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REGULAR ARTICLE

Effects of soil metals on pollen germination, fruit production, and seeds per fruit differ between a Ni hyperaccumulator and a congeneric nonaccumulator

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Abstract

Aims Developmentally distinct soil formations (e.g., serpentine vs. non-serpentine soils) can vary markedly in chemical composition, including elements known to alter plant reproduction. Here we evaluate the effects of soil Ca and Ni on pollen germination and fruit and seed production for two species: *Streptanthus polygaloides*, a serpentine endemic and Ni hyperaccumulator, and *S. tortuosus*, a non-endemic, non-Ni-accumulating, serpentine-tolerant species.

Methods In a factorial experiment, we grew each species in soils that were amended to have high or low concentrations of Ca and Ni. We then performed hand pollinations and measured pollen germination, fruit production, and seeds per fruit.

Results While Ca soil supplementation did not affect reproduction for either species, soil Ni had a strong influence, which varied by species. For *S. tortuosus*, the high Ni recipient treatment resulted in reduced pollen germination and fruit/seed production. For *S. polygaloides*, recipient effects were detected, but in the opposite direction of *S. tortuosus*: high Ni recipient treatment had a neutral (or slightly positive) effect on *S. polygaloides* pollen germination and fruit/seed production.

Conclusions Soil chemistry can greatly influence plant reproduction by altering pollen-pistil interactions, and may determine patterns of plant species abundance and distribution on novel soils by directly affecting pollen germination and subsequent reproductive success.

Keywords *Streptanthus* · Nickel · Calcium · Hyperaccumulation · Serpentine · Pollen-pistil interactions

Introduction

Serpentine-derived soils represent a nutritionally stressful growing environment for most plants because of a low Ca:Mg ratio, deficiency of nutrients (e.g., N, P, K), and high levels of heavy metals (e.g., Ni, Co, Cr; Brooks 1987; Brady et al. 2005; Kazakou et al. 2008). High Ni and low Ca concentrations, in particular, are thought to be key in generating adaptation to serpentine soil (Lazarus et al. 2011; Kazakou et al. 2008), and serpentine affinity (i.e., endemic vs. non-endemic) is known to affect both Ni and Ca accumulation into vegetative and reproductive plant tissues (Nagy and Proctor 1997; Burrell et al. 2012; DeHart et al. 2014; Meindl et al. 2014a). Because of the toxic effects of Ni, and the beneficial effects of Ca, differential acquisition of these elements by plants growing in serpentine soil may result in differential fitness between taxa that vary in element accumulation.

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Calcium is an essential nutrient for plants, and contributes towards a variety of functions, including intercellular signaling, cell wall structure, and pollen grain germination (Broadley et al. 2012). Calcium is particularly important for plant reproduction, as it is required for pollen germination and tube growth (Brewbaker and Kwack 1963; Broadley et al. 2012). Specifically, Ca in the pistil influences the direction of pollen tube growth, as pollen tubes grow chemotropically along an increasing Ca gradient from the top to the bottom of the pistil (Mascarenhas and Machlis 1962, 1964; Rosen 1968; Chichiriccò et al. 2002). Calcium is also involved in pollen formation (Chen et al. 2008; Kuang and Liao 2014), thus Ca accumulation by both maternal (i.e. in pistils) and paternal (i.e. in anthers and pollen) plants may be important regulators of plant fitness. However, plants that vary in serpentine affinity are known to differentially acquire this element from serpentine soils. For example, in a study of three confamilial pairs of endemic and non-endemic plant species, DeHart et al. (2014) found that serpentine endemics accumulated higher concentrations of Ca into tissues compared to non-endemics when growing on Ca-poor serpentine soil. Similarly, Rajakaruna et al. (2002) found that serpentine races of *Lasthenia californica* (Asteraceae) accumulated higher concentrations of Ca compared to non-serpentine races in common garden experiments. Because Ca concentrations in pistils and pollen regulate pollen grain germination (Broadley et al. 2012; Chen et al. 2008; Kuang and Liao 2014), plant fitness may vary across species that differentially acquire this limiting element from serpentine soils, particularly in the early stages of soil colonization.

Similarly, serpentine endemics and non-endemics differ in Ni accumulation, as endemics are known to accumulate lower concentrations of Ni compared to non-endemic species (Nagy and Proctor 1997; Burrell et al. 2012; Meindl et al. 2014a). Differences in Ni accumulation between endemic and non-endemic species can be most pronounced for reproductive organs (i.e. anthers and pistils), where serpentine endemics accumulate lower concentrations of Ni than non-endemics (Meindl et al. 2014a). Nickel accumulation into reproductive organs may decrease both male and female components of plant fitness for unadapted species. For example, Ni is known to reduce pollen germination when present exogenously (Breygina et al. 2012), thus Ni accumulation in pistils may reduce pollen germination and subsequently seed and fruit production. In addition, heavy metal accumulation can lead to developmental abnormalities of both anthers and pollen (Yousefi et al. 2011). Therefore, the fitness consequences of growth on serpentine soil may differ across species that vary in Ni accumulation into reproductive organs.

A relatively small number of serpentine taxa are known to accumulate extremely high concentrations of heavy metals (i.e. hyperaccumulation; van der Ent et al. 2013; Pollard et al. 2014). Metal hyperaccumulation refers to the uptake and sequestration of soil metals by plants into above ground tissues in concentrations that are orders of magnitude higher than typical plants (reviewed in van der Ent et al. 2013). Hyperaccumulators of the heavy metal Ni, for example, exhibit shoot Ni concentrations >1000 mg kg^{-1}, while most plants contain <5 mg kg^{-1} (van der Ent et al. 2013). However, the direct effects of floral metal hyperaccumulation on plant fitness have rarely been characterized. A preliminary study of the Ni hyperaccumulator *Alyssum inflatum* (Brassicaceae) found that plants are more likely to flower, and produce larger floral displays, when grown in Ni-supplemented soils (Ghasemi et al. 2014). While defense against plant antagonists is thought to be a primary benefit of metal hyperaccumulation (i.e. elemental defense; Boyd 2007), some metal hyperaccumulators not only tolerate otherwise toxic concentrations of heavy metals, but directly benefit from metal hyperaccumulation in terms of increased fitness. However, the extent to which hyperaccumulating species benefit from these elements in their environment is largely unknown; therefore, it is important to characterize the effects of floral metal hyperaccumulation in traits crucial to plant fitness, such as pollen germination, fruit, or seed production.

In this study, we employ a fully factorial experiment to test for the effects of Ni and Ca on pollen germination, fruit production, and seed production for two closely related species that vary in their affinity to serpentine soil and the degree to which they accumulate the heavy metal Ni (*Streptanthus polygaloides* [serpentine endemic, Ni hyperaccumulator]; *S. tortuosus* [non-endemic, non-accumulator, but considered an ‘indicator’ of and thus tolerant to serpentine soils; Safford et al. 2005]. We predict that plant reproduction will be maximized when soil treatments most closely reflect the chemical composition of the native soil to which each species is associated. For example, *S. tortuosus* typically grows on non-serpentine soil, which tends to be relatively high in Ca and low in Ni; therefore, we predict that this species will achieve higher reproductive success in a high Ca treatment (White 2003), while this species will exhibit toxic effects when grown in high Ni soil (Breygina et al. 2012; Yousefi et al. 2011). Conversely, we predict that *S. polygaloides*, which is a serpentine soil endemic and a Ni-hyperaccumulator, will not respond as positively to high Ca soil treatments or negatively to high Ni treatments, as this species grows natively on soils with both low Ca (thus soil Ca supplementation will not benefit this species; White 2003) and high Ni concentrations. Furthermore, given the positive effects of elevated soil Ni for other hyperaccumulator species (Ghasemi et al. 2014), Ni supplementation may actually increase reproductive success for *S. polygaloides*. Specifically, we asked the following questions: (1) Do soil Ni and Ca concentrations in either the maternal or paternal soil environment affect pollen germination, fruit production, and seed production? (2) Does the response to soil Ni and Ca concentrations in either the maternal or paternal soil environment differ between species?

Materials and methods

Study species

Streptanthus polygaloides and *S. tortuosus* are closely related taxa (sister species *sensu* Mayer and Soltis 1994; Cacho and Strauss 2014) in the mustard family (Brassicaceae). *Streptanthus tortuosus* is common and can be found throughout California and Oregon, while *S. polygaloides* is restricted to the Sierra Nevada of northern California, where the ranges of the two species overlap (Baldwin et al. 2012). *Streptanthus polygaloides* is a Ni hyperaccumulating, annual endemic to serpentine soil (Baldwin et al. 2012; Reeves et al. 1981). *Streptanthus tortuosus* is a non-Ni hyperaccumulating perennial that can occur either on or off of serpentine soil, though it is more frequently found off serpentine (Baldwin et al. 2012). Both species are spring-flowering herbs that are at least partially self-compatible (Meindl et al. 2014b; Wall and Boyd 2002; Preston 1994).

Experimental design

We bulk-collected and pooled seeds from >20 individual plants from a single natural population per species in northern California (*S. polygaloides*: 39°48′53.5″ N 121°34′44.5″ W [Butte County, CA]; *S. tortuosus* [a non-serpentine population]: 39°59′33.9″ N 121°16′ 53.6 W [Plumas County, CA]) in the summer of 2012. In the fall of 2013, we sowed seeds directly into potting soil and treated seeds for two weeks with 4 °C cold and dark conditions. Following germination, we transplanted seedlings to 27 in^3 pots (Deepots, Stuewe and Sons, Inc., Tangent, OR, USA) filled with standard potting soil (Fafard #4, Sun Gro Horticulture, Agawam, MA, USA) and supplied with six Nutricote® NPK 13–13-13 time-release fertilizer pellets (Arysta LifeScience Corporation, New York, NY, USA). Previous work determined the perennial *S. tortuosus* requires a vernalization treatment to flower within a single growing season (Meindl, unpublished). Thus, 1 month after transplanting, we subjected *S. tortuosus* plants to a 4 °C cold treatment for 1 month at 8D:16N (D = day; N = night). Subsequently, we grew both *S. tortuosus* and *S. polygaloides* under controlled conditions of 12D:12N and between 21.1 and 26.7 °C until flowering in the greenhouse at the University of Pittsburgh.

One month after transplanting (*S. polygaloides*), or 1 week after cold treatment (*S. tortuosus*), we divided plants into four treatment groups ($N = 20$ plants / species / treatment; total $N = 160$ plants): (1) high Ni and high Ca, (2) high Ni and low Ca, (3) low Ni and high Ca, or (4) low Ni and low Ca. We allowed plants to become fully-grown and approach flowering before applying treatment solutions in order to avoid any effects of soil treatments on overall plant size. We introduced soil treatments by top watering plants with 40 mL of treatment solution once per week for the duration of the experiment (eight weeks). High Ni solutions contained 500 mg kg^{-1} Ni, while low Ni solutions contained 5 mg kg^{-1} Ni. High Ca solutions contained 2200 mg kg^{-1} Ca, while low Ca solutions contained 220 mg kg^{-1} Ca. Both Ni and Ca solutions were prepared using metal nitrates ($Ni[NO_3]_2$-$6H_2O$ or $Ca[NO_3]_2$-$4H_20$). We added ammonium nitrate (NH_4NO_3) to treatment solutions (2)–(4) to control for additional nitrogen applied to plants in the high Ni/high Ca treatment (N from both ammonium and nitrate was considered in our efforts to equalize N concentrations across treatments). Our soil treatment solutions reflect realistic levels of bioavailable Ni and Ca because bioavailable fractions of Ni in serpentine soils generally range from 50 to 500 mg kg^{-1}, while those in non-serpentine soils generally range between 0.05 and 5 mg kg^{-1} (L'Huillier and Edighoffer 1996; Chardot et al. 2005; Broadley et al. 2012). Likewise, bioavailable fractions of Ca in non-serpentine soils generally range from 2000 to 4000 mg kg^{-1}, while those in serpentine soils generally range from 100 to 600 mg kg^{-1} (Whittaker 1954; DeHart et al. 2014). Of the 160 experimental plants, we randomly assigned half to be pollen donors, and the other half to be pollen recipients, to determine the degree to which both the paternal and maternal soil environments affect plant reproduction.

To determine the effect of soil treatments on plant Ca and Ni concentrations each week we collected pollen and pistil samples from 40 flowers pooled across all donor plants for each treatment and species separately. We separated pollen grains from anthers on filter paper (Whatman® Grade 1; GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), then weighed the pollen to the nearest 0.0001 g on an AE200 Mettler® analytical balance (Mettler-Toledo; LLC, Columbus, OH, USA). We determined Ni and Ca concentrations using Inductively Coupled Plasma Mass Spectrometry (ICP-MS, NEXION 300X, PerkinElmer, Waltham, MA, USA) at the University of Pittsburgh following Meindl and Ashman (2014), and concentrations are reported as mg kg^{-1}.

To determine the effects of donor and recipient soil treatments on pollen germination and fruit and seed production we performed hand-pollinations. For each species separately, we bulk-collected pollen from donor plants of each treatment once per week. We collected whole anthers, placed them in open microcentrifuge tubes, and allowed them to dehisce overnight. The following day, we performed pollinations on each of the recipient plants. Once per week, we pollinated four to eight flowers per recipient plant (N = 4–16 flowers pollinated per plant, N = 596 total flowers pollinated). Each flower received pollen from one of the four donor treatments in random order. For half of these flowers, we collected styles 24 h after performing pollinations and fixed the styles in 70% ethanol. We then softened and stained styles with analine blue (Dafni 1992; Arceo-Gómez and Ashman 2011) and observed them with the aid of an epifluorescence microscope (Axioskop, Carl Zeiss Microscopy, LLC, Thornwood, NY, USA). For each style, we recorded the total numbers of pollen, germinated pollen, and pollen tubes that reached the ovary. To determine if the likelihood of pollen germination was affected by either maternal or paternal soil treatments, we calculated the percentage of pollen applied that germinated (number of germinated pollen grains / total number of pollen grains). To determine whether subsequent pollen tube growth was affected by either maternal or paternal soil treatments, we calculated the percentage of germinated pollen to reach the ovary (number of pollen tubes that reached ovary / number of germinated pollen grains). We recorded whether the remaining pollinated flowers matured fruits, and for those that did we determined the number of fertile seeds (i.e., those seeds that were large and swollen, rather than small and deflated). Because individual plants had multiple flowers pollinated per donor treatment, we calculated average values of all responses.

We conducted statistical analyses in SAS (version 9.3; SAS Institute Inc., Cary, NC, USA). To evaluate the effect of soil donor and recipient Ni and Ca treatments on the percentage of pollen applied that germinated, the percentage of germinated pollen to reach the ovary, and seeds produced per fruit, we used mixed-model ANCOVA (PROC MIXED). To evaluate the effect of soil donor and recipient treatments on the likelihood of pollinated flowers to mature fruits, we used log-linear analysis (PROC GLIMMIX) and specified a binary distribution (SAS Institute 2011). We included the total number of pollen grains applied to stigmas as a covariate in pollen germination models. We also used mixed model ANOVA (PROC MIXED) to compare Ni and Ca concentrations in pistils and pollen across species and soil treatments, with species, soil Ni treatment, soil Ca treatment, floral tissue (i.e., pistil or pollen) and their interactions as fixed effects, and sampling week as a random effect. Final statistical models did not include 4- or 5-way interactions, but, as determined by AIC values, our reduced models produced a better fit for the data compared to full models (percent pollen germination model: full model AIC = −32.6, reduced model AIC = −68.2; percent of germinated pollen to reach ovary model: full model AIC = 3, reduced model AIC = −13.3; seed set model: full model AIC = 36.4, reduced model AIC = 21.5). Furthermore, we explored various stepwise variable selection methods (i.e., forward, backward, stepwise, lasso, and lar methods; SAS Institute 2011) to determine which effects should be retained in final models, and the effects chosen by these stepwise variable selection methods did not include 4- or 5-way interactions. Denominator degrees of freedom for F-tests were determined using the Kenward-Roger approximation, which is preferred for small sample sizes and unbalanced data (Bell et al. 2014). We included individual plant (nested within species) as a random effect in all models.

Least squares means and standard errors are reported throughout.

Results

Pistil and pollen chemistry

Nickel treatment level affected Ni concentrations across pollen and pistils in both species, but the magnitude of the response varied by both species and tissue (Ni Treatment x Species x Tissue Type: $F_{1,36} = 29.1$, $P < 0.0001$). Specifically, Ni concentrations in pistils were nearly 17 times higher for plants in the high relative to the low Ni treatment for the hyperaccumulating serpentine endemic, *S. polygaloides* (1380 ± 59.8 vs. 82.2 ± 59.8 mg kg^{-1}, respectively). Ni concentrations in pistils were approximately seven times higher for plants in the high Ni relative to the low Ni treatment for the non-serpentine species, *S. tortuosus* (189 ± 84.6 vs. 27.9 ± 84.6 mg kg^{-1}, respectively). Furthermore, Ni concentrations in pollen were nearly nine times higher for plants in the high Ni relative to the low Ni treatment for *S. polygaloides* (156 ± 53.5 vs. 18.0 ± 53.5 mg kg^{-1}, respectively), while Ni concentrations in pollen were approximately 13 times higher for plants in the high Ni relative to the low Ni treatment for *S. tortuosus* (107 ± 84.6 vs. 8.3 ± 84.6 mg kg^{-1}, respectively).

Ca concentrations were slightly elevated overall for plants in the high Ca treatment relative to the low Ca treatment (6710 ± 440 vs. 6320 ± 440 mg kg^{-1}, respectively); however, these differences were not statistically significant, and none of the interactions involving treatment and species were significant either (All $P > 0.05$).

Ni treatment level did not significantly influence tissue Ca concentrations (Ni Treatment: $F_{1,31.6} = 2$, $P = 0.16$), despite Ca concentrations being slightly elevated in plants that received the high Ni treatment relative to plants that received the low Ni treatment

Table 1 Results from mixed model ANOVAs of (A) the percentage of pollen that germinated, (B) the percentage of germinated pollen that reached the ovary, (C) the likelihood of pollinated flowers to produce fruit, and (D) the number of seeds produced per fruit for *S. polygaloides* and *S. tortuosus* (Species) that were grown in one of four recipient soil treatments (Recipient Ni [low Ni vs. high Ni], Recipient Ca [low Ca vs. high Ca]) and whose flowers were pollinated with pollen produced by plants grown in one of four donor soil treatments (Donor Ni [low Ni vs. high Ni], Donor Ca [low Ca vs. high Ca])

Fixed effects	(A) Percentage of pollen that germinated		(B) Percentage of germinated pollen that reached ovary		(C) Likelihood to produce fruit		(D) Seeds per fruit	
	df	*F*	*df*	*F*	*df*	*F*	*df*	*F*
Total Grains	1, 258	14.81**	1, 257	24.49**	NA	NA	NA	NA
Species	1, 68.2	0.66	1, 65	9.56**	1, 58.7	0.33	1, 47.8	0.72
Recipient Ni	1, 61.2	1.84	1, 58.6	0.94	1, 58.8	1.33	1, 47.8	1.34
Recipient Ca	1, 58.9	0.33	1, 56.6	0.71	1, 58.7	3.16	1, 47.8	0.02
Donor Ni	1, 195	1.58	1, 192	0.21	1, 438	0.39	1, 137	0.04
Donor Ca	1, 195	3.71	1, 193	0.06	1, 438	0.3	1, 136	0.06
Species*Recipient Ni	1, 59.3	4.19*	1, 56.9	17.66**	1, 58.8	24.69**	1, 47.8	9.4**
Species*Recipient Ca	1, 58.8	0.11	1, 56.5	0.54	1, 58.7	1.64	1, 47.8	0.46
Species*Donor Ni	1, 194	5.33*	1, 192	3.05	1, 438	0.44	1, 137	0.14
Species*Donor Ca	1, 196	1.44	1, 193	0.36	1, 438	1.95	1, 136	0.02
Recipient Ni*Recipient Ca	1, 59.1	0.05	1, 56.8	0.08	1, 58.7	0.47	1, 47.8	0.99
Donor Ni*Donor Ca	1, 194	0.94	1, 191	0.76	1, 438	0.01	1, 136	3.35
Species*Recipient Ni*Recipient Ca	1, 58.8	0.01	1, 56.5	0.01	1, 58.7	2.09	1, 47.8	0.06
Species*Donor Ni*Donor Ca	1, 195	0.48	1, 192	1.67	1, 438	0.29	1, 136	1.36
Random Effect		*Z*		*Z*		*Z*		*Z*
Plant ID (Species)		3.79**		3.94**		NA		3.85**

The total number of pollen grains applied (Total Grains) was included as a covariate in models (A) and (B)

*$P < 0.05$; **$P < 0.01$

(6900 ± 440 vs. 6130 ± 440 mg kg^{-1}, respectively). Similarly, Ca treatment level did not significantly influence tissue Ni concentrations (Ca Treatment: $F_{1,36} = 2.32$, $P = 0.14$), despite Ni concentrations being slightly elevated in plants that received the high Ca treatment relative to plants that received the low Ca treatment (285 ± 36 vs. 207 ± 36 mg kg^{-1}, respectively).

Pollen germination

Recipient Ni treatment level did not strongly influence the percentage of pollen to germinate for *S. tortuosus* (high Ni: 67 ± 4%, low Ni: 70 ± 4%), but pollen germination was elevated for recipient plants in the high Ni treatment relative to the low Ni treatment for *S. polygaloides* (72 ± 4% vs. 59 ± 4%, respectively; Species x Recipient Ni Treatment: $F_{1,59.3} = 4.19$, $P < 0.05$; Table 1A; Fig. 1a). Conversely, while donor Ni treatment level did not influence the percentage of pollen to germinate for *S. polygaloides* (high Ni: 67 ± 3%, low Ni: 65 ± 3%), pollen germination was reduced for *S. tortuosus* plants pollinated with pollen from donor plants in the high Ni-treatment (high Ni: 65 ± 3%, low Ni: 72 ± 3%; Species x Donor Ni Treatment: $F_{1,194} = 3.33$, $P < 0.05$; Table 1A).

Relative to the low Ni treatment (67 ± 4%), there was a 34% reduction in the amount of germinated pollen to reach the ovary for *S. tortuosus* in the high Ni treatment (44 ± 4%). Alternatively, in *S. polygaloides* the amount of germinated pollen reaching the ovary was elevated for recipient plants in the high Ni treatment relative to the low Ni treatment by 23% (76 ± 4% vs. 62 ± 5%, respectively; Species x Recipient Ni Treatment: $F_{1,56.9} = 17.66$, $P < 0.01$; Table 1B; Fig. 1b). The total number of pollen grains applied and plant identity also significantly affected both metrics of pollen germination (Table 1A, B).

Neither donor nor recipient Ca treatment levels affected either metric of pollen germination (Table 1A, B).

Fruit and seed production

Both the likelihood of pollinated flowers to mature a fruit and the number of seeds produced per fruit were strongly influenced by recipient Ni treatment level (Table 1C, D; Figs. 2 and 3). For *S. polygaloides*, 74% of flowers of recipient plants in the high Ni treatment produced fruit, while only 45% of those in the low Ni treatment did. For *S. tortuosus*, 26% of flowers of recipient plants in the high Ni treatment produced fruit, compared to 81% of flowers for recipient plants in the low Ni treatment (Species x Recipient Ni Treatment: $F_{1,58.8} = 24.69$, $P < 0.01$; Table 1C; Fig. 2). While recipient high Ni treatment reduced seed production for *S. tortuosus* (high Ni: 3.5 ± 1.9, low Ni: 11.0 ± 1.4), seed production was elevated for plants in the high Ni treatment relative to recipient plants in the low Ni treatment for *S. polygaloides* by 48% (high Ni: 10.5 ± 1.3, low Ni: 7.1 ± 2.3; Species x Recipient Ni Treatment: $F_{1,47.8} = 9.4$, $P < 0.01$; Table 1D; Fig. 3). Plant identity also significantly influenced pollen germination (Table 1D).

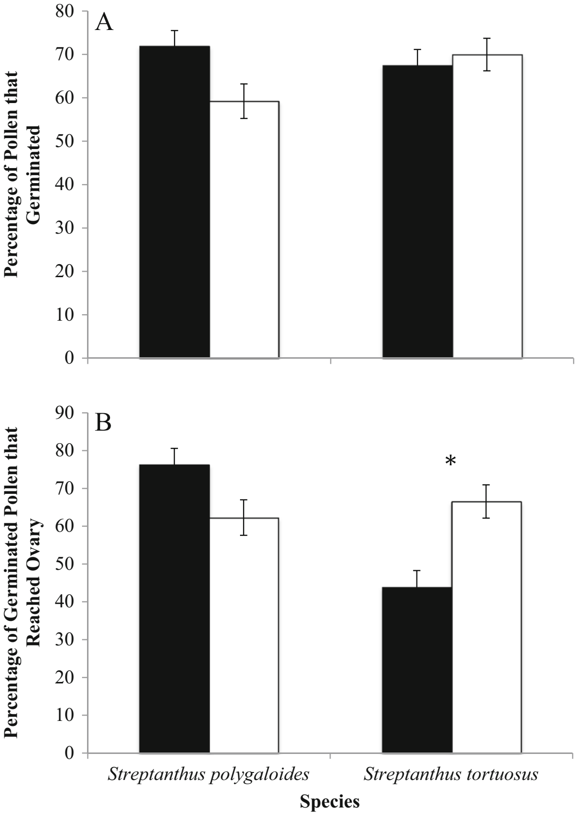

Fig. 1 The percentage of pollen that germinated (**a**) and the percentage of germinated pollen that reached the ovary (**b**) for *Streptanthus polygaloides* and *S. tortuosus* that were either grown in high-Ni (black bars) or low-Ni (white bars) soils. Bars represent lsmeans and associated standard errors. Asterisks indicate significant (i.e., adjusted *P*-value < 0.05) pairwise Tukey tests

Calcium treatment levels did not affect fruit or seed production for either species (Table 1C, D).

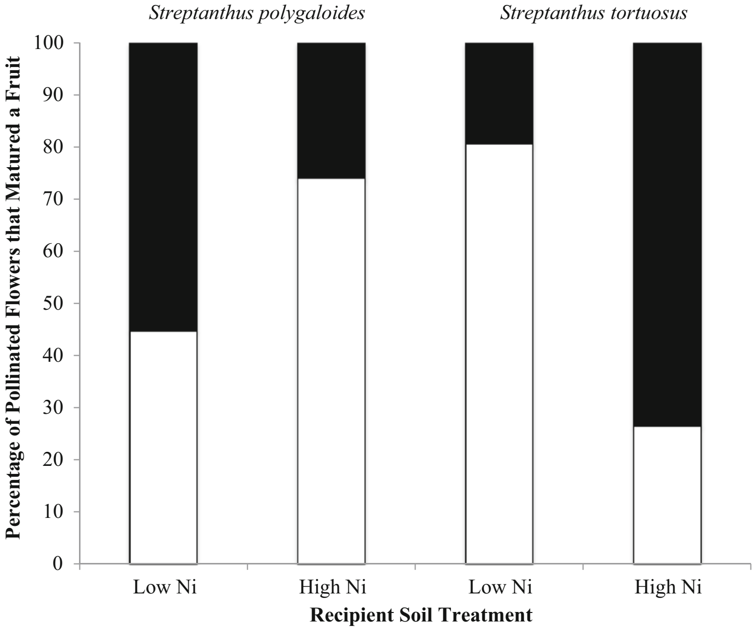

Fig. 2 The percentages of pollinated flowers that matured fruits for *Streptanthus polygaloides* and *S. tortuosus* plants that were either grown in high-Ni or low-Ni soils. White portions of bars represent flowers that matured a fruit, whereas black sections of bars represent flowers that did not mature a fruit

Discussion

Serpentine soils are an abiotically stressful growing environment for plants, in large part due to low available Ca and elevated Ni concentrations (Brady et al. 2005; Kazakou et al. 2008). While both elements in isolation are known to increase (Ca) or decrease (Ni) pollen germination and fruit and seed production (Ruane and Donohue 2007; Breygina et al. 2012), ours is the first study to simultaneously test for effects of both elements on pollen germination, fruit production, and seed production for species that are known to vary in serpentine affinity and floral metal accumulation. While Ca may generally be important for pollen grain germination and tube growth, our study suggests that the elevated Ni concentrations in serpentine soil are more likely to affect plant reproduction for non-endemic species and thus limit their ability to reproduce on these harsh soils. In addition, our study suggests that soil metals may increase the reproductive potential

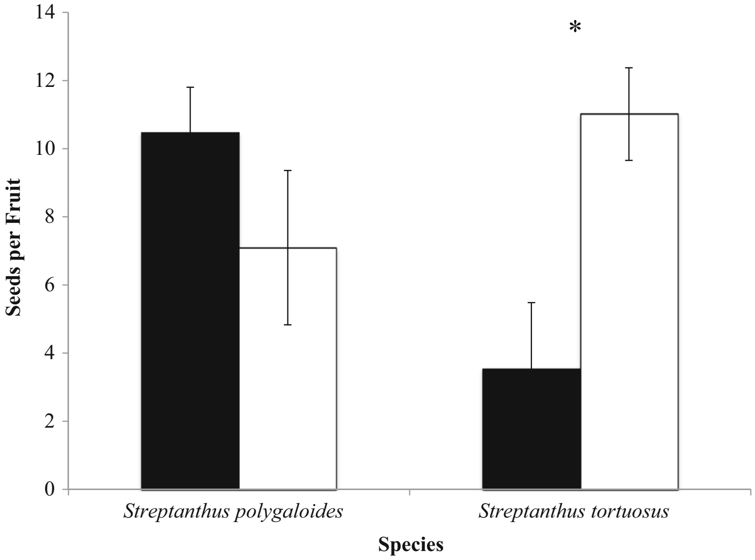

Fig. 3 The number of seeds produced per fruit for *Streptanthus polygaloides* and *S. tortuosus* plants that were either grown in Ni-treated soils (black bars) or soils that were not Ni-treated (white bars). Bars represent lsmeans and associated standard errors. Asterisks indicate significant (i.e., adjusted *P*-value < 0.05) pairwise Tukey tests

of metal hyperaccumulating plants, adding new insights to the potential adaptive value of this trait.

While Ca is known to be an important nutrient for plant reproduction (Brewbaker and Kwack 1963; Broadley et al. 2012), in this study we did not observe an effect of soil Ca treatment on pollen germination, fruit production, or seed production. Adequate Ca concentration in plant tissues varies tremendously, ranging between 1000 and 50,000 mg kg^{-1} in different species (White 2003). Tissue Ca concentrations were above 6000 mg kg^{-1} across all treatments in this study; therefore, Ca may not have been limiting for plants in any of the treatments. Furthermore, Ca and Ni may compete for uptake by plant roots, though studies have documented both negative and positive correlations between the accumulations of these two elements by plants (Robinson 1999; Chaney et al. 2008). Any potential beneficial effects of increased tissue Ca concentrations could have been negated by increased Ni concentrations, as Ca and Ni concentrations in pistils and pollen were positively correlated in this study.

Our results provide further evidence that the elevated heavy metal concentrations of serpentine soils present a significant barrier to plant colonization for non-adapted plant species (Brady et al. 2005; Kazakou et al. 2008). Here, we found that both paternal and maternal components of plant reproduction were inhibited for *S. tortuosus*, a non-endemic serpentine species, when grown in Ni-rich soils. Specifically, pollen from *S. tortuosus* plants grown in Ni-treated soils displayed decreased germination rates relative to pollen from plants grown in low-Ni soils. Furthermore, *S. tortuosus* plants grown in Ni-treated soils were less likely to produce fruits, and the fruits that were produced contained fewer seeds, relative to plants grown in low-Ni soils. Therefore, non-serpentine plants that are not adapted or tolerant to the high heavy metal concentrations of serpentine are unlikely to successfully reproduce on serpentine soils, as Ni accumulation into reproductive organs limits overall plant fitness. As heavy metals are generally known to reduce plant fitness (e.g., Hancock et al. 2012), limiting floral metal accumulation is likely a key adaptation to plant growth on serpentine soils for endemic plant species that are not known to hyperaccumulate heavy metals (DeHart et al. 2014; Meindl et al. 2014a).

Results from this study suggest that metal hyperaccumulators may directly benefit from metal hyperaccumulation in terms of increased fitness. There are several hypotheses regarding the adaptive value of metal hyperaccumulation, including defense against herbivores and pathogens, and elemental allelopathy against other plant species (reviewed in Boyd and Martens 1992; Rascio and Navari-Izzo 2011). However, recent research has suggested that hyperaccumulating species may achieve higher fitness when grown in metal-rich soils, suggesting a more direct benefit of increasing metal concentrations in aboveground tissues. For example, *Alyssum inflatum* (Brassicaceae), a Ni hyperaccumulator native to serpentine soils in Anatolia, was more likely to flower, and produced more inflorescences and more flowers, when grown in Ni-supplemented soils compared to Ni-free control soils (Ghasemi et al. 2014). In another Ni hyperaccumulating species, *Alyssum murale* (Brassicaceae), germination rates for seeds produced by maternal plants grown in high-Ni soils were twice as high compared to seeds derived from parents grown on low-Ni soils (M. McKenna, pers. comm.). Similarly, reproductive benefits have been documented for the selenium (Se) hyperaccumulator *Stanleya pinnata* (Brassicaceae), as pollen germination was observed to be higher when germinated on artificial media that contained Se, relative to media that did not (Prins et al. 2011). In this study, we found that the Ni hyperaccumulator *S. polygaloides* exhibited higher rates of pollen germination, was more likely to produce fruit, and produced more seeds per fruit when maternal plants were grown in high-Ni soils relative to low-Ni soils. The mechanism that produced these patterns is unknown, but may relate to the beneficial effects of Ni on plant N metabolism (Polacco et al. 2013). Regardless of the mechanism, however, our study provides additional evidence of the beneficial effects of metal hyperaccumulation on plant reproduction for metal hyperaccumulating species (Ghasemi et al. 2014). These patterns may help to explain why the vast majority of metal hyperaccumulating plants are entirely restricted, i.e., endemic, to soils with elevated metal concentrations (Pollard et al. 2014). Specifically, our study provides evidence that metal hyperaccumulators achieve higher fitness when grown in metal-rich soils, suggesting that they are specifically adapted to these environments (i.e., the specialist model of edaphic endemism; Meyer 1986; Palacio et al. 2007). A growing number of studies are finding that hyperaccumulating plants concentrate hyperaccumulated elements into reproductive tissues (Quinn et al. 2011; Meindl et al. 2014b), which may directly increase their fitness and help explain their patterns of distribution, often exclusively on metal-rich soils.

While soil heavy metals have been previously implicated in fostering reproductive isolation between populations on vs. off metal-rich soil indirectly via changes in floral phenology (Antonovics 2006), our study provides evidence for a more direct mechanism through which soil metals may impart reproductive isolation between populations. Specifically, we found that pollen from *S. tortuosus* plants germinated at a lower frequency when on the stigmas of plants in high-Ni soils relative to plants grown in low-Ni soils. This suggests that pollen arriving from a non-serpentine plant to the stigma of a serpentine plant is unlikely to be successful in siring progeny, as the Ni concentrations in the pistils of maternal plants may limit pollen grain germination and/or pollen tube growth towards ovules. In a series of experiments with *Mimulus guttatus* (Phrymaceae), Searcy and Mulcahy (1985) and Searcy and Macnair (1990) showed that copper (Cu) in the pistils of plants could act as a selective filter since seed production was reduced when conspecific pollen donors were not tolerant to soils with elevated Cu levels. If floral metal accumulation interferes with interspecific crosses in the same manner as was observed for conspecific crosses in this study, then floral Ni accumulation may provide a selective barrier to gene exchange between serpentine and non-serpentine populations, and thus provide a prezygotic isolating mechanism between populations that vary in floral metal concentrations and/or metal tolerance.

While our study provides compelling evidence that metals found in serpentine soils can directly impact plant fitness, there is still much to learn regarding the impacts of soil metals on edaphic tolerance and distributions of plants. In the present study, we utilized standard potting soil such that we could specifically manipulate and vary our two elements of interest: Ca and Ni. However, serpentine soils are known to deviate from non-serpentine soils in several other ways, including Ca:Mg ratios, heavy metal concentrations other than Ni (e.g., Co and Cr), and Na (Brady et al. 2005). Documenting variation in uptake of the suite of these nutrients, and associated impacts on plant reproduction, will contribute greatly to our understanding of plant distributions across chemically variable soils. Therefore, the use of field-collected soils, rather than potting soils, would allow for a more realistic assessment of the impacts of the serpentine environment on plant reproduction and fitness. Furthermore, plant species display a gradient of adaptation to serpentine soils, ranging from intolerant to indifferent to strongly endemic (Safford et al. 2005). Studies of plant fitness that include plant species from each of these three categories of plants will offer great insight into the process of serpentine adaptation and, specifically, metal tolerance.

Our results join a growing body of work that shows how soil chemistry affects plant reproduction. Soil chemistry is known to affect pollinator visitation (Meindl and Ashman 2014), flowering phenology (Gardner and Macnair 2000; Antonovics 2006), flower morphology (Gardner and Macnair 2000; Meindl et al. 2013), gamete function (Searcy and Mulcahy 1985; Searcy and Macnair 1990; this study), and pollen interactions on the style (Wipf et al. 2016). Taken together these studies indicate a key role for soil chemistry, specifically soil metals, in spurring adaptation in plants.

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