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Soil calcium and plant disease in serpentine ecosystems: a test of the pathogen refuge hypothesis

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Abstract Ecologists have long sought mechanistic explanations for the patterns of plant distribution and endemism associated with serpentine soils. We conducted the first empirical test of the serpentine pathogen refuge hypothesis, which posits that the low levels of calcium found in serpentine soils provide associated plants with a refuge from attack by pathogens. We measured the range of soil calcium concentrations experienced by 16 wild population of California dwarf flax (*Hesperolinon californicum*) and experimentally recreated part of this range in the greenhouse by soaking serpentine soils in calcium chloride solutions of varying molarity. When flax plants grown in these soils were inoculated with spores of the rust fungus *Melampsora lini* we found a significant negative relationship between infection rates and soil calcium concentrations. This result refutes the pathogen refuge hypothesis and suggests that serpentine plants, by virtue of their association with low calcium soils, may be highly vulnerable to attack by pathogens. This interaction between plant nutrition and disease may in part explain demographic patterns associated with serpentine plant populations and suggests scenarios for the evolution of life history traits and the distribution of genetic resistance to infection in serpentine plant communities.

Keywords *Hesperolinon californicum* · *Melampsora lini* · Pathogen refuge hypothesis · Serpentine soil · Soil calcium

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Introduction

One of the central goals of ecological research is to understand the relative importance of the abiotic environment and species interactions in determining the distribution and abundance of organisms. While the mechanisms involved are complex and context specific, two general modes of influence have been delineated (Real and Brown 1991). Research predicated on an autecological perspective has stressed the role of organismal physiology, often examining systems in which species are excluded from certain localities because of an inability to tolerate the abiotic conditions present there. Conversely, studies conducted within a synecological framework have emphasized the role of variation in species interactions in determining patterns of distribution and abundance. Natural systems in which both mechanisms interact synergistically, and which therefore offer an excellent opportunity to bridge this dichotomy, are plant communities associated with serpentine soils.

Serpentine soils, found worldwide in areas of seismic activity and tectonic uplift, are characterized by a unique suite of biotically stressful edaphic properties that include coarse rocky texture, low water-holding potential, high concentrations of magnesium, iron,

silica, and various heavy metals (e.g., nickel, chromium), and extreme deficiencies in nutritive elements including calcium, nitrogen, phosphorus, and potassium (Proctor and Woodell 1975; Brooks 1987). Serpentine outcrops have long been of interest to botanists and ecologists because they support distinct floristic assemblages consisting of rare, unique, and highly specialized taxa (Baker et al. 1991; Roberts and Proctor 1992). Many of these species are endemic to serpentine soils and their distributional patterns effectively demarcate the often sharp boundaries of insular serpentine patches. In contrast, most plant species found on adjacent soil types are excluded from serpentine soils. The striking edaphic differences between serpentine and nonserpentine soils, and the marked biological discontinuity in areas where they abut, suggest strong selective pressures imposed on plants by serpentine soils.

Results of transplant and soil manipulation experiments have implicated low calcium availability as a primary factor underlying the floristic partitioning characteristic of serpentine ecotones (Kruckeberg 1954; Whittaker 1954; Kruckeberg 1967). Calcium concentrations in these soils can be up to 17 times lower than levels measured in nonserpentine and agricultural soils (Mehlich and Tewari 1974). Although calcium availability undoubtedly represents a force critical in driving ecological and evolutionary dynamics in serpentine plant communities, the specific mechanism(s) by which calcium exerts its effects remain unresolved. While the hypothesis that a tradeoff between competitive ability and tolerance of serpentine conditions (especially low calcium) has received the most support (Kruckeberg 1954; Proctor and Woodell 1975; Rice 1989; Jurjavcic et al. 2002), few other mechanistic possibilities have been investigated in great detail (Brady et al. 2005). One such alternative is the hypothesis that low calcium serpentine soils may provide a refuge from pathogen attack (Kruckeberg 1992). This "pathogen refuge" hypothesis posits that under conditions of low calcium availability characteristic of serpentine soils, plants should experience reduced pathogen pressure either because infection rates are lower or because symptom-associated damage is reduced. Greater disease on higher calcium, nonserpentine soils may impede the colonization of these soils by serpentine plants, and thereby contribute to the pattern of serpentine endemism. The hypothesis has never been explicitly tested and is difficult to address anecdotally because little is known about the diseases of serpentine plants. The handful of studies that have considered plant pathogens in serpentine ecosystems have all focused on hyperaccumulators, plants that have unusually high tissue concentrations of certain selectively absorbed heavy metals (e.g., nickel, selenium). While a few studies have documented reduced rates of infection in hyperaccumulators (Boyd et al. 1994; Davis et al. 2001; Hanson et al. 2003), this defensive strategy is taxonomically rare and characteristic of only a fraction of serpentine-associated plants (Brooks 1998). Exposure to low soil calcium concentrations is a condition experienced by all serpentine flora, so effects of calcium availability on interactions with pathogens should be pervasive among the nonhyperaccumulating members of these plant communities.

While predictions of the pathogen refuge hypothesis are consistent with patterns of floristic endemism observed in serpentine systems, they run counter to results of research on calcium-mediated plant disease development in agricultural systems. Calcium has been shown to play a key role in the pathways used by plants to recognize and respond to abiotic environmental stressors (Reddy 2001; Sanders et al. 2002) and to attack by plant pathogens (Lamb et al. 1989; Blumwald et al. 1998; Grant and Mansfield 1999). Activation of calcium-binding sensor molecules often appears necessary to initiate defensive responses of plants (Scheel 1998), and the application of calcium to soils has been shown to exert a suppressive effect on a wide range of agricultural plant pathogens including species in the genera *Aspergillus*, *Erwinia*, *Fusarium*, *Plasmodiophora*, *Pseudomonas*, *Pythium*, *Rhizoctonia*, *Sclerotium*, and *Verticillium* [reviewed in Engelhard (1989)]. Contrary to the pathogen refuge hypothesis, these findings suggest that calcium deficiency should impair a plant's ability to defend against attacking pathogens, making serpentine flora more vulnerable to infection.

The goal of this study was to investigate these conflicting predictions through an experimental test of the pathogen refuge hypothesis. We conducted a greenhouse experiment in which the calcium concentration of field-collected serpentine soil was augmented via the application of calcium chloride solutions of varying molarity. Using this technique we recreated part of the range of soil calcium concentrations associated with natural populations of California dwarf flax, *Hesperolinon californicum*. By inoculating flax plants grown in these experimental soils with spores of *Melampsora lini*, a pathogenic fungus that infects *H. californicum* in the wild, we investigated the relationship between soil calcium concentrations and rates of infection by a pathogen and thereby assessed the potential for a pathogen refuge effect to contribute to floristic partitioning in serpentine systems.

Materials and methods

Host and pathogen

California dwarf flax, *H. californicum* Small (Linaceae), is a diminutive annual, generally 20–40 cm tall at flowering, with thin stems and leaves (Sharsmith 1961). It is endemic to California, growing primarily in the Coast Range Mountains but also occurring in scattered populations on the margins of the eastern Sacramento and western San Joaquin valleys. *H. californicum* is considered a serpentine generalist, or bodenvag taxon as it grows on soils that vary widely in their degree of serpentine influence and is sometimes found on non-serpentine soils (Kruckeberg 1954; Sharsmith 1961). It is unlikely that *H. californicum* is a hyperaccumulator given that metal hyperaccumulation has never been reported in the Linaceae (A. J. M. Baker, personal communications). *Melampsora lini* Persoon is a macrocyclic, wind dispersed, autecious rust fungus (Uredinales) that forms urediospores in pustules on the stems and leaves of infected plants (Flor 1954). It is specific to hosts in the family Linaceae, and while infection of 12 of the 13 species of *Hesperolinon* has been observed in the field (Y. P. Springer, personal observations), it is unclear whether species-specific strains of *M. lini* have evolved. As with most rusts, infections are nonsystemic and result in loss of plant vigor or death via destruction of photosynthetic tissue and increased desiccation through damaged cuticle surfaces (Littlefield 1981). *M. lini* infection has been shown to significantly decrease seedling survivorship and adult fecundity in *H. californicum* (Y. P. Springer, in preparation).

Quantifying soil calcium concentrations in natural *H. californicum* populations

We determined the biogeographic range of *H. californicum* using a monograph on the genus *Hesperolinon* (Sharsmith 1961), herbarium records at the University of California Berkeley Jepson Herbarium (http://www.calflora.org), and interviews with local naturalists and plant ecologists. We selected 16 study populations that collectively span the latitudinal extent of this range as completely as possible (Fig. 1). Populations are separated from each other by at least 1 km and range in size from approximately 1,000 to more than 20,000 individuals. We collected soil samples from each of the 16 study populations in 2002 to characterize the natural range of soil calcium concentrations experienced by *H. californicum*. Depending on the spatial area of each population, we collected between six and 18 soil samples per population. Each sample consisted of four bulked scoops (~250 ml each) from the upper 10 cm of soil taken from each of the corners of a 0.25-m^2 quadrat. We placed quadrats haphazardly within populations, attempting to sample the spatial extent of local plant distributions as evenly as possible. In the laboratory, soil samples were oven dried at 60°C for 48 h and sieved using a 2-mm screen. After large organic particles (leaves, twigs, seeds) had been removed using forceps, we performed two liquid extractions using protocols adapted from those used by the ANR Analytical Laboratory (University of California Davis, Davis, Calif.). Diethylene triamine pentaacetate (DTPA) extractions (10 g soil + 20 ml of DTPA solution: 1 l = 1.96 g DTPA, 14.92 g TEA, 1.47 g calcium chloride, Millipure water, pH = 7.3) were used to quantify soil concentrations of aluminum, barium, cadmium, cobalt, chromium, copper, iron, manganese, nickel, lead, silicon, strontium, and zinc. Ammonium acetate (AA) extractions (2 g soil + 20 ml AA solution: 1 l = 57 ml glacial acetic acid, 68 ml ammonium hydroxide, millipure water, pH = 7.0) were used to quantify soil concentrations of calcium, magnesium, potassium, and sodium. Extraction mixtures were sealed in individual 50-ml Nalgene Oak Ridge polypropylene copolymer tubes, placed on an orbital shaker (260 r.p.m. at room temperature) for 2 h (DTPA) or 30 min (AA), briefly centrifuged, and the supernatant filtered by gravity into clean 15-ml Falcon tubes using Whatman 2 V qualitative filter paper. Filtered solutions were analyzed on a Perkin Elmer Optima 4300 DV internally coupled plasma optical emissions spectrometer using 10-p.p.m. scandium and yttrium internal standards to control for instrument drift. The spectrometer calculated elemental concentrations (mg kg^{-1} dry soil) of the focal analytes and standards in each sample by averaging measurements taken in five sequential subsamples. A sample was rerun if the SD of these subsamples exceeded 2.8% of the mean for either of the internal standards.

Collection of materials

We collected serpentine soils, flax seeds, and rust spores used in the experiment between April 2003 and August 2003. Soil samples were collected from the *H. californicum* populations in the southern (soil source 1), central (soil source 2), and northern (soil source 3) regions of the species' biogeographic range at those sites where the lowest soil calcium concentrations had been recorded (Fig. 1). Regional range divisions were based on natural discontinuities in the host species' distribution. Soil was collected to a depth of 10 cm within *H. californicum* patches, sieved to obtain

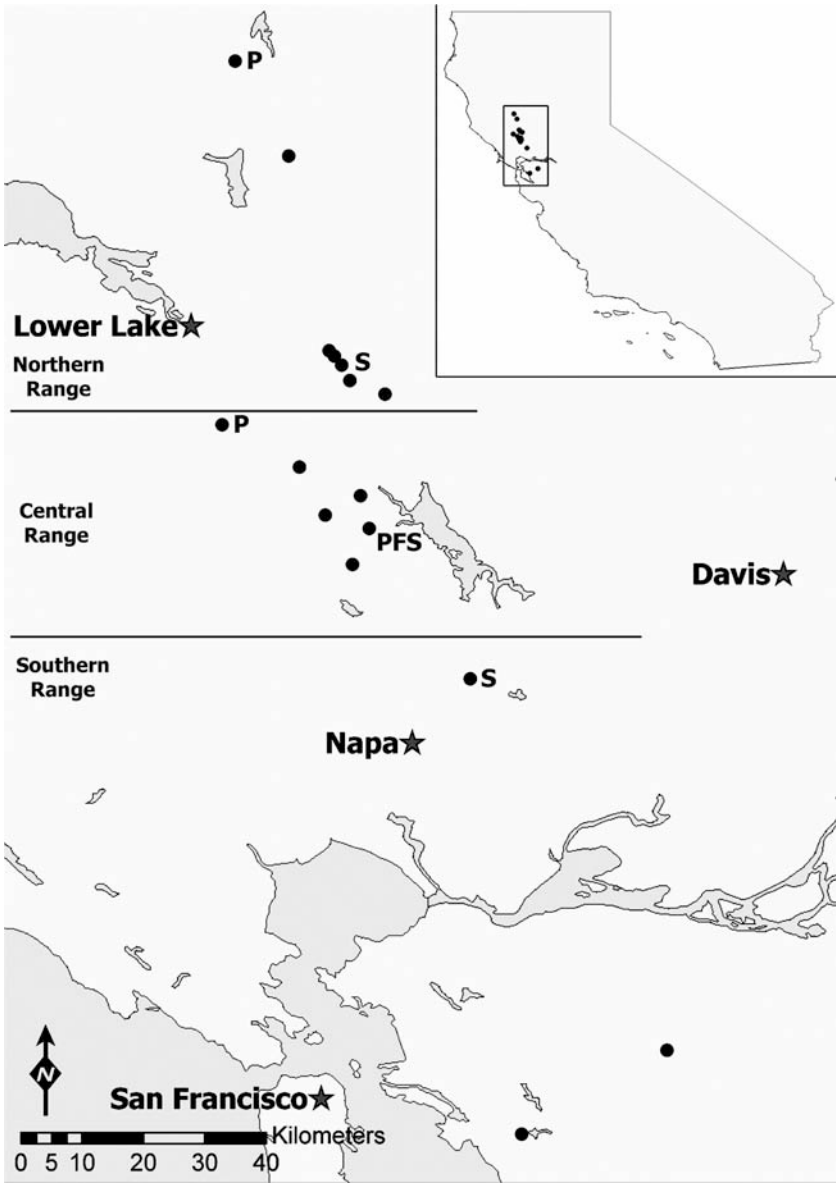

Fig. 1 Distribution of *Hesperolinon californicum* study populations (*filled circles*). Locations of plant (*P*), fungal (*F*), and soil (*S*) collection are indicated *symbolically*

the 2-mm fraction, and gamma irradiated (40.4–60.7 kGy dose intensity) by Sterigenics (Hayward, Calif.) to kill bacteria, fungi, and seeds. We removed large organic particles using forceps and prepared a 1:1 volumetric mix of serpentine soil and horticultural grade Perlite (Therm-O-Rock West, Chandler, Ariz.) for each of the three soil sources. We used these mixes to fill 66-ml pine cell Cone-tainers (Stuewe and Sons, Corvallis, Ore.) into which a synthetic cotton puff had been placed to prevent soil loss out of drainage holes. We collected *H. californicum* seeds from eight maternal lines in each of three populations, and harvested *M. lini* spores from multiple *H. californicum* plants at one of these sites (Fig. 1). In the laboratory, spores were amplified on greenhouse-grown *H. californicum* plants from the source population, collected using a spore vacuum (G-R Manufacturing, Manhattan, Kan.), lyophilized for 6 h (25 millitor), and stored at −80°C.

Greenhouse experiment

We conducted an inoculation experiment in the research greenhouses at the University of California Santa Cruz between April 2004 and July 2004. The experimental design consisted of 18 treatments including all combinations of three serpentine soils sources and six calcium chloride treatment solutions. Using the results of a preliminary treatment-level determination experiment (Y. P. Springer, unpublished data), we selected six calcium chloride treatment solution concentrations that produced soil calcium levels spanning the natural range experienced by *H. californicum* without

exceeding concentrations that were too osmotically stressful: 0.00 (DI water control), 0.68, 1.36, 3.40, 6.12, and 10.90 mM. Results from this preliminary experiment indicated that irrigation with calcium chloride solutions was associated with significant increases in soil strontium concentrations (r^2adj = 0.72, MS = 0.045, $F_{1,14} = 37.46$, $P < 0.0001$), but that these increases were proportional to the trace concentration of strontium in the calcium chloride salt used to make the treatment solutions. The experimental technique did not significantly alter the concentrations of any of the other 15 measured analytes. We quantified the electrical conductivity of calcium chloride treatment solutions (mS cm^{-1}) with a Denver Instruments model 250 pH/ISE conductivity meter (Denver Instrument, Göttingen, Germany).

Nine *H. californicum* maternal lines produced enough seedlings to be planted in each of the 18 soil source/calcium chloride treatment groups, and the remaining 13 lines were randomly assigned to groups with the goal of a balanced design among calcium chloride treatments within soil sources (seeds from two maternal lines were inviable). Within maternal lines seedlings were randomly assigned to soil sources and calcium chloride treatments and planted individually in Cone-tainers. We randomly assigned each conetainer to a location in a greenhouse grid where it was held upright over a 13-cm plastic watering dish by an inverted paper cup with a hole cut through the bottom. Plants were bottom-watered by filling watering dishes with calcium chloride solutions of the assigned concentrations. Visual and tactile assessments of soil moisture were made every other day to determine the soaking schedule of each conetainer, and Cone-tainers were removed from soaking solutions for 3–5 days per week to allow soils to dry. Plants were grown under conditions of 14-h day length and temperatures of 21°C day/13°C night for 48 days, at which point the majority had attained a height characteristic of plants in the field when rust infection is first observed (3–6 cm). After removing unhealthy or dying individuals we measured the height (cm) and number of leaves of each of the remaining plants, which were then placed in conetainer racks. We thawed and rehydrated the lyophilized *M. lini* spores and added them to a 150-ml solution of autoclaved nanopure water containing 0.05% Tween 20 surfactant (Acros Organics, N.J.) to increase wetability of plant tissue. We removed twelve 0.5-ml aliquots of the inoculation solution to estimate spore concentration (using a hemacytometer) and viability (by scoring presence/absence of fungal germination tubes on agar-coated microscope coverslips 24 h after solution preparation). We sprayed experimental plants to runoff with the inoculation solution using a Preval disposable aerosol sprayer (Precision Valve, New York) and then sealed conetainer racks in humidity chambers at 100% relative humidity for 24 h. Following this period, plants were returned to their pre-inoculation positions in the greenhouse and the number of rust pustules on each plant was scored 14 days later.

Verifying effects of experimental calcium addition

To confirm that irrigation with calcium treatment solutions had the desired effect and measure the soil calcium concentrations experienced by experimental plants, we quantified soil calcium concentrations in two to three randomly selected Cone-tainers from each of the 18 treatment groups at the end of the experiment. Perlite was manually removed and AA analyses were performed as previously described. To verify that experimental increases in soil calcium concentrations resulted in concomitant changes in plant tissue calcium levels, tissue calcium concentrations were measured for five plants from each of the six soil treatment levels. Tissue analyses were performed by the ANR Analytical Laboratory (UC Davis) using nitric acid/hydrogen peroxide microwave digestion followed by inductively coupled plasma atomic emission spectrometry.

Statistical analyses

Calcium concentrations in natural soils, experimental soils, and experimental plant tissue

Variation in calcium concentrations of field soils was visualized using histograms. We used linear regression to characterize the relationship between calcium chloride treatment solution molarity and soil calcium concentration measured in experimental soils. Log_{10}-transformations of soil calcium concentrations and calcium chloride treatment solution molarity (hereafter "calcium treatment") were used to meet assumptions of normality. Linear regression was also used to assess the effect of calcium treatment on plant tissue calcium concentrations. Finally, we performed an analysis of covariance (ANCOVA) to quantify the contribution of soil source, calcium treatment, and their interaction to soil calcium concentrations.

Effects of soil source, maternal line, and calcium levels on infection rates

We used nominal logistic regression to analyze the probability of infection of each plant with respect to: (1) soil source, (2) seed source population, (3) maternal line (nested within seed source population), (4) calcium treatment, and (5) greenhouse table location. To

determine the best-fit model we constructed the full model, which contained each of these single-factor effects and all possible interaction terms, and reduced it by iteratively dropping the nonsignificant effect of the highest order and rerunning the reduced models until all remaining terms were significant at the $P < 0.05$ level (Kleinbaum 1994). Explanatory power of the best-fit model was quantified using the concordance score (percentage of correctly predicted observations) and the area under the receiver operating characteristic curve (*C* score) (Hanley and McNeil 1982). The latter statistic is similar to the nonparametric Wilcoxon statistic and represents the probability that a randomly chosen diseased subject is correctly rated, or ranked with greater suspicion, than a randomly chosen nondiseased subject. We conducted separate post hoc tests of the influence of plant height and number of leaves on infection rates by adding these effects (square root transformed to meet assumptions of normality) to the best-fit model and comparing the resulting concordance and *C* scores with those of the original solution. We used linear regression to test for a relationship between the severity of infection (number of pustules associated with sick plants) and calcium treatment. All analyses were performed in JMP v5.1.1 (SAS Institute, Cary, N.C.).

Results

Calcium concentrations in natural soils, experimental soils, and tissue of experimental plants

Across 121 soil samples taken from the 16 *H. californicum* populations in the field, soil calcium concentrations varied from 206.0 to 6,209.8 mg kg^{-1} (mean ± SD = 1,198.2 ± 1,251.7). The frequency distribution of these field-measured calcium concentrations, $\log_{10}$-transformed for illustrative purposes, was essentially normal with a slight positive skew (Fig. 2). Results of linear regression showed that these calcium concentrations were an excellent proxy for the calcium:magnesium ratio, a commonly used measure of the degree of serpentine influence associated with serpentine soils (Brooks 1987) {$\log_{10}$ calcium:magnesium ratio = −4.26 + (1.30 × $\log_{10}$[calcium]), r^2adj = 0.79, MS = 22.58, $F_{1,120} = 443.76$, $P < 0.0001$}. The calcium concentrations of experimental serpentine soils, and the tissue calcium content of *H. californicum* growing in these soils, increased in proportion to the concentration of calcium chloride solution used. Experimental soil calcium concentrations ranged from 284.0 to 1361.9 mg kg^{-1} across the 18 soil source/calcium treatment combinations (Fig. 2). This range represents 18% of the absolute natural range but encompasses 75% of the 121 field-measured calcium concentration values. The mean soil calcium concentrations (mg kg^{-1}) associated with calcium treatments 1–6 were 383.5, 445.1, 509.3, 574.0, 759.8, and 907.9, respectively. Linear regression revealed a significant, positive relationship between calcium treatment and the calcium concentration (% dry weight) of plant tissue [% dry weight = 0.37 + (1.30 × calcium treatment), r^2adj. = 0.79, MS = 6.85, $F_{1,29} = 109.41$, $P < 0.0001$].

While soil calcium concentrations were tightly correlated to calcium treatment level within each soil source, ANCOVA results indicated that variation in soil calcium among the 18 treatment groups was a function of both experimental calcium treatment and soil

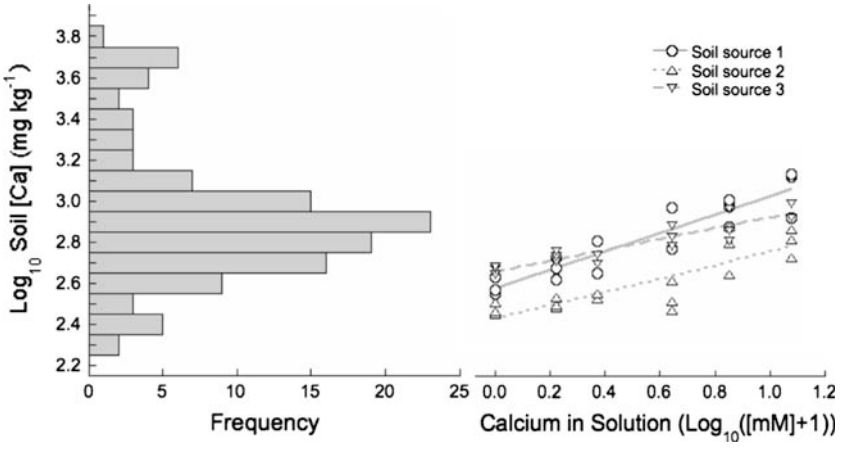

Fig. 2 $\log_{10}$-transformed soil calcium concentrations measured in 121 samples from 16 wild *H. californicum* populations (*left*) and in experimental soils soaked in one of six calcium chloride treatment solutions (*right*). Regression lines are soil source 1 (*solid line*): $\log_{10}$ soil calcium = 2.58 + (0.44 × calcium treatment), r^2adj = 0.84, MS = 0.47, $F_{1,15} = 81.44$, $P < 0.0001$. Soil source 2 (*dotted line*): $\log_{10}$ soil calcium = 2.46 + (0.31 × calcium treatment), r^2adj = 0.53, MS = 0.23, $F_{1,17} = 20.38$, $P = 0.0004$. Soil source 3 (*dashed line*): $\log_{10}$ soil calcium = 2.66 + (0.26 × calcium treatment), r^2adj = 0.83, MS = 0.15, $F_{1,16} = 78.21$, $P < 0.0001$

source. Results of analysis of covariance indicated that the best-fit, three parameter model (soil source, calcium treatment, soil source by calcium treatment interaction) explained 79% of the variation in soil calcium concentrations (MS = 0.25, $F_{5,50} = 38.38$, $P < 0.0001$). While soil calcium levels were positively correlated with calcium treatment (MS = 0.79, $F_{1,50} = 122.90$, $P < 0.0001$), the three soil sources differed significantly in their intrinsic calcium concentrations (MS = 0.40, $F_{2,50} = 30.79$, $P < 0.0001$) and in the rate at which these concentrations increased across calcium treatment levels (MS = 0.041, $F_{2,50} = 3.20$, $P = 0.05$) (Fig. 2). These results suggested that the soil sources differed both qualitatively and quantitatively in their calcium related properties, so we included calcium treatment and soil source as separate effects in logistic regression analyses of infection rates rather than using soil calcium concentrations for each soil source/calcium treatment level combination.

Effects of soil source, maternal line, and calcium levels on infection rates

H. californicum plants growing in high-calcium soils experienced significantly lower infection rates than conspecifics in low-calcium soils (Fig. 3). Overall, the best-fit infection rate model produced by logistic regression explained infection patterns well ($r^2 = 0.39$, −loglikelihood full model = 74.53, reduced model = 117.06, $df = 26$, $\chi^2 = 85.06$, $P < 0.0001$). Concordance of the model was 0.86 (206 of 239 observations correctly predicted) and the area under the receiver operating characteristic curve (*C* score) was 0.90. Infection rates declined significantly with calcium treatment (log rank $\chi^2 = 11.91$, $df = 1$, $P < 0.001$) and varied significantly across soil sources (log rank $\chi^2 = 10.23$, $df = 2$, $P < 0.01$, Fig. 3) and maternal lines (log rank $\chi^2 = 45.94$, $df = 21$, $P < 0.01$). There was also a significant interaction between soil source and calcium treatment (log rank $\chi^2 = 9.75$, $df = 2$, $P < 0.01$). When added separately ex post facto to the model the effects of both number of leaves (log rank $\chi^2 = 3.54$, $df = 1$, $P = 0.060$) and plant height (log rank $\chi^2 = 2.96$, $df = 1$, $P = 0.085$) were nearly significant, but neither improved the model's predictive ability (concordance or *C* score) by more than 1.3%.

Overall, of the 351 seedlings planted, 239 survived to be inoculated (68.1%) and 46 of these (19.2%) became infected. The number of maternal lines and individual plants in each soil source/calcium treatment combination is given in Table 1. There were significant positive relationships between calcium treatment and plant height, pre-inoculation mortality rate and treatment solution conductivity (Table 1). While there was no association between infection severity and calcium treatment, the high degree of variability in the number of pustules per sick plant (mean ± SD = 4.0 ± 4.1) may have compromised our ability to detect a relationship. The average spore concentration of the inoculation solution aliquots (mean ± SE) was 9,167 ± 751 spores ml^{-1}, and spore germination rates were high (mean ± SD = 76.0 ± 6.4%).

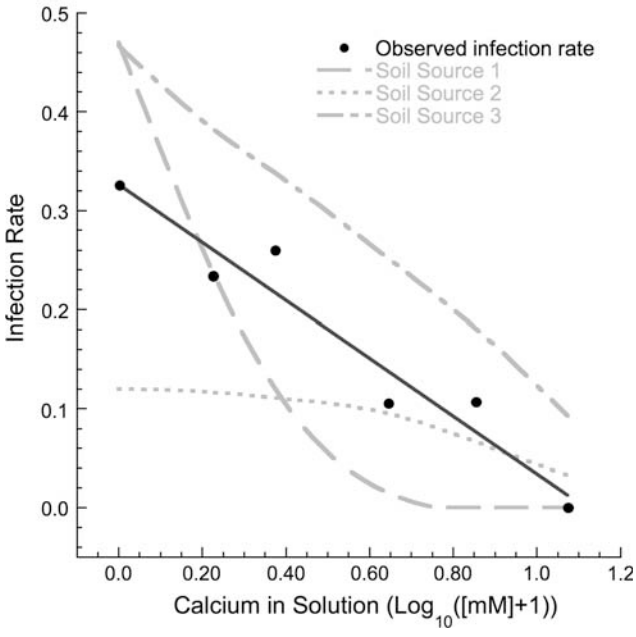

Fig. 3 Rates of rust infection as a function of calcium treatment level. Observed infection rates were calculated by pooling data across soil sources for each of the six calcium treatment levels (r^2adj. = 0.92, MS = 0.069, $F_{1,5} = 59.20$, $P = 0.0015$). Predicted infection rates for each soil source, depicted in *gray*, were generated from best-fit infection rate model equation [maternal line (ML), soil source (SS)]: probability of infection = 1.94 + (SS1 × 0.90) + (SS2 × 0.24) + (ML1 × −0.74) + (ML2 × 0.15) + (ML3 × −1.38) + (ML4 × 9.80) + (ML5 × −1.31) + (ML6 × −0.48) + (ML7 × 9.14) + (ML8 × −1.43) + (ML9 × −2.43) + (ML10 × 0.01) + (ML11 × −2.70) + (ML12 × −2.35) + (ML13 × −4.01) + (ML14 × −2.54) + (ML15 × −3.13) + (ML16 × 0.14) + (ML17 × −3.22) + (ML18 × 9.77) + (ML19 × −0.47) + (ML20 × −1.80) + (ML21 × −0.73) + (calcium treatment × 3.10) + {4.33 × [SS1 × (calcium treatment −0.46)]}−{3.65 × [SS2 × (calcium treatment−0.46)]}

Discussion

Experimental manipulations elevated the calcium concentrations of both serpentine soils and plant tissue, and *H. californicum* infection rates declined significantly with increasing calcium treatment concentration. This result runs counter to the predictions of the pathogen refuge hypothesis and is consistent with multiple lines of evidence generated by agriculturally

Table 1 Calcium chloride solution concentration and conductivity, plant sample size[a], and plant mortality rate[b] associated with the six calcium treatments

Concentration (log 10 mM + 1)	Conductivity (mS cm^{-1})	Plant mortality rate	Number of maternal lines		
			Soil source 1	Soil source 2	Soil source 3
0.0	0.0	8.0	16	15	15
0.23	0.18	4.1	15	16	16
0.37	0.31	8.0	15	15	16
0.64	0.52	23.0	15 (16)	11 (13)	15 (18)
0.85	0.94	46.2	11	6	9 (11)
1.08	1.25	71.9	11 (14)	1	9 (10)

[a] For sample size data, the number of maternal lines is equal to the number of individual plants inoculated except when multiple plants from one or more maternal lines were included. In these instances the number of plants inoculated is indicated *in parentheses*

[b] Mortality rates are based on the number of plants that survived to be inoculated

based research that suggest a central role of calcium in plant recognition of and defensive responses to attacking pathogens. First, cytosolic free calcium concentrations increase when plants are challenged by pathogens and pathogen-derived elicitor compounds (Dixon et al. 1994; Gelli et al. 1997; Zimmermann et al. 1997). Second, the involvement of calcium-binding sensor molecules in plant defenses has been demonstrated (Romeis et al. 2000; Lee and Rudd 2002; Yang and Poovaiah 2003), and the activation of these molecules can be integral in initiating specific responses including oxidative burst (Harding et al. 1997; Xing et al. 2001), the hypersensitive response (Levine et al. 1996; Xu and Heath 1998), and expression of defense-associated genes (Hahlbrock et al. 1995). Experimentally inactivating these sensors, or inhibiting calcium influx, can prevent the initiation of plant defenses (Jabs et al. 1997; Grant et al. 2000). Third, crop plants receiving a calcium-augmented nutrition regimen often have higher tissue calcium concentrations and experience reductions in pathogen damage via enhanced resistance to infection or more moderate symptom expression (McGuire and Kelman 1984; Volpin and Elad 1991; Engelhard 1989; Yamazaki and Hoshina 1995).

Of the 16 other soil analytes examined, only strontium concentrations increased across experimental treatment levels. In contrast to the aforementioned evidence implicating important contributions of calcium in plant–pathogen interactions, there is to our knowledge no evidence for a role of strontium in plant–pathogen interactions. Furthermore, strontium is not considered an essential element for plants, nor is it associated with any plant nutrient deficiency (Zeiger and Taiz 2002). Because of similarity in atomic properties strontium can compete with calcium in cellular uptake and binding pathways (Rediske and Selders 1953; Bowen and Dymond 1956), but this interference should have had a negligible impact on observed patterns since soil strontium concentrations were on average 3 orders of magnitude lower than those of calcium. In light of these points, and the large body of evidence implicating calcium's role in infection-related processes, we conclude that experimental increases of soil calcium concentrations caused significant reductions in *H. californicum* infection rates.

The relationship observed between soil calcium concentrations and plant infection rates is somewhat surprising considering two other observed patterns that should have led to elevated infection rates under high soil calcium conditions. First, although plant height and number of leaves did not significantly improve the fit of the best-fit infection rate model when added ex post facto, separate regression analyses indicated that plants grown under higher soil calcium concentrations were significantly taller and tended to have more leaves relative to their low soil calcium counterparts. Because of their greater surface area, these larger plants would be expected to receive a greater number of spores and exhibit higher infection rates. Second, significantly higher pre-inoculation mortality rates experienced by plants growing under higher soil calcium conditions suggest that these plants experienced more edaphically imposed stress. Given the significant positive correlation between molarity and conductance of the calcium treatment solutions it seems likely that osmotic stress arising from irrigation with salt solutions contributed to observed patterns of plant mortality (Maas 1986). This conjecture is supported by the observation of aboveground symptoms associated with root-burn in some dead plants. Because *H. californicum* will readily grow in commercial potting soil with calcium concentrations 10 times higher than those measured in our experimental soils, we conclude that observed mortality was caused not by toxicity of calcium itself but rather by the osmotic stress associated with irrigation of soils with salt solutions. Exposure to environmental

stresses such as this should have weakened plants and made them more susceptible to infection. Evidence from the agricultural literature suggests that many fungal pathogens are highly tolerant of salts in culture (Tresner and Hayes 1971) and that irrigation with salt solutions can increase the incidence and/or severity of infection associated with a variety of plant pathogens including species of *Fusarium* (Turco et al. 2002; Triky-Dotan et al. 2005), *Phytophthora* (MacDonald 1984; Blaker and Macdonald 1986; Swiecki and Macdonald 1991; Sanogo 2004), *Pythium* (Rasmussen and Stanghellini 1988), and *Verticillium* and *Alternaria* (Nachmias et al. 1993) (but see Elmer 1992; Brac De La Perriere et al. 1995; Elmer 1997, 2002). The fact that the opposite pattern was documented suggests that mechanisms that reduce infection rates must have acted in opposition to these two factors to produce the observed patterns of disease.

Under some natural conditions *H. californicum* resistance responses may be physiologically constrained by the low calcium availability characteristic of strongly serpentine soils. Experimentally augmenting soil calcium may have bolstered host plant defenses and increased resistance to rust infection. Alternatively, higher soil calcium conditions may have resulted in changes in host tissue biochemistry that reduced rust virulence by interfering with spore germination, host recognition or penetration, or infection (Flego et al. 1997; Sebghati et al. 2000). Irrespective of the mechanism(s) that led to reduced infection rates, the pattern is intriguing because it occurred over a relatively low and narrow range of soil calcium concentrations well within the range experience by *H. californicum* in its natural environment. This suggests that physiological processes manifest in the experiment may also be important in the ecology and evolution of *H. californicum/M. lini* interactions in the wild. Additionally, because many serpentine-associated plants experience similar levels of calcium availability, these results may have implications for many other serpentine plants.

The observation of significant variation in infection rates among plants growing in the three serpentine soil sources suggests that although the soils were all serpentine derived, they differed fundamentally in ecologically relevant characteristics. These differences were further evidenced by variation in how the soils responded to the experimental addition of calcium: soil source × calcium treatment interaction term also had a significant effect on observed infection rates. While the underlying geochemical cause(s) of this edaphic variation could not be identified, our results suggest the need for more cautious use of the term "serpentine" to loosely identify a group of soils that are qualitatively similar in their geologic derivation but may be highly variable quantitatively in their physical and chemical properties. The dichotomy between serpentine and nonserpentine soils often used in the serpentine plant ecology literature can create an illusion of clearly delineated edaphic categories, each defined by fairly consistent properties. Such generalizations can obscure edaphic variability among "serpentine" soils that may contribute in important ways to ecological and evolutionary patterns observed in serpentine plant communities.

Significant differences in infection rates among the different maternal lines used in the experiment are probably due to variation in the distribution of disease-resistance genes. Based on studies of flax/flax rust interactions in the domesticated flax *Linum ustitatissimum* (Flor 1956), and the wild flax *L. marginale* (Burdon and Thrall 2000), both of which are infected by *M. lini*, it seems likely that this resistance manifests as a gene-for-gene compatibility system. In these systems, host resistance and pathogen virulence are determined through the expression of a few genes with large effect, and infection is only possible when a given host and pathogen contain "matching" resistance and virulence alleles (Thompson and Burdon 1992). Our results suggest that genes conferring resistance may be fairly common in *H. californicum* (overall resistance rate was 80.8%) but that the distribution of these genes is variable among individual maternal plant lineages.

Plant ecologists have long sought mechanistic explanations for the striking patterns of plant distribution in areas where serpentine soils occur (Proctor and Woodell 1975; Kruckeberg 1984). Attempts to elucidate the processes responsible for maintaining the observed floristic discontinuity remain inconclusive but suggest the following explanations (Walker 1954; Kruckeberg 1984). Most plants growing on nonserpentine soils are physiologically incapable of tolerating the edaphic conditions characteristic of serpentine soils. While the biochemical mechanisms responsible for this exclusion probably vary taxonomically, calcium deficiencies and the inability to tolerate them are believed to play central roles in excluding these species. Why serpentine-associated plants are found obligately or almost exclusively on these harsh soils remains much more uncertain. Because serpentine plants will often grow readily on nonserpentine soils in the greenhouse, physiological restrictions do not seem to limit their distributions to serpentine soils. Explanations involving species interactions have received more support, largely based on observational evidence. The most widely accepted theory posits that serpentine-associated plants are poor competitors that are excluded from more benign

nonserpentine soils by their more vigorous nonserpentine counterparts (Whittaker 1954). An alternative proposition, that serpentine-associated plants may find a refuge from pathogens when growing on serpentine soils, suggests that biotic factors other than competition may be important drivers in maintaining the serpentine floristic "syndrome" (Kruckeberg 1992). Our results indicate that the opposite may be true. Serpentine-associated plants growing on calcium-deficient soils may be more vulnerable to infection than plants growing on soils where calcium is present at higher concentrations.

The results of our study suggest some novel insights into patterns of species distribution in serpentine plant communities. Ironically, while our findings do not explain patterns of edaphic endemism of serpentine plants, they may in part explain the inability of nonserpentine plants to colonize serpentine patches. When nonserpentine plants colonize low calcium serpentine soils they may be faced with both abiotic physiological challenges and increased disease-related morbidity and mortality. In addition, calcium/infection interactions may have implications for the distribution of pathogens in these systems. If serpentine plant species, by virtue of their association with low-calcium soils, represent more vulnerable hosts than their nonserpentine counterparts, then pathogens able to exploit them may have higher fitness than those infecting nonserpentine hosts. This could result in a greater number of and/or more specialized pathogens being associated with serpentine flora.

Our findings offer some intriguing scenarios for plant evolution in serpentine ecosystems. First, competitive exclusion from nonserpentine patches may restrict serpentine species to an edaphic niche where disease is more frequent and perhaps severe, effectively rendering serpentine patches coevolutionary "hotspots" with regard to plant–pathogen interactions (Thompson 1994). Second, greater vulnerability to pathogen attack could affect demographic patterns and life history evolution in serpentine flora. Like *H. californicum*, many herbaceous serpentine species are diminutive in size and have a short lifespan. Because of these life history traits they may be subject to significant disease-induced declines in survivorship and fecundity. Such is the case for *H. californicum*, which experiences dramatic fitness reductions when infected by *M. lini* (Y. P. Springer, in preparation). This could contribute to the low levels of abundance and density commonly associated with serpentine herbs. Alternatively, these "fast" life history traits may represent an evolutionary response to disease pressure, allowing host plants to quickly take advantage of periods when conditions are unfavorable for pathogen dispersal or establishment. Third, soil-driven variation in the frequency of plant–pathogen interactions could manifest over a relatively narrow range of natural soil calcium levels. This may generate small-scale variation in selection for and distribution of genetic resistance to infection. The documented relationship between soil calcium concentrations and infection rates, coupled with potentially severe effects of infection on fitness, suggests that many serpentine annuals may be under strong selection to acquire genetic resistance. When serpentine plants grow on calcium-deficient soils in the presence of pathogens there should be strong selection for genetic resistance to infection. Alternatively, when these plants can colonize edaphically benign habitats with higher calcium, increases in calcium-mediated, environmentally conferred resistance may reduce the strength of selection favoring genetic resistance. This trade-off between resistance mechanisms could result in a spatial mosaic in the distribution of genetic resistance that is inversely related to the calcium levels in associated soils. Such a mosaic could manifest spatially among populations of a single host species or taxonomically among serpentine plant species that vary in their competitive vigor and resulting ability to colonize higher calcium soils. Results of our study suggest that effects of calcium-mediated plant–pathogen interactions should be given greater consideration when assessing the mechanisms that shape the demographic, genetic, and biogeographic patterns documented in serpentine ecosystems.

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