explore several causal pathways through which soil fungi could impact plant fitness and floral evolution in a model system for pollinator-mediated selection. In doing so, we shed light on the potential

for linkages between above- and belowground communities to influence ecological and evolutionary processes.

Soil communities are comprised of mutualistic and antagonistic fungi, and plants can interact with both simultaneously. The direction and magnitude of fungal effects on floral interaction traits and reproduction likely depend on the type of plant–fungus interaction. In this study, we were particularly interested in the effects of arbuscular mycorrhizal fungi (AMF), root symbionts that provide their hosts with soil resources in exchange for photosynthate (Smith and Read, 1997). These interactions are generally viewed as mutualisms, although AMF can function as parasites depending on the host–fungus combination and environmental conditions (Johnson et al., 1997; Hoeksema et al., 2010). Given the role of mycorrhizal fungi in nutrient uptake, AMF are hypothesized to positively impact plant traits important to pollination and reproduction.

Animal pollinators are essential for sexual reproduction in most plant species and factors that alter plant–pollinator interactions have implications for plant fitness and population dynamics (Ashman et al., 2004). Soil fungi, including AMF, could impact plant–pollinator interactions and their fitness consequences indirectly by mediating overall plant size (Fig. 1A). In particular, larger, more apparent plants may be more attractive to pollinators (Gange and Smith, 2005). Larger plants may also allocate more resources to reproduction, promoting pollen limitation of seed set and opportunities for pollinator-mediated selection on floral traits.

Alternatively, soil fungi could impact pollinator visitation and plant reproduction by modifying the quality or magnitude of floral signals and rewards for pollinators (Fig. 1B). Investing

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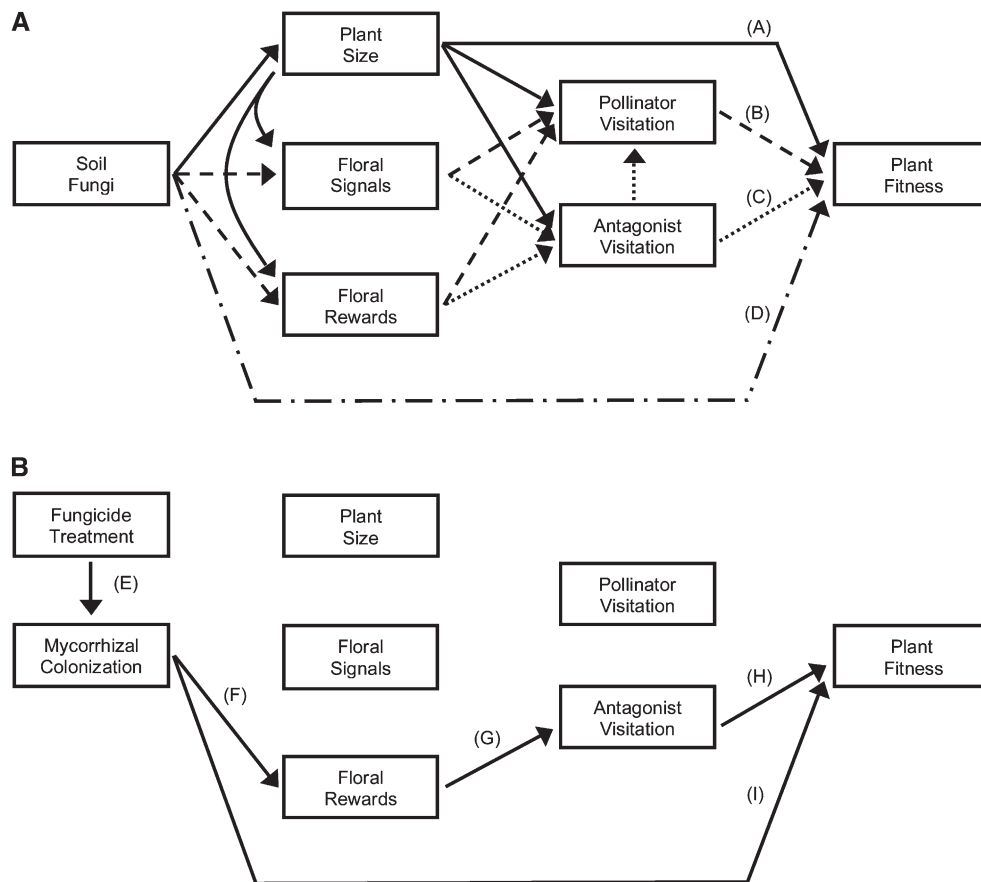


Fig. 1. Panel A: Four hypothesized pathways through which soil fungi may alter plant reproduction and animal-mediated selection on floral traits. (A) Fungal effects on resource allocation to plant growth may in turn affect apparency to floral visitors and/or the plant's resource budget for reproduction (—). (B) Soil fungi may alter floral signals and rewards exploited by pollinators (---) or (C) floral antagonists (-----). (D) Direct effects of soil fungi on plant fitness through nutrient provisioning to seeds or pollen may reduce the opportunity for selection by pollinators and floral antagonists (- - -). The direction and magnitude of these effects likely differ for antagonistic and mutualistic soil fungi. Panel B: Pathways supported by the observed fungicide effects at the krummholz site in 2009. Fungicide reduced (E) AMF colonization, but increased (F) nectar sugar content, (G) the likelihood of ant damage and (H) seed set. Fungicide also increased (I) seed set independent of plant size, pollen receipt, or ant damage.

in floral signals and rewards increases pollination success, but at a resource cost, setting the stage for both positive and negative fungal effects on these floral traits (Pyke, 1991; Obeso, 2002). Larcenists, animals that consume nectar without pollinating the flower, are common floral antagonists that often co-opt floral traits intended to attract pollinators (Galen, 1999; Irwin et al., 2004). Some larcenists indirectly affect plant fitness by altering the behavior of legitimate pollinators (Irwin and Brody, 1998). Others have little effect on pollinator behavior, but reduce plant fitness by damaging reproductive organs (Galen, 1983). Consequently, soil fungal effects on pollinator signals and rewards may also impact larcenist visitation, an ecological cost of pollination mutualisms (Fig. 1C).

Finally, soil fungi could impact plant reproduction by directly altering resource allocation to pollen and seeds (Fig. 1D). In some studies, mycorrhizal plants allocate more nutrients to pollen and seeds, increasing male and female fitness as well as offspring vigor (Lewis and Koide, 1990; Nuortila et al., 2004). Through these mechanisms, soil fungi could reduce pollinator or larcenist impacts on plant fitness and trait evolution.

The mechanisms just considered (Fig. 1) focus on ecological pathways linking soil fungi to floral evolution via their effects on the behavioral choices of selective agents (i.e., pollinators

and larcenists) or the fitness consequences of those choices (Herrera et al., 2002; Bronstein et al., 2006). Soil fungi could also influence floral evolution by affecting the amount of environmentally induced variation in a given trait. If variation in floral traits is causally linked to fungal colonization, then soil fungi could alter the range of floral phenotypes and the opportunity for animal-mediated selection. Moreover, inequalities in trait sensitivity to fungal inputs could restrict correlated responses to selection from aboveground mutualists or antagonists.

In this study, we examined linkages between above- and belowground interactions and their potential to influence fitness and floral evolution in *Polemonium viscosum*. Specifically, we used a fungicide to reduce fungal colonization in two subpopulations of *P. viscosum* on Pennsylvania Mountain (Park County, Colorado, USA). In doing so, we evaluated the effects of soil fungi on (1) floral signals and rewards, (2) pollination, (3) damage inflicted by floral larcenists, (4) seed set, and (5) plant size. To more directly assess the effects of AMF on *P. viscosum*, we evaluated the relationship between AMF colonization and phenotypic variation in floral traits. Our results suggest that soil fungi impact ecological costs of pollination mutualisms and that spatial and temporal variation in multispecies effects may restrict the evolution of specialized interaction traits.

MATERIALS AND METHODS

Study system and experimental design—*Polemonium viscosum* Nutt. (Polemoniaceae) is a long-lived, perennial wildflower that grows above timberline in the Rocky Mountains (Galen and Kevan, 1980). Individuals flower intermittently and produce 6–20 flowers per reproductive episode. Volatiles emitted by calyx trichomes generate variation in *P. viscosum* floral fragrance, and both sweet and skunky floral scent morphs occur in the Rocky Mountains (Galen and Kevan, 1980). We did not discriminate between the two morphs in this experiment although ~70% of the experimental plants were of the sweet morph.

Polemonium viscosum is self-incompatible, and a single species of alpine bumblebee (*Bombus balteatus*) accounts for 80–90% of seed production by *P. viscosum* at high elevation tundra sites (Galen and Kevan, 1980; Galen, 1996; Galen and Geib, 2007). Solitary bees, syrphid flies, and anthomyiid flies are common co-pollinators, particularly at lower elevations in krummholz habitat near treeline. Larcenist ants (*Formica neorufibarbis*) also visit *P. viscosum* flowers to forage for nectar, often disrupting female fitness by severing the style from the ovary (Galen, 1983). Ants also reduce pollen fertility, with potential costs to male fitness (Galen and Butchart, 2003). Ant abundance decreases, while bumblebee abundance increases with elevation above treeline (Galen and Kevan, 1980; Galen, 1983). Flower size, shape, scent, and nectar rewards influence visitation by bumblebees and ants; both visitors prefer larger, sweet-scented flowers and are known to track nectar rewards in *P. viscosum* (Galen, 1983; Galen and Newport, 1987; Galen et al., 1987; Cresswell and Galen, 1991; Galen, 1999).

This study was conducted from June through August 2008 and 2009 in subpopulations of *P. viscosum* located in the krummholz (3500 m a.s.l.) and ~1.5 km away in the tundra (3700 m a.s.l.) on Pennsylvania Mountain. A diverse community of soil fungi, including several species of arbuscular mycorrhizal fungi (AMF), colonizes *P. viscosum* plants at this location (Becklin, 2010). Historically, the krummholz subpopulation experiences more drought stress than the tundra subpopulation due to its earlier flowering schedule during the dry month of June (Galen et al., 1999). Subpopulations also differ in floral traits including flower size, which is greater in the tundra subpopulation, and the prevalence of the skunky scent, which is more common in the krummholz subpopulation (Galen and Kevan, 1980; Galen, 1996). At both sites, we haphazardly selected 15 replicate patches (3 m radius), each containing six *P. viscosum* plants with green, unopened flower buds. Since most plants from 2008 did not flower the following year, we selected six new flowering plants per patch in 2009. Within each patch, plants were randomly assigned to fungicide (F) and control (C) treatments. Because some buds aborted or were grazed during the experiment, the sample size was reduced to 12–15 plants per treatment at each site and year. Every 3 weeks during the growing season, we applied a 40% w/w aqueous iprodione solution at a rate of 2 g/m² (United Phosphorus, King of Prussia, Pennsylvania, USA) to the fungicide-treated plants and an equivalent amount of water to the control plants. Individual plants were at least 0.5 m away from each other, and treatments were applied directly to the root zone of individuals, so it is unlikely that the fungicide inadvertently affected control plants.

To ascertain the efficacy of the fungicide treatment, we collected root samples from two nonexperimental plants per patch in 2008. In 2009, we sampled roots from the plants used to evaluate floral signals (see below). Roots were cleared in 10% KOH, acidified in 0.1 N HCl, and stained in 0.05% trypan blue (Phillips and Hayman, 1970). AMF colonization was measured using the magnified intersections method at 400× magnification (McGonigle et al., 1990).

General fungicides can affect the abundance or composition of other soil organisms, including pathogenic fungi (Newsham et al., 1994). Consequently, experimental results based on a fungicide treatment may reflect the effects of both mycorrhizal and pathogenic fungi. We did not assess colonization by non-mycorrhizal fungi in this study, so it is unclear whether the fungicide treatment impacted plant–pathogen interactions. Fungicide treatments may also alter the composition of soil fungi since not all fungi are equally susceptible to fungicides (Helgason et al., 2007). Given the importance of fungal identity, particularly in mycorrhizal associations (Helgason et al., 2002; Sikes et al., 2009), fungicide-induced changes in fungal composition could generate significant effects on plant traits even if net colonization changes very little. Despite the limitations, we argue that at present fungicides represent the most feasible way to manipulate fungal colonization in remote field locations while minimizing overall disturbance. This approach also allows for comparisons with previous studies that used fungicides to manipulate mycorrhizal fungi in the field (e.g., Gange et al., 1990; Carey et al., 1992; Gange and Smith, 2005; Cahill et al., 2008).

Soil cores (2 × 15 cm) were collected from the center of each replicate patch in 2010 to evaluate soil conditions at the krummholz and tundra sites. Slow mineralization rates in alpine ecosystems likely limit the amount of interannual variation in soil nutrient content (Körner, 2003); thus, we used these samples to

compare nutrient availability between sites. Samples were analyzed for pH, cation exchange capacity, percentage organic matter, percentage total Kjeldahl nitrogen, Bray I phosphorus concentration, calcium concentration, magnesium concentration, and potassium concentration (Soil and Plant Testing Laboratory, University of Missouri, Columbia, Missouri, USA).

Floral signals and rewards—In each patch, we randomly selected two plants to assess fungicide effects on floral signals and pollinator rewards. For signaling traits, we recorded flowering duration, scape height, flower number, flower shape, flower size, fragrance intensity (emission rate), and fragrance diversity (number of volatile organic compounds, VOCs). For rewards, we measured nectar sugar content, which is the primary reward for bumblebee pollinators, and pollen production, which is an additional reward for co-pollinators (Galen and Kevan, 1980). With the exception of flowering duration and pollen production (estimated from predehiscent anthers of early, male phase flowers), all traits were measured when the plants were in full bloom. Scape height, flower shape, and fragrance traits were measured in 2009 only; all other traits were recorded in both years.

Since floral traits are highly repeatable among flowers in *P. viscosum* (Galen, 1996) flower shape, flower size, and nectar sugar content were measured for one fully expanded flower per plant. Flower shape was calculated as the ratio of corolla width to length. Flower size (corolla surface area) was determined from a pressed flower using a CID 202 leaf area meter (CID Bio-Science, Camas, Washington, USA). Floral fragrances were collected under clear skies and at least 24 h after nectar collections (see below) to avoid contamination with wound volatiles. During that 24 h period, plants were covered with pollination bags to exclude pollinators. We used a dynamic headspace method to collect floral fragrances, standardizing all aspects of our protocol to remove nonbiological sources of variance (Raguso and Pellmyr, 1998). Headspace chambers were created from oven bags (Reynolds, Richmond, Virginia, USA), cut and resealed to 8 × 12 cm (Raguso et al., 2006). Bags were placed over each inflorescence, and clean air was pulled over the flowers into absorbent traps consisting of glass Pasteur pipettes packed with 10 mg of SuperQ absorbent 80–100 mesh (W. R. Grace, Columbia, Maryland, USA) between plugs of quartz wool at a flow rate of 250 mL/min for 90 min using PAS-500 portable battery-operated vacuum pumps (Spectrex, Redwood City, CA, USA). Traps were transported on ice to the laboratory where they were eluted with 300 µL of GC-MS grade hexane (Burdick & Jackson GC2). The eluate was stored at –20°C in Teflon-capped borosilicate glass vials until analyzed at Cornell University (Ithaca, New York, USA). Before GC-MS analysis, we used a brief flow of gaseous N₂ to concentrate samples to 50 mL, then added 5 mL of 0.03% toluene (23.6 ng) as an internal standard. Aliquots (1 mL) of each sample were injected into a Shimadzu GC-17A equipped with a Shimadzu QP5000 quadrupole electron impact MS detector (Shimadzu Corp., Columbia, MD, USA). All GC-MS analyses were done using split-less injections on a polar GC column (diameter 0.25 mm, 1.30 m, film thickness 0.25 mm [EC WAX]; W. R. Grace). GC-MS operating conditions and temperature programs were as described by Raguso et al. (2003). The threshold for peak detection was 100 pg of methyl stearate on single ion mode. Peak areas of total ion chromatograms were integrated using Shimadzu's GC-MS Solutions software, then divided by the peak area of the internal standard to normalize slight variation in final sample volume. Fragrance intensity (total VOC emission rate in toluene equivalents; Raguso et al., 2006) was measured as nanograms per inflorescence per hour because the relationship between emission rate and the number of open flowers was not significant ($R^2 = 0.01$, $P = 0.41$). Fragrance diversity was measured as the total number of floral VOCs.

To measure nectar sugar content, we excluded pollinators from the flowers for 24 h then excised one fully expanded flower and rinsed out the nectar with distilled water. Nectar samples were frozen for transfer to the laboratory where we assayed for total sucrose equivalents using colorimetric analysis (anthrone test; Cresswell and Galen, 1991). Pollen production was determined by collecting one anther per plant prior to anther dehiscence. Anther pollen was suspended in a 1% saline solution. We then counted the number of pollen grains produced per anther using an Elzone 280PC particle counter (Micromeritics Instrument, Norcross, Georgia, USA) with a 500 µL volumetric tube and a 95 µm diameter orifice. Minimum and maximum particle detection range was set to 25–55 µm.

Costs and benefits of pollinator attraction—In each patch, two plants were randomly selected to assess fungicide effects on the costs and benefits of pollinator attraction. In *P. viscosum*, self-incompatibility occurs during pollen germination; thus, we counted the number of germinated pollen grains per stigma as a measure of pollinator benefits (pollen receipt; Galen and Geib, 2007). To evaluate pollen receipt, we collected the pistil from a haphazardly selected

flower on each plant after the corolla wilted and preserved it in a 3:1 solution of glacial acetic acid and ethanol. In the laboratory, we cleared the pistils with 8 mol/L NaOH before staining them with aniline blue. Using a Leica MZFLIII stereomicroscope (Leica Microsystems, Bannockburn, Illinois, USA), we counted the number of germinated pollen grains on each stigma. Since ant visitors detach the style from the ovary during foraging (Galen, 1983), missing styles were used as a measure of ant damage (cost of pollinator attraction). Every 2–3 d during flowering, we examined each plant for cumulative ant damage.

Reproductive fitness—To evaluate whether the fungicide treatment affected plant fitness by altering pollinator visitation or resource availability, we compared seed set of open-pollinated and hand-pollinated plants in fungicide and control treatments. Plants used to measure pollen receipt (see above) were open to insect pollination, and two additional plants per patch (one per treatment) were randomly selected for hand pollination. Donor anthers from *P. viscosum* plants outside the experimental arrays were brushed against each stigma on the hand-pollinated plants until they were visibly coated with pollen. Hand pollinations were repeated daily until the flowers wilted. We collected seeds from open- and hand-pollinated plants in late August.

Plant size—To ascertain whether the fungicide treatment affected overall plant size, we counted the number of leaves and measured maximum leaf length of hand-pollinated plants twice during the growing season (peak flowering and seed set). Total leaf length was calculated as the product of leaf number and maximum leaf length.

Statistical analyses—All statistical analyses were conducted using the program JMP 8.0 (SAS Institute, Cary, North Carolina, USA). To improve normality and homoscedasticity, AMF colonization rate was arcsine-square root transformed; count data and fragrance measurements were square root transformed; flower size, flower shape, nectar sugar content, and total leaf length were log transformed. Soil characteristics (pH, cation exchange capacity, percentage organic matter, percentage total Kjeldahl nitrogen, Bray I phosphorus concentration, calcium concentration, magnesium concentration, potassium concentration) were compared between sites using multivariate analysis of variance (MANOVA) with site as a fixed factor. Since the MANOVA for soil characteristics showed a significant overall difference between sites (see *Results: Soil characteristics*), we analyzed each soil component separately using analysis of variance (ANOVA) with site as a fixed factor. Treatment efficacy was evaluated using ANOVA with AMF colonization as the dependent variable; year, site, treatment, and their interactions as fixed factors; and patch as a random factor. Since treatment efficacy differed between years (see *Results: AMF colonization*), we conducted all subsequent analyses separately for 2008 and 2009. Treatment effects on floral signals (flowering duration, scape height, flower size, flower shape, flower number, total VOC emission, total VOC diversity) and pollinator rewards (nectar sugar content, pollen production per anther) were analyzed using separate MANOVAs with site, fungicide treatment, and their interaction as fixed factors. Since the MANOVA for pollinator rewards showed significant differences (see *Results: Floral signals and rewards*), we analyzed nectar sugar content and pollen production separately using ANOVA with site, fungicide treatment, and their interaction as fixed factors. For the 2009 data only, we analyzed correlations between AMF colonization and floral traits using multiple regression. As above, separate analyses were conducted for floral signals (flowering duration, scape height, flower size, flower shape, flower number, total VOC emission, total VOC diversity) and pollinator rewards (nectar sugar content, pollen production per anther). Control and fungicide treatments were pooled in the multiple regression models to provide the greatest range of colonization. Colonization rates were significantly different for krummholz and tundra plants, so separate regression models were conducted for each site. Since the multiple regression model for floral signals at the tundra site showed a significant overall correlation (see *Results: Floral signals and rewards*), we examined trait-specific correlations between AMF colonization and each floral signal using linear regression. We tested whether fungicide affected the likelihood of ant damage (cost of pollinator attraction) using ordinal logistic regression with site, fungicide treatment, and their interaction as fixed factors. Pollen receipt per pistil (benefit of pollinator attraction) was analyzed using ANOVA with site, fungicide treatment, and their interaction as fixed factors. Seed set (seeds per flower) by hand-pollinated and open-pollinated plants was analyzed using ANOVA with site, fungicide treatment, pollination treatment, and their interactions as fixed factors. For the krummholz site where fungicide significantly impacted seed set and ant damage (see *Results: Reproductive fitness and Costs and benefits of pollinator attraction*), we tested the relationship between seeds per flower and the number of

damaged flowers per plant using analysis of covariance (ANCOVA) with fungicide treatment as a fixed factor. Finally, we tested whether fungicide affected overall plant size by subjecting total leaf length of hand-pollinated plants to repeated-measures ANOVA with site, fungicide treatment, and their interaction as fixed factors. Posthoc power analyses (conducted at $\alpha = 0.05$ using G*Power; Faul et al., 2007) were used to assess whether nonsignificant treatment effects were attributable to low sample size (N) or rather reflect negligible fungicide impacts (effect size, f^2) on floral traits and reproduction.

RESULTS

Soil characteristics—Overall soil characteristics differed between sites (MANOVA: $F = 25.09$, $df = 8$, $P < 0.0001$). Soil pH, percentage total Kjeldahl nitrogen, calcium concentration, and magnesium concentration were significantly higher at the tundra site, while potassium concentration was significantly higher at the krummholz site (P -values listed in Appendix S1, see Supplemental Data with the online version of this article). Bray I phosphorus concentration, cation exchange capacity, and percentage organic matter did not differ between sites (P -values listed in Appendix S1).

AMF colonization—AMF colonization varied spatially and temporally; specifically, colonization was significantly higher in 2009 compared to 2008 ($F_{\text{year}} = 41.56$, $df = 1$, $P < 0.0001$), and in tundra plants compared to krummholz plants ($F_{\text{site}} = 42.69$, $df = 1$, $P < 0.0001$). The fungicide treatment reduced AMF colonization in both years of the experiment (Table 1); however, this effect was significantly greater in 2008 than in 2009 ($F_{\text{year} \times \text{fungicide}} = 53.61$, $df = 1$, $P < 0.0001$). AMF colonization was reduced in fungicide-treated plants by an average of 68% in 2008 and 12% in 2009.

Floral signals and rewards—Treatment effects on floral signals were relatively weak, while effects on rewards were trait-specific and context-dependent. Neither fungicide nor the site by fungicide interaction affected floral signals in 2008 (MANOVA: $P = 0.26$ and 0.36 ; Appendix S2, see online Supplemental Data) or 2009 (MANOVA: $P = 0.09$ and 0.40 ; Appendix S2). Excluding the traits measured only in 2009 (i.e., flower shape, scape height, total VOC emission, and total VOC diversity) did not alter the results for that year. Power analyses indicate that we had insufficient statistical power to detect a treatment effect on floral signals in either year (power = 0.35 and 0.70 for 2008 and 2009, respectively). The treatment effect size was substantially larger in 2009 than in 2008 ($f^2 = 0.35$ and 0.08 , respectively). Low statistical power in 2009 may be due to insufficient sampling effort (increasing the total sample size by 10 individuals would have increased our statistical power to 0.8).

In 2009 the fungicide treatment significantly affected pollinator rewards, but only at the krummholz site (MANOVA:

TABLE 1. Mean (SE) proportion of root length colonized by arbuscular mycorrhizal fungi in control and fungicide treatments in 2008 and 2009.

| Year | Site | Control | Fungicide | % Reduction |
|------|-----------|-------------|-------------|-------------|
| 2008 | Krummholz | 0.86 (0.01) | 0.20 (0.03) | 76.7 |
| | Tundra | 0.86 (0.03) | 0.35 (0.06) | 59.3 |
| 2009 | Krummholz | 0.65 (0.06) | 0.57 (0.06) | 12.3 |
| | Tundra | 0.96 (0.01) | 0.85 (0.02) | 11.5 |

Note: % Reduction = $100 \times (\text{Control} - \text{Fungicide}) / \text{Control}$

$F_{\text{site} \times \text{fungicide}} = 3.42, df = 2, P = 0.04$; Appendix S2). At that site, fungicide affected nectar sugar content, which was significantly higher in fungicide-treated plants than controls (ANOVA: $F = 7.51, df = 1, P = 0.01$, Fig. 2). In contrast, pollen production in the krummholz habitat was unaffected by treatment (ANOVA: $F = 1.38, df = 1, P = 0.25$). Neither fungicide nor the site by fungicide interaction affected pollinator rewards in 2008 (MANOVA: $P = 0.87$ and 0.10 ; Appendix S2). Power analyses indicate that we had adequate statistical power to detect a site by fungicide interaction effect on pollinator rewards in both years of the experiment (power = 0.83 and 0.94 for 2008 and 2009, respectively).

Although the fungicide treatment did not affect floral signals in 2009, chemical signal traits were significantly correlated with AMF colonization at the tundra site (multiple regression: $R^2 = 0.31, P = 0.008$). At that site, total VOC emission and diversity decreased with AMF colonization (linear regression: $R^2 = 0.29, P = 0.006$, Fig. 3A; and $R^2 = 0.28, P = 0.007$, Fig. 3B). Floral signals were not correlated with AMF colonization at the krummholz site (multiple regression: $R^2 = 0.01, P = 0.74$, power = 0.06). Pollinator rewards were not correlated with AMF colonization at either site (multiple regression: krummholz, $R^2 = 0.04, P = 0.58$, power = 0.19; and tundra, $R^2 = 0.13, P = 0.19$, power = 0.48).

Costs and benefits of pollinator attraction—Treatment effects differed for antagonistic and mutualistic aboveground interactions. *Polemonium viscosum* plants were more likely to be damaged by ants in 2009 than 2008 ($\chi^2 = 11.85, df = 1, P = 0.001$, Fig. 4). The likelihood of ant damage in 2008 was independent of the fungicide treatment ($\chi^2 = 1.41, df = 1, P = 0.23$). In 2009, fungicide-treated plants experienced more ant damage than control plants in the krummholz but not in the tundra ($\chi^2 = 3.89, df = 1, P = 0.05$ and $\chi^2 = 0.69, df = 1, P = 0.41$, Fig. 4). Treatment did not affect pollen receipt in either year (2008: $F = 0.3, df = 1, P = 0.59$, power = 0.08; and 2009: $F = 0.61, df = 1, P = 0.44$, power = 0.13). Low statistical power was likely due to the extremely small effect size for pollen receipt ($f^2 = 0.01$ for both years).

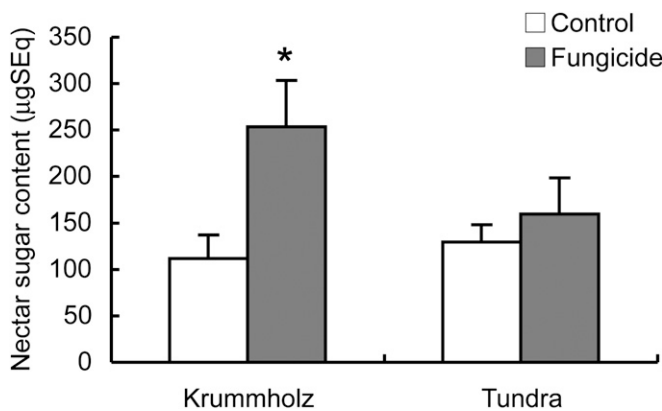


Fig. 2. Nectar sugar content (μg of sucrose equivalents, μgSEq) for fungicide-treated (solid bars) and control (open bars) plants at the krummholz and tundra sites in 2009. Brackets show standard errors and asterisks indicate sites with a significant treatment effect ($P \leq 0.05$).

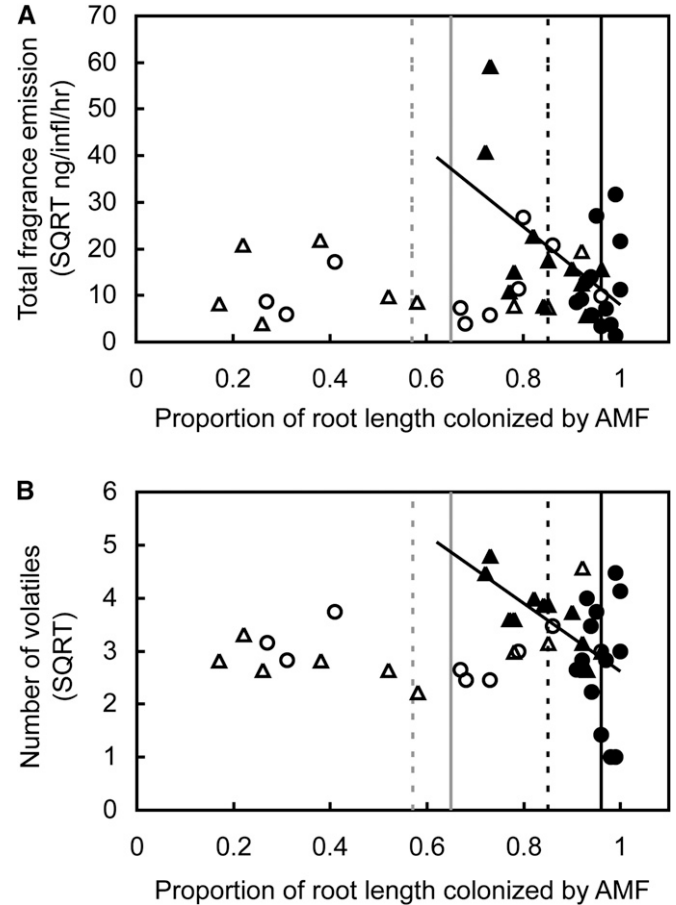


Fig. 3. Relationship between mycorrhizal fungal colonization and (A) total fragrance emission rate and (B) the number of floral volatiles produced by krummholz (open symbols) and tundra (closed symbols) plants. Triangles represent control plants and circles represent fungicide-treated plants. Vertical lines show mean AMF colonization of control (dashed) and fungicide-treated (solid) plants at krummholz (gray) and tundra (black) sites. Negative relationships were significant at the tundra site ($R^2 = 0.29, P = 0.006$ and $R^2 = 0.28, P = 0.007$).

Reproductive fitness—Seed set was likely limited by both pollination and resource availability. In 2009, both fungicide and control plants were significantly pollen-limited ($F_{\text{pollination}} = 8.11, df = 1, P = 0.006$; Appendix S3, see online Supplemental Data), and the fungicide effect on seed set differed between sites ($F_{\text{site} \times \text{fungicide}} = 5.87, df = 1, P = 0.02$; Appendix S3). Interestingly, open-pollinated fungicide-treated plants produced significantly more seeds per flower in the krummholz ($F = 8.32, df = 1, P = 0.005$, Fig. 5). In contrast, fungicide did not affect seed set in the tundra ($F = 1.35, df = 1, P = 0.25$, power = 0.20, Fig. 5). In the krummholz, the relationship between seed set and the number of flowers damaged by ants differed between treatments ($F = 4.30, df = 1, P = 0.05$). Specifically, seed set by control plants decreased with the number of damaged flowers ($R^2 = 0.55, P = 0.01$, Fig. 6) whereas seed set by fungicide-treated plants was not correlated with ant damage ($R^2 = 0.04, P = 0.5, \text{power} = 0.10$, Fig. 6). Fungicide did not affect seed set of open- or hand-pollinated plants in 2008 ($F = 1.62, df = 1, P = 0.21$; Appendix S3). Low statistical power in that year was likely due to a small effect size and not sampling effort ($f^2 = 0.01, \text{power} = 0.23$).

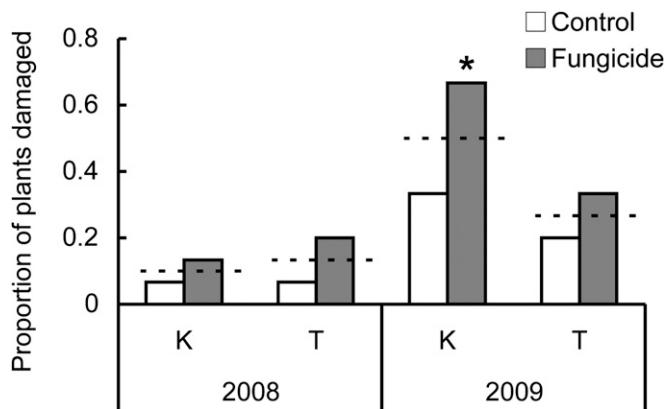


Fig. 4. Proportion of fungicide-treated (solid bars) and control (open bars) plants with flowers damaged by ants at the krummholz (K) and tundra (T) sites in 2008 and 2009. Dashed lines show the expected proportion given random foraging. Asterisks indicate treatments where the observed proportion of damaged plants was significantly greater than expected at random ($P \leq 0.05$).

Plant size—Total leaf length during flowering and seed set did not differ between control and fungicide-treated plants in either year (2008: $F = 2.78$, $df = 1$, $P = 0.10$, power = 0.07; and 2009: $F = 0.01$, $df = 1$, $P = 0.97$, power = 0.05; Appendix S4, see online Supplemental Data). Thus, it is unlikely that the observed fungicide effects on pollinator rewards and seed set were due to differences in plant size. Low statistical power was likely due to the small effect size for this trait ($f^2 = 0.05$ and 0.01 for 2008 and 2009, respectively).

DISCUSSION

This study explored indirect interactions between species in above- and belowground communities and their potential to impact floral evolution in a shared host plant. The observed fungicide effects on floral traits in *P. viscosum* were highly variable among traits, between years, and between subpopulations, indicating that linkages between above- and belowground communities are

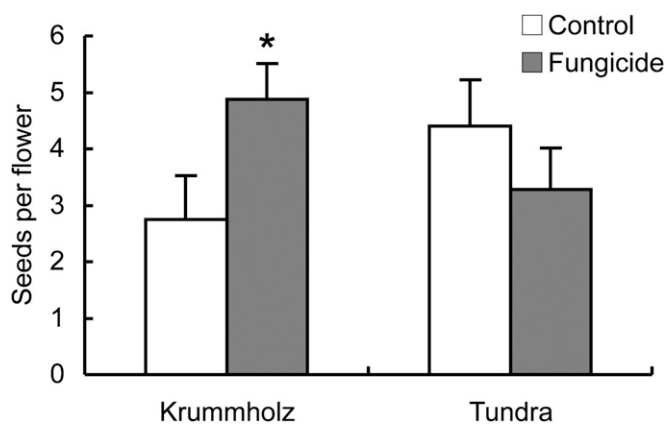


Fig. 5. Mean number of seeds produced per flower by open-pollinated plants at the krummholz and tundra sites in 2009. Solid bars represent fungicide-treated plants and open bars represent control plants. Brackets show standard errors and asterisks indicate sites with a significant fungicide effect ($P \leq 0.05$).

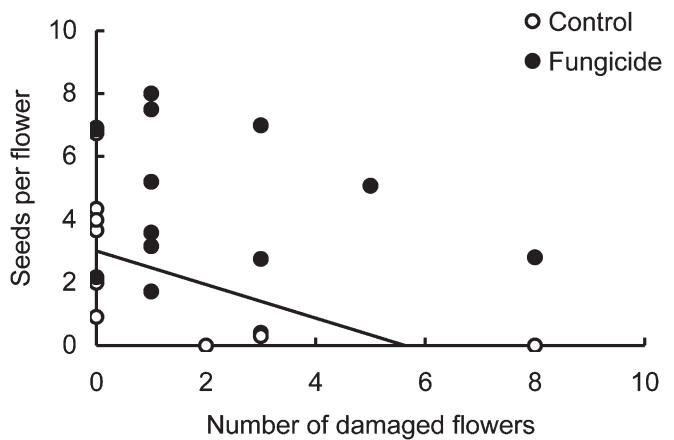


Fig. 6. Relationship between seeds per flower and the number of flowers damaged by ants at the krummholz site in 2009. Open and closed symbols represent control and fungicide-treated plants, respectively. The negative relationship was significant for control plants ($R^2 = 0.55$, $P = 0.01$).

context-dependent and trait-specific in this system. Such dynamic multispecies effects may generate shifting selection pressures that impact the evolution of specialized interaction traits.

Spatial and temporal variation in environmental conditions, nutrient availability, and fungal colonization may have contributed to variation in the observed fungicide effects. At our study site, 2008 was a relatively wet year with greater spring snowpack and summer precipitation compared to 2009 (National Resources Conservation Service, <http://www.co.nrcs.usda.gov>). Since nutrient availability is correlated with snowpack and water availability in alpine ecosystems (Bowman, 1992; Bowman et al., 1993), decreased precipitation may have enhanced resource limitation in 2009, potentially strengthening mycorrhizal associations in that year. Differences in soil fertility and fungal colonization also may have contributed to spatial variation in belowground effects at the tundra and krummholz sites. In particular, overall soil nutrient content and average mycorrhizal fungal colonization were higher at the tundra site. Furthermore, low soil pH in the krummholz likely limits phosphorus availability, which is a particularly important determinant of mycorrhizal function (Alloush et al., 2001; Johnson, 2010). Small changes in fungal colonization may be more meaningful at nutrient-limited sites and at lower colonization rates.

This study may have underestimated soil fungal effects on plant traits and fitness due to several caveats of our experimental approach. First, soil fungi can differentially affect male and female fitness (Pendleton, 2000; Poulton et al., 2001b), and in some cases fungal costs to the parental generation may be offset by benefits to the offspring (Lewis and Koide, 1990; Nuortila et al., 2004). Future studies should consider the effects of soil fungi on multiple components of plant fitness. Second, *P. viscosum* is a perennial plant and the effects of multispecies interactions on fitness or floral evolution may accumulate over time. Following individuals in perennial populations for multiple years could provide insight into the long-term effects of belowground interactions. Third, the fungicide treatment did not eliminate soil fungi; rather it reduced mycorrhizal fungal colonization by 12–68% (Fig. 1E). Since we did not assess colonization by pathogenic fungi, it is unclear whether the fungicide treatment had a similar effect on those organisms. Given the

low treatment efficacy in 2009, results for that year should be interpreted with some caution. However, significant and ecologically meaningful fungal effects on plant traits and insect visitation were observed in other studies in which fungicide efficacy was low (Gange et al., 1990; Carey et al., 1992; Cahill et al., 2008). Finally, general fungicides may mask some mycorrhizal fungal benefits by also reducing potential negative effects of fungal pathogens (Carey et al., 1992; Newsham et al., 1994).

Floral signals and rewards—Soil fungi impact many traits that function as signals to floral visitors. For example, mycorrhizal fungi increased flower production in a number of host species (Lu and Koide, 1994; Pendleton, 2000; Poulton et al., 2001a; Gange and Smith, 2005), but had no effect on flower production by *Lythrum salicaria* (Philip et al., 2001) and decreased flower production by *Campanula rotundifolia* (Nuortila et al., 2004). Other studies identified mycorrhizal fungal effects on flowering duration, flower size, and inflorescence number or structure, all of which may function as visual signals to insect visitors (Bryla and Koide, 1990; Lu and Koide, 1994; Pendleton, 2000; Gange and Smith, 2005). As with flower number, the effect of mycorrhizal fungi on these floral traits varies among host species (Bryla and Koide, 1990; Gange and Smith, 2005). Thus, although most studies indicate that mycorrhizal fungi positively impact floral signals, the magnitude and direction of these effects are generally context-dependent. In contrast to mycorrhizal fungi, there is surprisingly little information about soil pathogen effects on floral traits other than seed set (Marr and Marshall, 2006). Teasing apart mycorrhizal and pathogenic fungal effects may shed light on the degree to which mutualistic and antagonistic belowground organisms act as selective agents.

In contrast to previous studies, floral signals were largely unaffected by fungicide application in this experiment, indicating that soil fungi have little impact on these traits in *P. viscosum*. Much of the work examining mycorrhizal fungal effects on floral traits has focused on annual or cultivated host species (e.g., Bryla and Koide, 1990; Lu and Koide, 1994). Studies of perennial hosts in natural communities suggest that mycorrhizal fungal effects are subtler in these systems due to complex ecological interactions and physiological tradeoffs that determine plant phenotypes and reproductive success (Gange and Smith, 2005; Cahill et al., 2008). Given the small impact of fungicide on floral signals in this study, increased sampling effort might have improved our ability to detect a significant treatment effect. However, plasticity of several floral traits in *P. viscosum* is likely limited by genetic and developmental factors. For example, flower size is highly heritable in this host, so much of the variation in flower size is genetically based (Galen, 1996). In contrast, flower number is a plastic trait, but *P. viscosum* plants flower determinately with a predetermined bud number. Consequently, both of these traits are likely buffered from belowground effects. Soil fungi may be more likely to impact traits that exhibit greater environmentally based phenotypic variation (e.g., nectar production).

Floral fragrance has largely been overlooked in the context of plant–fungus interactions, although some authors hypothesized a role for soil fungi in fragrance emission based on the relationship between colonization and induced defensive volatiles (Laurie-Berry et al., 2006; Kempel et al., 2010; Varga and Kytoviita, 2010). Our results indicate that spatial and temporal variation in AMF colonization may contribute to phenotypic variability in *P. viscosum* fragrance as both fragrance intensity

and diversity declined with AMF colonization in the tundra habitat. Total VOC emission correlates significantly with emission rates of 2-phenylethanol, a VOC influencing pollinator and larcenist behavior ($R^2 = 0.87$, $P < 0.0001$; Galen et al., 2011), and cinnamic acid-derived compounds potentially shared between above- and belowground signaling pathways ($R^2 = 0.78$, $P < 0.0001$; Herrmann and Weaver, 1999; Ponce et al., 2009). Accordingly, if the relationship between AMF colonization and fragrance properties is causal, mycorrhizal fungi could represent a source of environmentally induced variation in floral fragrance, reducing the efficacy of insect-mediated selection on ecologically important VOCs in *P. viscosum*. Furthermore, different floral volatiles are known to function as attractive or defensive compounds; by affecting fragrance diversity, mycorrhizal fungi may influence their host's pollination niche breadth or susceptibility to floral antagonists (Kessler and Baldwin, 2007; Kessler et al., 2008). Exploring mechanisms linking soil fungi to differences in floral fragrance in *P. viscosum* and other host species may provide insight into the function and evolution of these complex signals.

The quantity and quality of floral rewards is particularly important to pollinator visitation and overall pollination success in many flowering plants (Mitchell, 1994). Mycorrhizal fungi generally increase pollen production, which can function as a reward for some pollinators (Philip et al., 2001; Poulton et al., 2001a; Varga and Kytoviita, 2010). In contrast, mycorrhizal fungal effects on nectar rewards range from positive to negative. Gange and Smith (2005) found that mycorrhizal fungi increase nectar sugar content in *Tagetes erecta*, but not in two other annual plant species. In contrast, Laird and Addicott (2007) found that mycorrhizal fungi decrease production of extrafloral nectaries in *Vicia faba*. Very little work has looked at the effects of fungal pathogens on pollinator rewards; however, Shykoff (1997) found that infection by a floral fungal pathogen did not impact nectar production or concentration in *Silene latifolia*. Our results suggest that soil fungi negatively affect pollinator rewards in *P. viscosum*, but this effect is context-dependent and differs for pollen and nectar rewards (Fig. 1F). The lack of a correlation between pollinator rewards and mycorrhizal fungal colonization suggests that changes in pathogenic fungi or mycorrhizal fungal composition may have contributed to the observed fungicide effect. Changes in nectar production are particularly important in this system since nectar is the primary reward for bumblebee pollinators (Galen and Kevan, 1980; Cresswell and Galen, 1991). Consequently, decreased nectar sugar content in control plants could reduce floral attractiveness and alter bumblebee foraging behavior. Since ants also forage for nectar rewards, decreased nectar sugar content may also discourage ant visitation and function as an indirect defense. Ultimately, our results suggest that spatial mosaics in soil fungal communities have the potential to influence the patchy distribution of nectar, but are unlikely to influence pollen rewards and visitation by pollen collecting insects.

Costs and benefits of pollinator attraction—Relatively few studies have directly examined soil fungal effects on pollinators. Results of these studies indicate that mycorrhizal fungi increase pollinator visitation, presumably by altering traits that function as signals or rewards (Gange and Smith, 2005; Wolfe et al., 2005). In some cases, the relationship between mycorrhizal fungal colonization and visitation depended on pollinator identity. For example, Cahill et al. (2008) found that disrupting mycorrhizal associations decreases visitation by large-bodied

bumblebees, but increases visitation by small-bodied bees and flies. In contrast, Varga and Kytoviita (2010) saw no difference in bumblebee visitation to mycorrhizal and nonmycorrhizal plants, but noted small changes in visitation by syrphid flies and other hymenopterans. Studies examining links between fungal pathogens and floral visitors has focused on floral diseases transmitted by animal pollinators (Shykoff, 1997; Pfunder and Roy, 2000). In contrast, soil pathogens do not rely on floral attractiveness and pollinators for dispersal; thus, soil pathogens are less likely to directly impact plant–pollinator interactions. However, since above- and belowground signaling pathways are linked (Bezemer and van Dam, 2005) selection for resistance to soil pathogens could have unexpected implications for pollination mutualisms. In the present study, we examined compatible pollen receipt as a proxy for the benefit of pollinator visitation. Pollen receipt was similar between treatments, indicating that soil fungi did not alter net pollinator services; however, pollen receipt may not reflect shifts in pollinator identity which could be important in natural communities where plants interact with diverse pollinator guilds (Cahill et al., 2008).

Floral larcenists have been well studied in the context of plant–pollinator interactions (reviewed by Irwin et al., 2001), but not in relation to indirect effects of plant–fungus symbioses. In the present study, fungicide-treated plants experienced more ant damage, but this effect was minimal in 2008 when ant abundance was low. In 2009, fungicide effects on floral larceny were limited to the krummholz site where ants are generally more abundant (Galen, 1983) and there is greater potential for soil fungi to impact ant behavior. We measured the net cost of ant visitation (i.e., floral damage) rather than visitation rate. Since it often takes multiple ant visits to dislodge the pistil from a *P. viscosum* flower (Galen and Geib, 2007), our measure of ant damage likely underestimates the difference in ant visitation to control and fungicide-treated plants. Overall, we hypothesize that, when ants are abundant, soil fungi may indirectly reduce ant visitation (Fig. 1G) by reducing floral rewards (Fig. 1F), which could ultimately impact plant fitness and trait evolution (Fig. 1H). Because ant visitation can reduce male fertility as well as female fitness in *P. viscosum* (Galen and Butchart, 2003), soil fungi could provide indirect benefits through both fitness components.

Reproductive fitness—Soil fungi are reported to impact components of reproductive fitness in numerous studies (Bryla and Koide, 1990; Newsham et al., 1994; Poulton et al., 2001b, 2002; Nuortila et al., 2004; Marr and Marshall, 2006). In most cases, mycorrhizal fungi increased plant fitness while pathogenic fungi decreased plant fitness; however, the magnitude of these effects depends on host identity and how fitness is measured. For example, mycorrhizal fungal effects on fruit and seed production ranged from neutral to positive in eight accessions of wild *Lycopersicon esculentum* (Bryla and Koide, 1990). In contrast, mycorrhizal fungi decreased overall seed set in *Campanula rotundifolia*, but increased offspring vigor in the next generation (Nuortila et al., 2004). Fungal effects on plant fitness are usually ascribed to differences in overall plant size (Lu and Koide, 1994). Yet few studies have attempted to distinguish direct effects via resource allocation from indirect effects via plant–insect interactions (Wolfe et al., 2005). In this study, we evaluated seed set of hand-pollinated and open-pollinated plants to tease apart the direct and indirect mechanisms. Results show that over both sites and years, *P. viscosum* plants were

generally pollen-limited, indicating that indirect effects of soil fungi on pollination could impact seed set. However, direct effects are still possible, since *P. viscosum* plants on Pennsylvania Mountain experience simultaneous pollen and resource limitation of seed set (Galen, 1985).

Our results suggest that for krummholz *P. viscosum*, direct negative effects of soil fungi on seed set (Fig. 1I) outweigh the indirect benefit of reduced floral larceny (Fig. 1G). Moreover, we hypothesize that soil fungi may reduce fecundity by restricting plant tolerance to ant damage. Ant visitation is highly variable within *P. viscosum* plants; consequently, some flowers on each plant typically escape damage (Galen and Geib, 2007). These “surplus” flowers can replace lost reproductive fitness from damage to other flowers as long as the plant has sufficient resources (Irwin et al., 2008). The finding that ant damage reduced seed set in control plants, but not in fungicide-treated plants supports this idea (Fig. 6). Because reduced attractiveness to larcenists benefits both pollen and seeds while tolerance only buffers seed set, costs and benefits of soil fungi in this system are likely not gender neutral. Instead, for *P. viscosum*, soil fungi appear more likely to benefit male fitness at a resource cost to seed set. Future measurements of male and female fitness under experimental manipulation of fungal colonization and ant access are needed to test this hypothesis.

Multispecies interactions and ecological specialization—In multispecies interaction webs, ecological specialization is predicted to evolve when interaction strengths are constant and a key interaction drives selection (Van der Putten et al., 2001). In nature, interaction strengths can be highly dynamic, which could result in shifting selection pressures over space and time. Thus, whether specialized traits and interactions evolve may depend on environmental conditions and the presence of both antagonistic and mutualistic partners. In this study, soil fungi influenced plant traits idiosyncratically, and the response of even sensitive traits varied in magnitude over time. Consequently, we hypothesize that soil fungi may alter selection pressures and slow the evolution of ecologically specialized aboveground traits and relationships. Furthermore, in krummholz *P. viscosum*, pronounced differences among floral traits in sensitivity to belowground effects should restrict correlated responses to pollinator-mediated selection on rewards and attractants. It follows that over time soil fungi could be an important driver of pollination niche breadth in this alpine wildflower. In other systems, specialized pollination mutualisms and mycorrhizal associations influence plant species coexistence and diversification (Waterman et al., 2011). Examining simultaneous above- and belowground effects on specialized traits may help elucidate how dynamic multispecies interaction webs influence ecological and evolutionary processes at a larger scale.

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