Calcium : magnesium ratio affects environmental stress sensitivity in the serpentine-endemic *Alyssum inflatum* (Brassicaceae)

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Abstract. Plants endemic to serpentine soils are adapted to harsh environmental conditions typical of those soils, particularly, low (<1) calcium (Ca) : magnesium (Mg) ratios. We compared survival of two perennial *Alyssum* species native to Iran under experimental manipulations of Ca : Mg ratio, including when Ca : Mg ratio was varied under conditions of high ammonium concentration and heat stress. *Alyssum inflatum* is a serpentine endemic capable of nickel (Ni) hyperaccumulation, whereas *A. lanceolatum* is found on non-serpentine soils and is not known to hyperaccumulate Ni. We grew plants of both species under four Ca : Mg ratios (0.4, 2, 20, 40) and tested survival when plants were exposed to elevated ammonium levels (0, 1 and 4 mM) and heat stress (control conditions vs a 5-h 36°C treatment daily for 5 days). *Alyssum lanceolatum* was more tolerant of Ca : Mg ratio variation (100% survival in all treatments), whereas *A. inflatum* survival was maximum at Ca : Mg = 2, reduced at Ca : Mg = 0.4, and very low for Ca : Mg ratios of 20 and 40. *Alyssum lanceolatum* also tolerated ammonium and heat stress, whereas survival of *A. inflatum* declined at higher Ca : Mg ratios when subjected to both stresses. We conclude that at higher Ca : Mg ratios, the serpentine endemic has reduced tolerance for these environmental stresses and may be more susceptible to human-driven climate change-associated stressors than the non-serpentine species.

Additional keywords: global climate change, heat stress, local adaptation, nitrogen deposition, serpentine endemism.

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Introduction

Soils are important habitats for many organisms; the physical and chemical properties of soils vary greatly and this variation can be an important factor that determines the distribution of species across the face of the planet (Rajakaruna and Boyd 2008). Serpentine soils are an excellent example of this principle (Harrison and Rajakaruna 2011). These soils develop from parent material generally classified as ultramafic rocks, having high concentrations of iron (Fe) and magnesium (Mg) and low concentrations of silicon (Si), and may contain elevated concentrations of some heavy metals such as cobalt (Co), chromium (Cr) or nickel (Ni) (Alexander *et al*. 2007). As a consequence of the chemistry of the parent rocks, serpentine soils possess a suite of characteristics (termed the serpentine syndrome; Kruckeberg 1984) that challenge the growth of many maladapted plant species. These features often include a low calcium (Ca) : magnesium (Mg) ratio, high levels of some heavy metals and, in Mediterranean regions, these soils are often

comparatively shallow and stony and more likely to create drought stress (Brady *et al*. 2005; Kazakou *et al*. 2008). Tolerance of a low soil Ca : Mg ratio is considered to be a significant feature of serpentine adaptation (Bradshaw 2005; Palm *et al*. 2012). However, how serpentine-adapted species tolerate low Ca : Mg ratios is still not well understood (see O'Dell and Rajakaruna 2011), although it is clear that the low Ca : Mg ratio severely limits the growth of plants not adapted to serpentine soil (Palm and Van Volkenburgh 2014).

The challenging environmental conditions of serpentine soils have promoted the evolution of ecotypes or species that possess adaptations to those conditions (Kay *et al*. 2011; O'Dell and Rajakaruna 2011). In fact, some species adapted to serpentine soils are sufficiently specialised so that they are endemic to them (Anacker 2011, 2014). Serpentine endemism may be an important facet contributing to global biodiversity; for example, in California (USA), a global biodiversity hotspot (Myers *et al*. 2000), there are 246 taxa from 103 genera and 41 families

endemic to serpentine soils (Safford *et al.* 2005; Anacker *et al.* 2011). Many serpentine endemics are known from other locations as well (e.g. Brooks 1987; Rajakaruna *et al.* 2009; Van der Ent *et al.* 2014).

The rapid expansion of human population on the planet over the past several hundred years is affecting habitats all over the globe. Many species have been or are being threatened by these impacts, so that some authors argue that we are facing a species extinction event of a magnitude that may rival the past five major extinction events through geologic time (Pievani 2014). Many factors are involved; however, two important factors include changes to climate (global climate change) and changes to the nitrogen (N) cycle. Global climate change includes changes in temperature, which in many areas of the planet is predicted to increase (Warren *et al.* 2011; Stocker *et al.* 2013). Nitrogen is an important nutrient that limits primary productivity in many natural (as well as agricultural) systems; however, planet-wide N fixation by humans has recently been estimated to exceed that of all natural sources on Earth (Galloway *et al.* 1995; Fowler *et al.* 2013).

How plant species in special edaphic situations (including serpentine soils) will be affected by human-caused changes is not known and is under active investigation (Harrison *et al.* 2009; Fernandez-Going 2014). As summarised by Damschen *et al.* (2012), climate change, including changes in temperature and precipitation, may have relatively pronounced effects on serpentine-endemic plant species. Effects of N deposition on serpentine species are less investigated, but Vallano *et al.* (2012) concluded that N deposition on a California serpentine grassland increased the vulnerability of the habitat to invasion by exotic species. Kazakou *et al.* (2008) pointed out that many factors may limit plant growth on serpentine soils, including low N concentrations. Although the situation on Iranian serpentine soils has not been investigated, some studies in temperate-zone serpentine areas (such as California, USA) have reported that N limitation (e.g. O'Dell and Claassen 2006) may be an important factor.

The present study explored the effects of Ca : Mg ratio, along with heat and N (in the form of ammonium) stress, on two *Alyssum* species, one of which is a serpentine endemic and the other a species found on non-serpentine soils. Our goal was to compare the Ca : Mg ratio responses of the two species and then to determine how Ca : Mg ratio may affect the response of each species to N enrichment or temperature stress, two key imminent environmental stressors worldwide.

Materials and methods

Study species

Alyssum inflatum Nyárády (Brassicaceae) is a serpentine-endemic plant from western Iran, belonging to section *Odontarrhena* (Ghasemi and Ghaderian 2009; Ghasemi *et al.* 2009*a*, 2009*b*). As a perennial, it is a fitting plant for long-term experiments and to examine survival and flowering under controlled conditions (Ghasemi *et al.* 2014). Seeds of *A. inflatum* were harvested from plants growing on serpentine soils of western Iran (35°13.625′N, 46°27.184′E) in September 2009 and stored at 4°C for at least 3 months to break dormancy.

Approximately 50 000 seeds were collected as a bulk sample from ~70 plants and mixed thoroughly.

The non-serpentine congener, *A. lanceolatum* Baumgartner, was used as a comparative species. It is also a perennial, but is classified in section *Alyssum* (Hedge and Rechinger 1968). Seeds of *A. lanceolatum* were harvested from plants growing on non-serpentine soils of central Iran located in the Karkas Mountains (33°32.364′N, 51°47.015′E). About 20 000 seeds were collected from ~100 plants. All seeds were stored at 4°C for at least 3 months to break dormancy.

Field soil conditions

Soil samples were collected from the two field sites to characterise their Ca : Mg ratios. At each site, soil was collected down to 20–30-cm depth near the locations of plants; 12 samples were collected from the *A. inflatum* serpentine soil and three from the *A. lanceolatum* non-serpentine soil.

Sample preparation for measurement of total and extractable soil concentrations of elements was based on the method of Reeves *et al.* (1999). For measurement of total elements, soil samples of 4–5 g were ground to pass through an 80-mesh (<190 μm) sieve and then oven-dried at 70°C. A further subsample of 0.5 g was transferred to a digestion tube for interaction with 10 mL of a 3 : 1 HCl–HNO_3 mixture. Tubes were left at room temperature overnight and were then placed in a heating block. Each tube was refluxed gently at 80°C for 2 h. After cooling, the digests were filtered through moistened Whatman No. #40 filter paper into a 50-mL volumetric flask. Flasks were then brought up to volume with distilled water. Measurement of Ni, Mg and Ca concentrations was performed by atomic absorption spectrophotometry (AAS, Philips, model: PU9100X, The Netherlands).

To measure the concentrations of ammonium nitrate-extractable soil elements, samples were prepared according to Gryschko *et al.* (2005). First, 20 g of air-dried (<2 mm sieved) soil was placed in a 100-mL screw-cap polythene bottle, 50 mL of 1 M NH_4NO_3 solution (pH 6) was then added, and the suspension was shaken for 2 h at 20°C in an end-over-end shaker. After shaking, soil suspensions were left to stand for 5 min and then filtered (Whatman No. #42 filter paper) into a clean bottle. Filtrates were then acidified with 0.2% HNO_3 for measurement of elements by AAS.

General experimental procedures

Experiments were performed by using solution culture infiltrated into the chemically inert substrate, perlite. Pots (450 mL) were filled with perlite and eight seeds were sown in each pot. After germination, seedlings were watered with modified Hoagland solution (pH 7), containing 1 mM $Ca(NO_3)_2$, 0.1 mM KH_2PO_4, 0.5 mM $MgSO_4$, 0.5 mM KNO_3, 0.2 μM $CuSO_4$, 0.2 μM $ZnSO_4$, 2 μM $MnSO_4$, 10 μM H_3BO_3, 0.1 μM Na_2MoO_4, 2 μM NaCl and 5 μM ferric ethylenediamine-di-2-hydroxyphenylacetate (FeEDDHA). Pots were placed in three replicate trays (one pot per tray) and Hoagland solution was poured into the trays (400 mL per tray). Evaporation was compensated by daily addition of distilled water to the solutions to keep them at a constant volume. The solutions under the pots were replaced every 5 days.

Experiments were performed in a semi-controlled glasshouse under natural light. Minimum and maximum temperatures were 23°C and 36°C in spring and summer, and 15°C and 25°C in late autumn and winter, respectively.

Survival of plants was determined by counting the number of live plants in each treatment category. Plants with wilted shoots were tested for living root cells by using Evan's blue stain (Mergemann and Sauter 2000). The roots were cut in lengths of ~5 cm (measured from the root tip) and were submerged in a 2% stain solution for 2 min. Stained roots were washed with the nutrient solution to prevent possible damage of cells via extreme change in osmotic pressure if distilled water was used. Staining of the root cells, which is a sign of high permeability and loss of function of plasma membrane and cell death, was checked using a brightfield microscope. Wilted plants whose roots took up stain were considered to be dead, and the date of death was recorded.

Ca : Mg ratio experiment

We used 1-month-old plants of both species for this experiment (24 plants per treatment: 3 pots × 8 plants per pot). We added varying amounts of Ca nitrate to the nutrient solutions (Ca concentrations of 0.2, 1, 10 and 20 mM) to yield a wide range of Ca : Mg ratios (0.4, 2, 20 and 40, respectively). Effects of Ca : Mg ratio on plants were determined at 25-day intervals for a period of 250 days (~8 months), beginning in early June and ending in January of the following year. The date on which a plant showed visible stress symptoms (wilting and drying of leaves) was recorded and the Evan's blue stain technique was used to confirm date of death for each plant that died during the experiment.

Ammonium stress experiment

Two factors, ammonium and Ca : Mg ratio, were manipulated in this experiment, using ammonium nitrate in concentrations of 0, 1 and 4 mM, and Ca nitrate in concentrations of 1, 5 and 10 mM (producing Ca : Mg ratios of 2, 10 and 20, respectively). These Ca : Mg ratios were chosen to include the optimum ratio for survival of *A. inflatum* (Ca : Mg = 2, as revealed by the Ca : Mg ratio experiment; see Results below), as well as two much greater ratios. Three replicates of each treatment (24 plants per treatment: 3 pots × 8 plants per pot) were used in a factorial complete block experimental design. This experiment was performed during autumn and winter (starting late September and finishing in early January), so that plants experienced relatively low average temperatures (compared with spring and summer).

Heat-stress experiment

The experiment examining the influence of Ca : Mg ratio on survival when plants were heat stressed used two concentrations of Ca, 1 mM and 10 mM, to produce solution Ca : Mg ratios of 2 and 20, respectively. As with the ammonium-stress experiment, the Ca : Mg ratios included the optimum for *A. inflatum* survival (Ca : Mg = 2) as well as a much higher ratio for comparison. Seeds were germinated in late September, using the basic medium, and 1-month-old plants (24 plants per treatment: 3 pots × 8 plants per pot) were placed in either of the two Ca : Mg ratio treatment conditions for 40 days. In late

November, plants were transferred to a growth cabinet with 16/8 h light/dark and 28°C/22°C temperature cycle for 2 days, and the Ca : Mg treatments were maintained for the duration of the experiment. After the 2-day acclimation period, during the following week, the temperature in the growth cabinet was raised to 36°C for 5 h during the light photoperiod. At the end of the week-long daily heat treatment, plants were evaluated as alive or dead, on the basis of the Evan's blue stain test of the roots of wilted plants.

Statistical analyses

In the Ca : Mg ratio experiment, *A. lanceolatum* plants grew well in all treatments and did not show signs of stress. Stress was observed only for *A. inflatum* plants. Therefore, we analysed data from *A. inflatum* to determine whether Ca : Mg ratio significantly affected the time it took for stress to result in plant mortality. Survival among treatments was compared with survival analysis, using the Kaplan–Meier estimate and the Peto–Peto–Wilcoxon test in StatView 5.0 (SAS Institute 2005).

For the ammonium-stress experiment, effects of the Ca : Mg ratio on plant survival were also analysed by survival analysis (SAS Institute 2005) for those ammonium treatments for which at least some plant mortality occurred. In those cases, we compared survival of plants to determine whether Ca : Mg ratio affected results. If it did, we then compared results for specific Ca : Mg treatments from the same ammonium treatment, using survival analysis in a pairwise fashion. We compared data from ratios of 2 and 10, as well as 10 and 20, to determine which result was contributing most to the overall significant Ca : Mg ratio effect.

For the heat-stress experiment, survival of plants in each pot after the week-long period of daily heat treatments was calculated as a decimal fraction and the resulting values were arcsine square-root transformed to ensure a more normal data distribution (Zar 1996). Transformed survival fractions were analysed using two-way ANOVA in StatView 5.0 (SAS Institute 2005), with Ca : Mg ratio and *Alyssum* species as the two main factors, and including the interaction term.

Results

Field soil conditions

As expected, the serpentine soil had a low Ca : Mg ratio and high concentrations of Ni compared with non-serpentine soil. Mean (s.e. in parentheses) Ca : Mg ratios for the 12 soil samples collected from serpentine sites in western Iran were 0.041 (0.004) for total Ca : Mg ratio and 1.6 (0.17) for ammonium nitrate-extractable Ca : Mg ratio, with total Ni concentrations of 1600 (38) mg kg^{-1}, and ammonium extractable of 1.6 (0.10) mg kg^{-1}. The Ca : Mg ratios for the three soil samples collected from the non-serpentine sites were 5.1 for the total Ca : Mg ratio and 14.5 for ammonium nitrate-extractable Ca : Mg ratio, with total Ni concentrations of 17 mg kg^{-1} (2.7) and ammonium extractable of 0.69 mg kg^{-1} (0.18).

Ca : Mg ratio experiment

The non-serpentine species, *A. lanceolatum*, did not show stress symptoms in any treatments and suffered no mortality (Fig. 1). In contrast, Ca : Mg ratio significantly affected survival of the serpentine species, *A. inflatum* (Peto–Peto–Wilcoxon test,
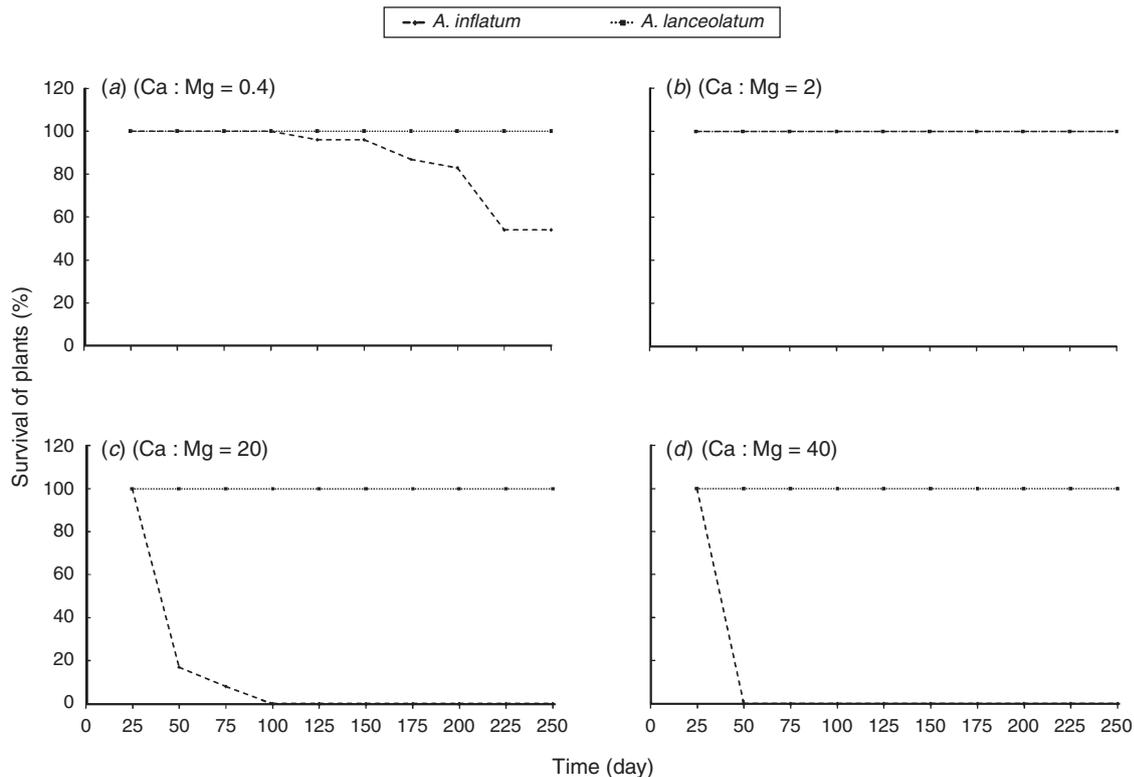
Fig. 1.   Effect of varying calcium (Ca) : magnesium (Mg) ratio on survival of serpentine *Alyssum inflatum* and non-serpentine *A. lanceolatum*. (*a*) Ca : Mg = 0.4, (*b*) Ca : Mg = 2, (*c*) Ca : Mg = 20 and (*d*) Ca : Mg = 40.

$\chi^2 = 109$, d.f. = 3, $P < 0.0001$; Fig. 1). At 250 days, no plants from the Ca : Mg = 2 treatment had died, whereas almost half (46%) of plants from the Ca : Mg = 0.4 treatment had died. Survival analysis showed that these responses were significantly different (Peto–Peto–Wilcoxon test, $\chi^2 = 11$, d.f. = 2, $P = 0.008$). High Ca : Mg ratios resulted in much more rapid mortality; comparison of data from Ca : Mg = 2 and Ca : Mg = 20 showed a highly significant difference (Peto–Peto–Wilcoxon test, $\chi^2 = 53$, d.f. = 1, $P < 0.0001$). Data from the Ca : Mg ratio = 40 treatment showed the most rapid onset of stress symptoms; comparison with data from Ca : Mg = 20 showed a significant difference (Peto–Peto–Wilcoxon test, $\chi^2 = 4.3$, d.f. = 1, $P = 0.039$).

Ammonium-stress experiment

The two species responded to these treatments very differently. No mortality was observed for the non-serpentine *A. lanceolatum* under any treatment in the experiment. In contrast, the serpentine endemic *A. inflatum* experienced no or low mortality for all Ca : Mg ratios under control (no ammonium) conditions, whereas mortality increased as Ca : Mg ratio increased for both 1 and 4 mM ammonium concentrations, with greatest mortality documented for plants in the highest Ca : Mg ratio and ammonium concentrations (Fig. 2).

For 1 mM ammonium, survival analysis indicated an overall Ca : Mg ratio treatment effect (Peto–Peto–Wilcoxon test, $\chi^2 = 49$, d.f. = 2, $P < 0.0001$), but there was no mortality at the lowest Ca : Mg ratio and very little for the Ca : Mg = 10 treatment; these

results did not differ significantly (Peto–Peto–Wilcoxon test, $\chi^2 = 0.66$, d.f. = 1, $P = 0.415$). However, at Ca : Mg = 20, mortality was 19% by the end of the experiment and survival analysis showed that the survival curves of the Ca : Mg = 10 and Ca : Mg = 20 treatments differed significantly (Peto–Peto–Wilcoxon test, $\chi^2 = 21$, d.f. = 2, $P < 0.0001$).

At 4 mM ammonium (Fig. 2), mortality was observed for only one plant in the entire experiment at Ca : Mg = 2; however, mortality was much more severe as Ca : Mg increased. Survival analysis showed a significant Ca : Mg effect for the entire 4 mM ammonium dataset (Peto–Peto–Wilcoxon test, $\chi^2 = 37$, d.f. = 2, $P < 0.0001$), but again, comparison of data from the treatments with Ca : Mg = 2 and Ca : Mg = 10 showed no significant difference (Peto–Peto–Wilcoxon test, $\chi^2 = 1.5$, d.f. = 1, $P = 0.225$). As with the 1 mM ammonium treatment, low survival of plants in the Ca : Mg = 20 treatment was driving the overall significant Ca : Mg effect, as shown by the highly significant difference between survival of plants in Ca : Mg = 10 and Ca : Mg = 20 treatments (Peto–Peto–Wilcoxon test, $\chi^2 = 15$, d.f. = 1, $P = 0.0001$).

Heat-stress experiment

Two-way ANOVA showed significant effects of all factors, namely, Ca : Mg ratio, species and the interaction ($F_{1,8} = 20$, $P = 0.0021$, for each factor and the interaction). All plants of both species survived after the week-long heat-stress period in the Ca : Mg ratio = 2 treatment (Fig. 3), whereas, for the Ca : Mg ratio = 20 treatment, all non-serpentine plants (*A. lanceolatum*)
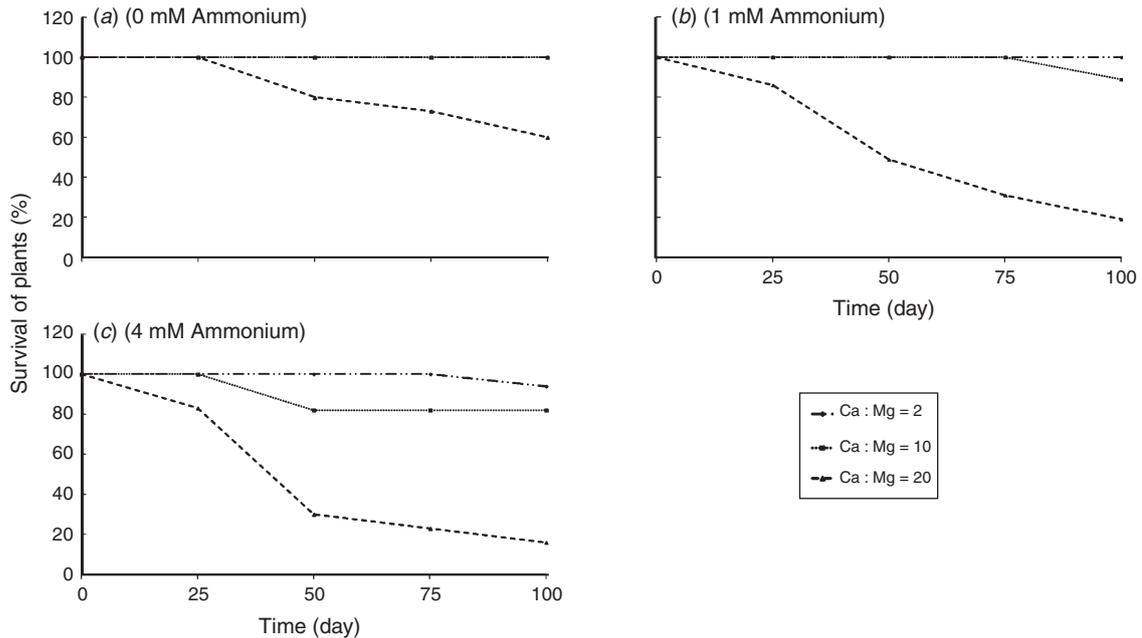
Fig. 2. Effect of ammonium treatments on survival of serpentine *Alyssum inflatum* grown at three calcium (Ca) : magnesium (Mg) ratios (Ca : Mg = 2, 10 and 20). (*a*) 0 mM ammonium, (*b*) 1 mM ammonium and, (*c*) 4 mM ammonium.
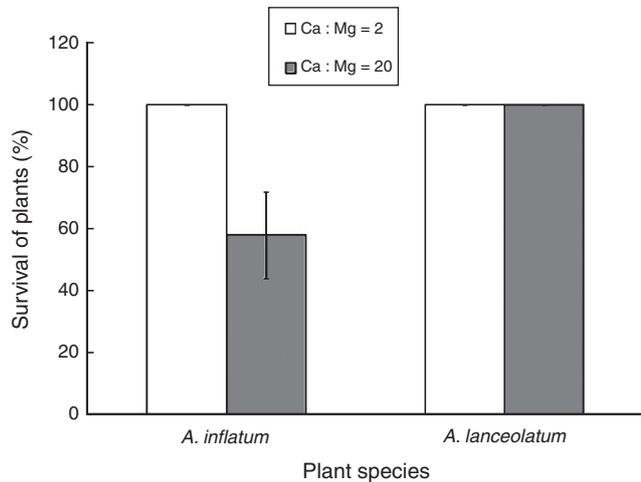

Fig. 3. Survival of serpentine *Alyssum inflatum* and non-serpentine *A. lanceolatum* after a week-long period of daily 5-h heat-stress treatments when grown at two calcium (Ca) : magnesium (Mg) ratios (Ca : Mg = 2 and 20). Error bars represent s.e. (bar present only for *A. inflatum* at Ca : Mg = 20, because survival was 100% for other bars).

survived, but only 63% of plants of the serpentine species (*A. inflatum*) survived (Fig. 3).

Discussion

Calcium and Mg are macronutrients (Epstein and Bloom 2004; Marschner 2011) and low Ca : Mg ratios, especially those <1 characteristic of serpentine soils, represent a physiological stress to most plants (Brady *et al.* 2005); plants grow best when the Ca : Mg of the soil is close to unity or greater (Lee 1998). A high proportion of plant Ca is located in the cell wall, bound to pectins

in the middle lamella (White and Broadley 2003; Marschner 2011). Pectins play an important role in plant cell-wall stability and cell-to-cell adhesion (Jarvis 1984; Jarvis *et al.* 2003). Typical symptoms of Ca deficiency are cessation of cell-wall extension, disintegration of cell walls and tissue collapse (necrosis) as a result of pectin breakdown (Demarty *et al.* 1984; Jarvis 1984; Marschner 2011). These symptoms are often seen in actively growing tissues, such as root tips, new shoots and leaves. The exceedingly low Ca : Mg ratios (often <0.7; Rajakaruna *et al.* 2009) typical of serpentine soils (and found in our analysis of the *A. inflatum* serpentine soil) require specialised physiological mechanisms to maintain adequate internal concentrations of Ca. Such mechanisms include a greater acquisition of Ca (Wallace *et al.* 1982; Tibbetts and Smith 1993; Asemaneh *et al.* 2007) and the exclusion of Mg (Madhok and Walker 1969; Sambatti and Rice 2007). Some serpentine-adapted plants are indifferent to elevated Mg (Rajakaruna *et al.* 2003; Asemaneh *et al.* 2007), whereas others have a greater Mg requirement (Madhok 1965; Madhok and Walker 1969).

Whatever the strategy the plants use to deal with the low Ca : Mg ratios typical of serpentine soils, our study showed that the serpentine-endemic species may have a narrow range of tolerance to soil Ca : Mg ratio. The endemic *A. inflatum* did best at Ca : Mg = 2, a ratio close to the extractable Ca : Mg ratio (1.67) of the serpentine soils from where the seeds were collected. Although it was clearly tolerant of the low (0.4) ratio, almost half (46%) of plants from the Ca : Mg = 0.4 treatment died within 7 months. It was also exceedingly sensitive to the high Ca : Mg ratios in our experiment, showing high mortality after about 1 month from the start of the experiment. Interestingly, the non-serpentine species was broadly tolerant, showing no mortality under the entire range of Ca : Mg ratios (including 0.4, a ratio typical of serpentine soils). It is, therefore, surprising to

not find *A. lanceolatum* on serpentine soils in Iran. Perhaps this is an indication of its lack of tolerance to other serpentine-related stressors, including heavy metals or drought.

Our study is the first effort exploring how plant performance under a range of Ca : Mg ratios can be influenced by N deposition and temperature rise, two environmental stressors that threaten some habitats worldwide. Nitrogen deposition is a severe threat to those plants adapted to low-N habitats and has had drastic effects on native plant diversity not only on serpentine outcrops (Going *et al.* 2009; Vallano *et al.* 2012; Eskelinen and Harrison 2014) but also in arid deserts in California (Schneider and Allen 2012), limestone forests in the Alps (Hülber *et al.* 2008) and N-limited bogs in New England, USA (Gotelli and Ellison 2002), primarily by contributing to invasions by high N-loving exotic grasses. Our study showed that increased N, in addition to encouraging the colonisation of high N-requiring invasive species, may also influence how serpentine-endemic species may respond to Ca : Mg ratios. The serpentine-endemic species was increasingly sensitive to the high-N treatments under the high Ca : Mg ratios, suggesting that under nutrient-rich conditions (i.e. high N and Ca), serpentine-adapted plants can be outcompeted by those species able to thrive under such nutrient-enriched conditions. Calcium is known to stimulate the absorption of ammonium in plants (Fenn *et al.* 1995; Fenn and Feagley 1999); therefore, the increased Ca : Mg ratio, under the higher ammonium concentrations, may have contributed to higher N in plant tissue, a feature that was beneficial to the non-serpentine species, but may have caused toxicity in the low N-adapted serpentine endemic. It is possible that the higher mortality was also induced by greater ionic strength in the solution (in response to increases in Ca and ammonium), in addition to specific ion effects of Ca and ammonium. Therefore, future experiments should maintain the ionic strength (i.e. osmotic conditions) of the treatment solutions by altering the concentrations of Ca and Mg, to confirm whether the increased mortality of the endemic taxon is in response to increased ammonium. However, it is interesting to note that the serpentine endemic was much more sensitive to the increases in Ca and ammonium than was the non-serpentine species.

Temperature increase, when combined with an increase in the Ca : Mg ratio, also appeared to have a detrimental effect on the serpentine endemic compared with its non-serpentine congener. Calcium uptake is stimulated under high temperature (Klein and Ferguson 1987); therefore, it is possible that the exposure to high temperature led to an increase in Ca uptake under the higher Ca : Mg ratios, a feature that was again beneficial to the non-serpentine taxon better adapted to deal with the higher Ca concentrations. Increased Ca uptake under higher temperature may have contributed to Ca toxicity in the serpentine endemic, leading to higher mortality under the high temperature and Ca : Mg treatments. Increased Ca is also known to aid in heat tolerance (Gong *et al.* 1998; Jiang and Huang 2001); therefore, in the taxon adapted to higher Ca, the increased Ca concentration may also have contributed to higher temperature tolerance. However, to confirm whether higher temperatures increase the Ca sensitivity of the serpentine-endemic species compared with its non-serpentine congener, both species should be grown at a constant high temperature (e.g. 25°C) and exposed to high Ca : Mg ratios (e.g. 20 and 40) in future studies.

It is generally accepted that competition plays a key role in restricting slow-growing and stress-tolerant species to less-fertile (low N, P) and ionically imbalanced (<1 Ca : Mg ratio) serpentine soils (Moore and Elmendorf 2011); however, the experimental evidence for the role of competition in such edaphic restrictions is mixed (Fernandez-Going and Harrison 2013). Recently, there has been much interest in examining how edaphic factors will interact with climatic factors (temperature and precipitation) in generating and maintaining patterns of edaphic endemism (Harrison *et al.* 2009; Fernandez-Going *et al.* 2013). Experimental evidence is limited (Anacker 2014), but is required to determine the mechanistic basis for how climate can influence edaphic restriction. Our study explored how a serpentine-adapted species and its non-serpentine congener respond under a range of Ca : Mg ratios to differing N and temperature regimes. The results suggested that the serpentine endemic has an extremely narrow range of tolerance to both edaphic (Ca : Mg and N) and climatic (temperature) variables tested, compared with its non-serpentine congener. Although the study was performed using artificial substrate (i.e. chemically amended perlite) and plants were not grown together to directly test for competition, our study suggested that in a warmer and N-rich future, the non-serpentine species could potentially outcompete the serpentine-endemic species, even on serpentine soils, and especially if it is tolerant of other serpentine-associated stressors (such as heavy metals). At the same time, the serpentine endemic will not be able to move into non-serpentine habitats because those soils are generally richer in nutrients and, as our study documents, it is maladapted to high-nutrient conditions, especially high concentrations of Ca and N.

A conceptual model of climate-edaphic influences on the future of edaphic floras (Harrison *et al.* 2009) predicts that, in regions where climate becomes warmer and wetter, serpentine endemics will be outcompeted by soil generalists. In this scenario, endemics will be restricted to the 'harshest' serpentine soils and become less common; this is clearly possible in the case of the *Alyssum* congeners we studied. The non-serpentine taxon is clearly better equipped to compete in high-Ca and -N soils under warming conditions and could potentially move into serpentine habitats in the future, especially if the soils become higher in N (via atmospheric N deposition; Phoenix *et al.* 2012) under the warming climate. The model also predicts that in regions where climate becomes warmer and drier, serpentine endemics will be less adversely affected than their non-serpentine congeners because the endemics are generally more adapted to drier conditions often characteristic of serpentine soils. In such cases, it is possible that endemics will expand their ranges into non-serpentine soils, even outcompeting soil generalist species. However, this appears to be an unlikely scenario in our case, at least with respect to temperature, because the endemic was less fit under higher temperature and in soils with higher Ca concentrations. It is also unclear how our congeners will respond to varying levels of water stress, because we did not test their relative performance under varying moisture treatments. Therefore, future studies should take into account temperature, moisture and nutrient enrichment and grow both taxa directly on and off serpentine soils, testing their relative performance under varying climatic and edaphic conditions. Such a study would add to the very limited number of experimental studies exploring how

climate change can influence patterns of serpentine endemism (Anacker 2014; Fernandez-Going 2014).

Acknowledgements

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