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Inducible tolerance to low Ca:Mg in serpentine ecotype of *Erythranthe guttata*

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ABSTRACT

In serpentine soils, the low level of calcium relative to magnesium (Ca:Mg) is detrimental to the growth of most plant species. Ecotypic variation in *Erythranthe guttata* allows for some populations to maintain high photosynthetic rates and biomass despite low Ca:Mg. In this study, the mechanism of tolerance was investigated by treating hydroponically grown plants with either high (1.0) or low (0.02) Ca:Mg growth solutions and assaying excised leaf discs for rates of photosynthesis and disc expansion, and for starch, Ca^{2+} and Mg^{2+} ion concentrations. Low Ca:Mg in the assay solutions reduced both photosynthesis and leaf disc expansion after one week of treatment. However, serpentine tissues show stable photosynthetic rates after one week and a recovery in leaf tissue expansion after two weeks exposure to low Ca:Mg conditions. Values for non-serpentine tissues continued to decline. Increased growth of low Ca:Mg treated discs supplied with exogenous sucrose suggests that growth in serpentine-exposed tissues is limited by availability of carbon products from photosynthesis. Serpentine leaves had higher vacuole Mg concentrations than non-serpentine leaves after three weeks of treatment with low Ca:Mg. The combination of elevated starch concentrations, reduced growth and lower vacuolar Mg concentrations in leaves of non-serpentine plants grown in low Ca:Mg indicate an inefficient use of carbon resources and starch degradation as an observed response to Mg toxicity. Together, these results suggest that serpentine *E. guttata* exhibits an inducible tolerance to low Ca:Mg through gradual compartmentalization of magnesium to maintain the production and metabolism of photosynthates necessary for growth.

1. Introduction

Serpentine soils are described as having low productivity, overall poor nutrient quality, low calcium-to-magnesium ratios, and high concentrations of heavy metals such as copper and zinc (Brooks, 1987; Kruckeberg, 2002; Brady et al., 2005). This set of physiochemical properties dramatically reduces the growth of non-tolerant plant species (Jenny, 1980; Kazakou et al., 2008), making serpentine soils a valuable system in which to study mechanisms of physiological adaptation (Eskandari et al., 2017; Meindl et al., 2021) and population genetics (Konečná et al., 2020). Low calcium:magnesium (Ca:Mg) itself is an abiotic factor that is inhibitory to the growth and persistence of many plant species (Sambatti and Rice, 2007; Harrison and Rajakaruna, 2011; Ghasemi et al., 2015). Similar to tolerance mechanisms found among plants exposed to salt stress (Wang et al., 2023; Delatorre-Herrera et al., 2021), the physiological mechanisms involved in adaptation to serpentine soil may be aided by Ca- and Mg-stress induced responses that differ between serpentine adapted and non-adapted populations. Ca and Mg are two essential divalent macronutrients for plants and symptoms of deficiency for both are well known. Ca is fundamental to growth of new tissues (White and Broadley, 2003) and as a signaling molecule for a wide variety of abiotic and biotic signals, including nutrient homeostasis (Behera et al., 2017; Tang and Luan, 2017) and pathogens (Monaghan et al., 2014). Mg is essential for photosynthesis as the central atom of chlorophyll molecules and as a key element modulating the rate of CO2-fixing RuBP carboxylase (Shaul, 2002). Reductions in photosynthetic rate, followed by declines in growth are evident when Mg is deficient in the soil (Meng et al., 2023; Farhat et al., 2015). The two ions cooperate to maintain ionic homeostasis within plant cells, including the induction of transient cytosolic peaks of Ca in response to elevated

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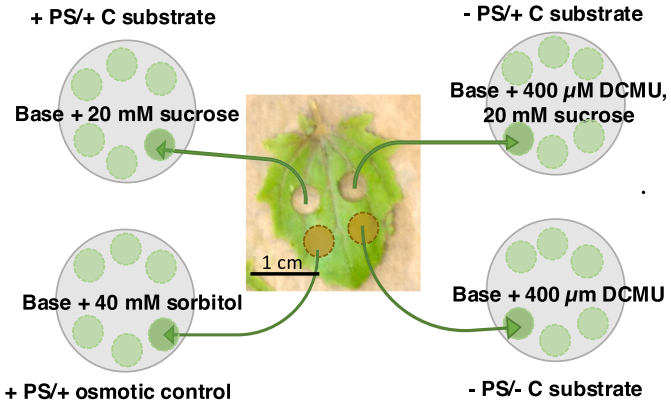
external Mg by coordinated release of Ca from the vacuole (Tang et al., 2015). When the Ca:Mg ratio of the growth medium falls below 0.7, as in the case of serpentine soil (Hewawasam et al., 2014), internal concentrations of Ca and Mg may reach levels of deficiency and toxicity, respectively, without the presence of ion-selective transport or sequestration mechanisms. Plant species that are tolerant to the extreme ion imbalances characteristic of serpentine soils (low Ca, high Mg, high heavy metals) are often found to cope through mechanisms of exclusion and sequestration. This is especially true for the heavy metals commonly found in serpentine soils. Some species avoid toxic accumulation of heavy metals, such as *Odontarrhena obovate* (Briq.) Cecchi & Selvi and *Pistacia atlantica* Desf., that restrict uptake of Cu and Ni at the roots (Tripti et al., 2021; Pakdaman et al., 2011), or limiting translocation of Zn and Ni to the shoots as in the serpentine-adapted *Thlaspi arvense* L. (Seregin et al., 2014). The low Ca:Mg of serpentine soil systems appears to be more complex and few studies have thoroughly described the mechanism(s) of Ca and Mg homeostasis under these conditions. Comparisons between tolerant populations with related non-tolerant ecotypes and species have attempted to elucidate a mechanism of tolerance: 1) selective uptake of Ca and Mg to avoid deficiency and toxicity, respectively (Palm et al., 2012; Walker et al., 1955); 2) amelioration or adjustment of internal concentrations of Ca and Mg through ion sequestration (Vicić et al., 2014; O'Dell et al., 2006); and 3) tolerance to or requirement for either elevated or suboptimal levels of these two macronutrients (Chatzistathis et al., 2019; Kolář et al., 2014; Madhok and Walker, 1969). In our previous study, we found that the serpentine-tolerant ecotype of *Erythranthe guttata* (Fisch. DC.) G. L. Nesom (formerly *Mimulus guttatus* Fisch. ex DC.) did not exclude Mg to a

(A)

(B)

Components	(Non serpentine) High Ca:Mg (mmol L-1)	(Serpentine) Low Ca:Mg (mmol)
Macronutrients		
$CaNO_3$ ($4H_2O$)	1	0.025
$MgNO_3$ ($6H_2O$)	0	0.975
$MgSO_4$ ($7H_2O$)	0.25	0.25
NH_3PO_4	0.5	0.5
KNO_3	1.5	1.5
Micronutrients		
NaCl	0.05	0.05
H_3BO_3	0.025	0.025
$MnCl_2$ ($4H_2O$)	0.002	0.002
ZnO	0.002	0.002
$CuCl_2$	0.001	0.001
Na_2MoO_4 ($2H_2C$	0.001	0.001
Fe-EDTA	0.25	0.25
Ca:Mg ratio	4	0.02

(C)



Base solutions for growth assay

High Ca:Mg (4.0)	Low Ca:Mg (0.02)
1.0 mM $CaCl_2$	0.025 mM $CaCl_2$
0.25 mM $MgCl_2$	1.225 mM $MgCl_2$
10 mM KCl	10 mM KCl

Fig. 1. Detailed explanation of the growing conditions for the experimental trial and leaf disc growth assay. A) The 12 randomly placed cuttings of serpentine (S) and non-serpentine (NS) ecotypes in the polyurethane tub. B) Composition of the non-serpentine (high Ca:Mg) and serpentine (low Ca:Mg) solutions used for the 3-week trial. C) Illustrative graphic describing the solutions used for leaf disc growth assay. High Ca:Mg base represents the non-serpentine condition, and low Ca:Mg, the serpentine condition. Abbreviations used: + PS: functioning electron transport of photosynthesis. – PS: photosynthetic electron transport inhibited. + C substrate: carbohydrate substance added (sucrose; 20 mM). DCMU (400 μM): 3-(3,4-dichlorophenyl)-1,1-dimethylurea, an inhibitor of electron transport between photosystems II and I. n = 6 discs for each ecotype and solution combination. This assay was performed in duplicate.

greater extent than the non-tolerant ecotype. Rather it maintained greater biomass production and rates of photosynthetic activity despite similar tissue Ca and Mg concentrations (Palm et al., 2012). The results did not explicitly conclude either low Ca or high Mg as the factor limiting growth and photosynthesis in the non-serpentine population. In contrast to Ca deficiency, the potential for Mg toxicity in nature is uncommon outside of serpentine systems and as a result, the effects of high internal concentrations of Mg are largely unknown. However, there is some evidence that high Mg in the cytosol can disrupt the rate of photosynthesis by altering ion homeostasis across the chloroplast membrane (Shaul, 2002; Marschner, 2012), and that as a divalent cation similar to Ca^{2+}, Mg^{2+} can replace it in the cell wall, potentially altering the capacity for growth and affecting the apoplastic and cytoplasmic concentrations of both ions. As leaves represent the nexus point between photosynthate production and metabolism and growth, the role of Ca and Mg homeostasis in leaves may be key to understanding the adaptation of serpentine *E. guttata* to low Ca:Mg. In the present study, which physiological process is primarily affected by the inhibitory Ca:Mg ratio – growth or photosynthesis – was investigated in serpentine and non-serpentine ecotypes of *E. guttata*. Experiments were conducted on excised leaf tissues to identify direct effects of Ca:Mg on photosynthesis or leaf growth rate. It was hypothesized was that high internal concentrations of Mg in non-serpentine plants have a detrimental effect on photosynthetic rates, limiting the availability of photosynthates needed for subsequent leaf expansion and overall biomass production.

2. Materials and methods

2.1. Experimental hydroponic culture of plant material

Plants of serpentine and non-serpentine ecotypes of *E. guttata* were used for all measurements described here from seeds originally collected in Lake County, CA from the McLaughlin Reserve and cultivated under greenhouse conditions as previously described (Palm et al., 2012).

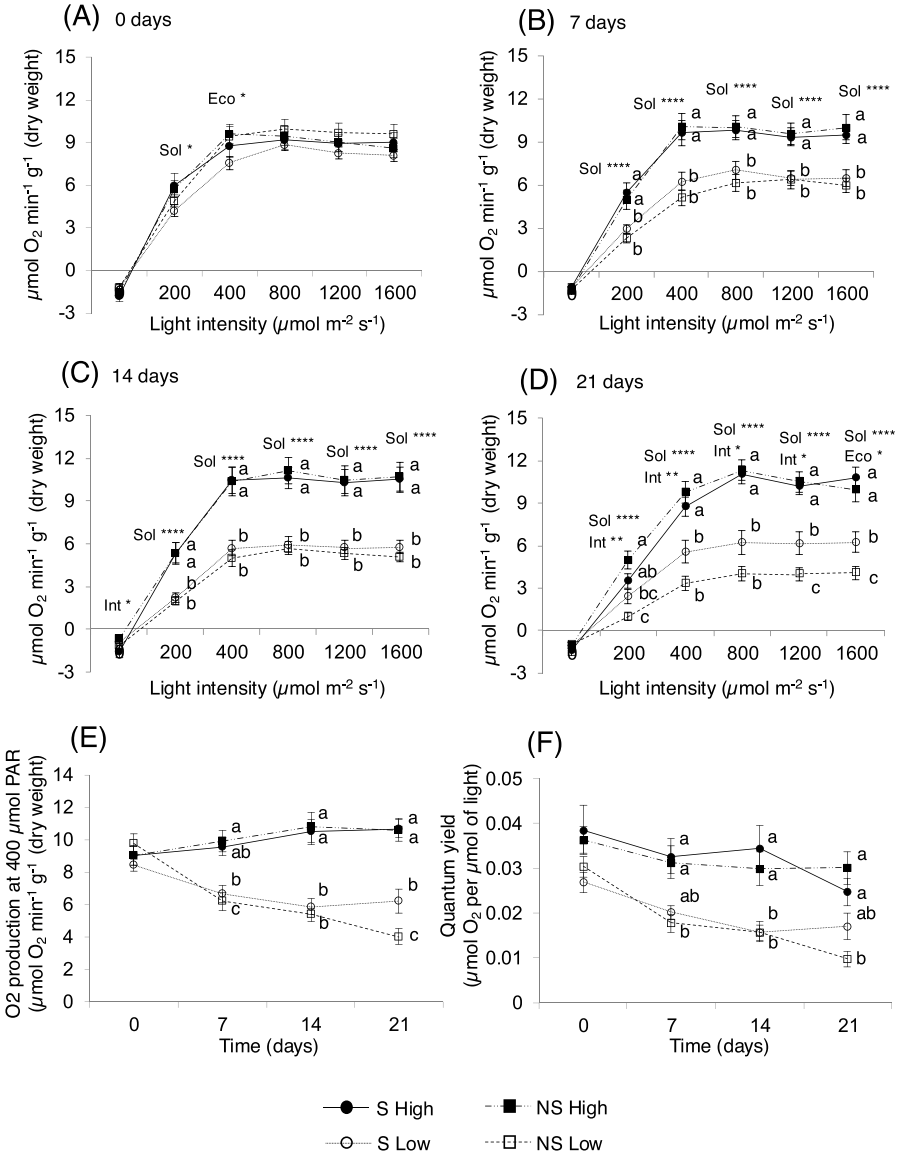


Fig. 2. Light response curves and photosynthetic rates in terms of oxygen production rate (μmol O_2 min^{-1}). Oxygen production rate over increasing light intensity of leaf tissue collected from plants exposed to either high or low Ca:Mg growth solutions for (A) 0 days, (B) 7 days, (C) 14 days, and (D) 21 days. (E) A comparison of O_2 production rate at a light intensity of 400 μmol PAR and (F) the quantum yield, or light-use efficiency, of the treatment combinations at the four time points. n = 6 discs for each data point. Small letters represent significant differences at each light intensity based on Tukey Test with a threshold of $p < 0.05$ following a 2-way ANOVA at least light intensity. Asterisks above individual light intensities indicate significant main effects (solution or ecotype) and interactions (*: <0.05; **: <0.01; ***: <0.001; ****: <0.0001).

Cuttings were made from parent plants grown in greenhouse potting soil and rooted in distilled water. Once roots had emerged, the cuttings were transferred to 15 L polyurethane opaque tubs containing 0.25x Hoagland's solution with a high Ca:Mg (4.0) to be grown hydroponically (Fig. 1A; Hoagland and Arnon, 1959). 12 cuttings per tub were used, 6 for each ecotype. After ten days, the tubs were filled with the same solutions previously described (Palm et al., 2012), either a non-serpentine, high Ca:Mg (4.0) or low Ca:Mg (0.02), serpentine-like solution (Fig. 1B). These concentrations were previously found to elicit the same phenotypes as with soil-grown plants (Palm et al., 2012). Measurements described below were obtained from assays using excised leaf tissues sampled 0, 7, 14, and 21 days after transfer of intact plants to the treatment solutions. Plants were grown in a controlled-environment growth chamber (Conviron; Winnepeg, Manitoba, Canada) with the following conditions: 14h light at 25 °C and 10h dark at 18.5 °C, with a maximum fluence rate of 400 μmol m^{-2} s^{-1} and an average relative humidity between 50 and 60%.

2.2. Photosynthetic rate assay

Photosynthetic properties were measured on leaf discs excised from intact plants 0, 7, 14, and 21 days after their exposure to high and low Ca:Mg growth (treatment) solutions. Excised leaf tissue was used for these measurements to isolate the effect of Ca:Mg ratios on the photosynthetic rate in the absence of the source-sink dynamics that are active at the whole-plant level (Paul and Foyer, 2001; Glans-Idan et al., 2020). An oxygen electrode (Rank Brothers, Ltd, Cambridge, England) was used to obtain photosynthetic rate data over a range of fluence rates: 0, 200, 400, 800, 1200, and 1600 μmol m^{-2} s^{-1}. The electrode was prepared and calibrated according to manufacturer instructions. Measurement solutions contained 2% $NaHCO_3$ with either 1 mM $CaCl_2$ and 0.25 mM $MgCl_2$ for high Ca:Mg or 0.02 mM $CaCl_2$ and 1.25 mM $MgCl_2$ for low Ca:Mg assay conditions. The Ca:Mg ratios of the measurement solutions matched those of the growth (treatment) solutions (whole plants) to avoid initial shock due to changes in concentration gradients of Ca and Mg from the growing solution. Following a protocol described by González et al. (2001), leaf discs were excised from fully mature leaves using a cork borer with a 12 mm diameter and incubated in the dark in the measurement solution for 30 min. Individual leaf discs were placed in the electrode chamber with 3 mL of fresh measurement solution. The amount of oxygen (in μmol min^{-1} g^{-1} of dry leaf tissue) consumed after 15 min in the dark (respiration rate) and produced after 6 min in the light (photosynthetic rate) at each fluence rate was recorded and used to create light response curves. The photosynthetic rate at 400 μmol m^{-2} s^{-1} (the ambient light intensity that was applied in the growth chamber) was used as a standard to compare across all treatment groups. Quantum efficiency – the amount of O_2 produced relative to the fluence rate supplied – was calculated as the slope of the curve between 0 and 400 μmol m^{-2} s^{-1} light.

2.3. Leaf expansion assay

To assess the effect of Ca:Mg on the growth of leaf tissue, a leaf disc assay was performed based on protocols previously described (Blum et al., 1992; Stahlberg and Van Volkenburgh, 1999). Tissue was collected at the end of the light cycle from young, expanding leaves at 1/3 full leaf area using a cork borer with a 4 mm diameter. Discs were placed in a Petri dish containing one of solutions briefly described below and illustrated in Fig. 1C, and incubated for 24 h in the same environmentally controlled growth chamber as previously described for intact plants with a 14h:10h light:dark cycle. Four leaf discs were collected from the same leaf (Fig. 1C). For the growth assay solutions, a base solution of 10 mM KCl was used, and $CaCl_2$ and $MgCl_2$ added to mimic the concentrations and ratios of the growth solutions. One of the four leaf discs was placed in each of the assay solutions with either the high or low Ca:Mg base solution (matching the treatment the tissue was sampled from) and one of the following: 40 mM sorbitol, 400 μM DCMU and 20 mM sucrose, 400 μM DCMU only, or 20 mM sucrose only. DCMU, (3-(3, 4-dichlorophenyl)-1,1-dimethylurea) was used due to its function as an herbicide that inhibits photosynthesis by blocking electron transport between photosystem II and photosystem I (Metz et al., 1986). The addition of 20 mM sucrose provided an energy source in the absence of photosynthesis and sorbitol was used as an osmotic control for the addition of sucrose with the DCMU treatment. A trial was performed prior to conducting the assay over a range of KCl, sucrose and DCMU concentrations in the absence of $CaCl_2$ and $MgCl_2$ to determine the adequate amount of KCl and sucrose needed to support leaf expansion and DCMU to inhibit photosynthesis (data not shown) and to avoid Cl^- toxicity. After 24 h, the leaf discs were photographed, and disc area was measured using ImageJ software.

2.4. Starch content

To evaluate the amount of starch produced and stored that could be utilized for leaf growth, leaf discs were collected each week from mature leaves at the end of the 14-h light period using a cork borer with an 18 mm diameter. Samples were weighed to obtain fresh weight (FW) and stored at −70 °C until analysis could be performed. Due to resource constraints, samples from 7 to 21 days were used for further analysis based on the results from the oxygen electrode photosynthesis and leaf growth assays. Samples were processed as described in Sun et al. (2011), extracting starch by first incubating the tissues in ethanol. The presence of free glucose was measured prior to the addition of amylogluosidase using a glucose hexokinase assay kit (Product no. GAHK20; Sigma-Aldrich Co, LLC) and used to adjust the subsequent values of glucose from starch. Following the enzymatic release of sugars with amyloglucosidase (Starch assay reagent, Product no. S9144; Sigma-Aldrich Co, LLC), the glucose content of each sample was analyzed with a glucose hexokinase assay kit as previously described, following the manufacturers' instructions. Absorbance was measured with a UV/VIS scanning spectrophotometer (Beckman Coulter; Brea, CA; USA) at 340 nm and used to calculate starch content (mg glucose eq.) per gram of leaf tissue.

2.5. Vacuole sap extraction for ICP-MS

Mature leaf tissue was harvested to evaluate vacuole sap osmolarity and concentration of Ca and Mg 7 and 21 days after exposure to high and low Ca:Mg treatment solutions. To ensure that free Ca^{2+} contained in the vacuole did not bind to the cell wall during the extraction process, leaves were first incubated in a 1 mM $SrCl_2$ solution to block open binding sites, following a protocol outlined in Bagshaw and Cleland (1993). Leaf tissue was harvested, midveins and petioles removed, and the remaining tissue weighed to obtain fresh weight. The leaves were cut into 1 mm wide strips and placed in an open 50 mL Falcon tube containing a 1 mM $SrCl_2$, 1 mM KCl solution (pH 6.0, adjusted with HCl). To increase the likelihood that the strontium would bind to open sites in the cell wall, the leaf tissue was vacuum infiltrated in a vacuum jar for 3 min at 400 mmHg. Each leaf sample was then incubated for 30 min. Leaf strips were rinsed 3 times in 1 mM KCl for 5 min and quickly dried with absorbent paper to remove excess surface moisture only. Tissue samples were frozen immediately in dry ice for 15 min. After briefly thawing, leaf tissue was squeezed between fingers and the expressed sap collected. From this, a 20 μL aliquot was taken to measure sap osmolarity using a freezing point osmometer (Advanced Instruments, Model 3300; Winborne, UK). The total volume of the extracted sap was measured and diluted up to 15 mL with dH_2O in a Falcon tube and stored in 4 °C until analysis of ion concentrations with ICP-MS could be performed.

2.6. Leaf tissue preparation for ICP-MS

The total leaf tissue concentrations of Ca and Mg were measured

using ICP-MS, following the protocol previously described (Palm et al., 2012). In the present study, mature leaves harvested at 7 and 21 days were striped of midveins and petioles and dried for 3 day at 70C. Dried leaf tissue (0.5g) was ground for each replicate and prepared for analysis by digesting in concentrated HNO_3 as previously described. Two replicates of NIST tomato leaf standard materials were digested alongside experimental samples as an internal control of the digestion protocol (National Institute of Standards and Technology; Product no. NIST1573a; Sigma Aldrich).

2.7. ICP-MS analysis of vacuole sap and leaf tissue

All ICP analyses of vacuole sap and leaf tissue Ca and Mg concentrations were conducted by Analytical Services (School of Environmental and Forestry Sciences (University of Washington, Seattle WA). Tomato leaf standards were also run alongside experimental samples. Values were provided as mg/L and converted to mM using the known biomass and dilution of individual samples.

2.8. Statistical analysis

Results for all measurements are presented as means with standard error. Prior to analysis, all data were tested for normality using the Shapiro-Wilk test, with datasets falling below the 0.05 p value threshold transformed before proceeding. 2-way ANOVA was performed at each light intensity for light curve data, and repeated measures 3-way ANOVA was used for all other datasets to test for differences among solution treatments and plant type with respect to time, as well as their interactions, using GraphPad Prism version 9.3.1 for MacOSX (GraphPad Software; San Diego, California). A *posthoc* comparison of means was performed via a Tukey test. Correlations between maximum photosynthetic rate and growth rate for leaves in the control high and low solutions (+sorbitol) and biochemical parameters (starch content, Ca and Mg localization and partitioning, and vacuole osmolarity) were assessed with data from weeks one and three. To determine whether there were significant differences between the treatment groups for both photosynthetic rate and growth rate, Welch's Two Sample T-test was used. A linear model was developed using the AIC Backward method to determine which variables explain the greatest amount of variation between the treatments with regard to photosynthetic rate and growth rate. For both Welch's T-test and correlation analyses, the statistical software R was used (R Core Team, 2021).

3. Results

3.1. Photosynthetic rates altered by low Ca:Mg ratio over time

Photosynthetic rates increased with fluence rate similarly for all leaf discs sampled at day 0 (Fig. 3A) regardless of the treatment solution. At this time point, all plants from which leaf tissue had been excised had been growing hydroponically on high Ca:Mg treatment solution. The 30-min incubation in low Ca:Mg for the assay had no effect on photosynthetic rate on either ecotype. After 7 days of treatment to low Ca:Mg, discs excised from both non-serpentine and serpentine (hereafter referred to as NS and S, respectively) plants showed a decrease in photosynthetic rate (Fig. 3B). This pattern continued for measurements conducted after 14 days of low Ca:Mg (Fig. 3C). After 21 days, S and NS plants continued to respond in a similar fashion in the high Ca:Mg

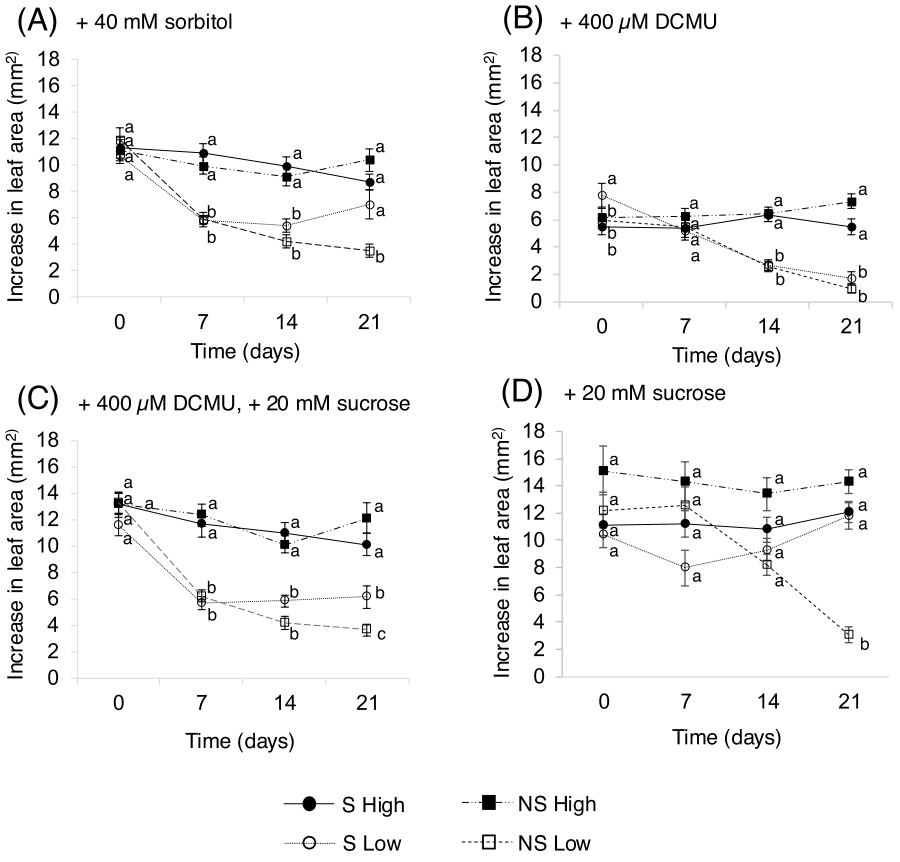


Fig. 3. Weekly growth analyses of leaf discs from high and low Ca:Mg growth solutions over three weeks. Leaf discs from four treatment combinations (ecotype x solution) were measured in response to (A) + sorbitol (40 mM; osmotic control), (B) + DCMU (400 μM; electron transport inhibitor), (C) + DCMU, + sucrose (400 μM; electron transport inhibitor + 20 mM; carbohydrate substrate) and (D) + sucrose (20 mM; carbohydrate substrate). Circles: serpentine ecotype, squares: non-serpentine ecotype; filled: high Ca:Mg solution and unfilled: low Ca:Mg solution. PAR: photosynthetically active radiation. n = 6 for each data point. Different small letters indicate significant differences for each time point based on *posthoc* Tukey Test with a threshold of $p < 0.05$ following a 3-way ANOVA.

solution with no significant difference ($p = 0.99$), but leaf tissue from NS plants in the low Ca:Mg solution showed further declines in maximum photosynthetic rate at the highest fluence rates, i.e. > 400 μmol m^{-2} s^{-1}. In contrast, leaf tissues from S plants in the low Ca:Mg solution maintained the same rate as 7 and 14 days after exposure (Fig. 3). Given the presence of $NaHCO_3$ in the assay solution, precipitation of $CaCO_3$ and $MgCO_3$ likely occurred due to the concentrations of these two ions, the reaction quotients and the K_{sp} values for the two precipitates, effectively reducing the availability of Ca and Mg during the assay. Though the absolute concentrations of either ion were not evaluated at the conclusion of the measurement, it was estimated that the concentrations were roughly 0.1 μM for both ions in both high and low Ca:Mg solutions, resulting in Ca:Mg ratios close to 1. Thus, the effect observed here on photosynthetic rates of excised leaf tissues is more likely due to the continued exposure of the whole-plant to either high or low Ca:Mg conditions. Assay photosynthetic rates at a fluence rate of 400 μmol m-2 s-1 (the same as the conditions in the growth chamber) for leaf discs excised at the four treatment times (days 0–21) stayed constant for both S and NS plants treated with high Ca:Mg (Fig. 3E). However, NS plants treated with low Ca:Mg showed rapid and steady decline in photosynthetic rates of assayed discs. Tissues excised from S plants treated with low Ca:Mg appear to have adapted to the treatment, maintaining photosynthetic capacity after day 14. Solution, ecotype and exposure time all demonstrated significant main effects, and interaction effects between solution and exposure time, as well as ecotype and exposure time, were found (Table 1). The photosynthetic efficiency (quantum yield) exhibits the same pattern as for photosynthetic rate at 400 μmol m^{-1} s^{-1}: S and NS plants in high Ca:Mg solution show no significant difference over the experimental time period, while NS plants in low Ca:Mg continually decline, and S plants initially decline but then stabilize at 14 days (Fig. 3F). By day 21, there was a significant difference ($p = 0.03$) in the response to low Ca:Mg between S and NS plants. Exposure time and solution are significant factors, as well as interactions between exposure time and ecotype (Table 1).

3.2. Growth rates of excised tissues in response to solution Ca:Mg

Discs excised from growing leaves may continue to expand at a rate similar to that observed prior to excision when provided sufficient nutrients in the incubation solution used for the growth assay (Van Volkenburgh and Cleland, 1980). Excised *E. guttata* leaf tissue expanded over 24 h at rates dependent not only on the Ca:Mg ratio of the treatment and assay solutions but also on their photosynthetic capacity and ability to utilize a supplemental source of carbohydrates (sucrose). S and NS leaf tissues from high Ca:Mg treated plants had similar expansion rates over the 21 days (Fig. 4A); these values were higher than those for discs excised from S and NS plants treated with low Ca:Mg (an average of 11 mm^2 day^{-1}) when photosynthesis was not blocked (+sorbitol; Fig. 4A). While NS leaf tissues from the low Ca:Mg treatment declined in expansion over the 21 day-trial, by day 14, S leaf tissues began to significantly increase their expansion, approaching values similar to those of high Ca:Mg leaves by day 21. Blocking the electron transport between PSII and PSI with DCMU reduced the expansion of all leaf tissues, but the reduction was most dramatic in S and NS leaves from plants treated with low Ca:Mg (Fig. 4B). Exposure time and solution had the most significant effect on the growth rate in this treatment, as well as the interaction between ecotype and exposure time, and ecotype and solution (Table 1). No recovery in the growth of S leaves was observed at 14 and 21 days as observed in the sorbitol control. Providing sucrose as a carbohydrate source in the absence of a functioning light energy transport system restored the expansion rates of S and NS leaf tissues in high Ca:Mg (Fig. 4C). The rates of S and NS leaf discs excised from the low Ca:Mg treatment were reduced as in the sorbitol assay, with a stabilization of S leaf expansion rate by day 14. Supplemental sucrose provided to discs able to photosynthesize generally increased disc expansion and altered the growth in response to the treatment Ca:Mg ratio (Fig. 4D). Initially, low Ca:Mg treatment had a negative effect on expansion of discs from S leaves (day 7), but over time they recovered expansion rates similar to S leaves in high Ca:Mg. In contrast, growth of NS leaf discs excided from leaves treated with low Ca:Mg declined dramatically after 7 days. In both the sorbitol and DCMU + sucrose treatments, the significant interaction effect between exposure time, ecotype and solution supports the fact that the difference in the response to solution between the two ecotypes is not immediate but develops over time (Table 1).

3.3. Starch content of leaves

By day 21 there was significant increase in the starch content of mature leaves of both S and NS plants in response to low Ca:Mg (Fig. 4A). The starch content of NS leaves was higher than serpentine leaves, but the difference was not significant ($p = 0.61$; Table 2). A correlation analysis demonstrates a negative relationship between starch content and both the photosynthetic rate at 400 μmol m^{-2} s^{-1} (Fig. 4B) and growth in the sorbitol growth assay solution (Fig. 4C).

3.4. Whole leaf and vacuole sap ion properties

Leaf and vacuole concentrations of Ca^{2+} and Mg^{2+} strongly mirrored the respective concentrations of the high and low Ca:Mg growth solutions, both at 7 and 21 days (Table 2). Leaf concentrations of Ca^{2+} were higher in S leaves grown in low Ca:Mg than NS leaves at 7 days, but this was significantly reversed at 21 days. At the same time, low Ca:Mg S leaves had both higher total leaf and vacuole Mg concentrations than low Ca:Mg NS leaves at days 7 and 21. The proportion of Ca^{2+} and Mg^{2+} ions in the vacuole of S leaves was higher in high and low Ca:Mg growth solutions than NS leaves at both days 7 and 21. At 7 days, there was no significant difference between the ecotypes in terms of the leaf and vacuole Ca:Mg ratios ($p = 0.3722$ and 0.8429, respectively) but the values were strongly dependent on the solution Ca:Mg ($p < 0.001$ for both leaf and vacuole). For both ecotypes, leaf and vacuole Ca:Mg ratios were highest in high Ca:Mg growth conditions. At day 21, the leaf Ca:Mg of S leaves grown in high Ca:Mg was significantly higher than that of NS leaves. However this did not extend to the vacuole Ca:Mg observed for either population, where no ecotype-dependent differences were found.

Table 1
Summary of results for repeated measures 3-way ANOVA for photosynthetic rate and growth rate measurements with a threshold of $p < 0.05$. ns indicates p-values above 0.05. PS: photosysnthetic rate at 400 μmol m^{-2} s^{-1}. DCMU: 3-(3,4-dichlorophenyl)-1,1-dimethylurea. Concentrations used in the assay: 20 mM sucrose; 40 mM sorbitol; 400 μmol m^{-2} s^{-1} DCMU.

	Oxygen production rate		Growth assay			
	PS at 400 μmol light	Quantum yield	sorbitol	sucrose + DCMU	DCMU	sucrose
Time	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Solution	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	ns
Ecotype	0.0483	ns	ns	ns	ns	ns
Time x Solution	<0.0001	<0.0001	0.0001	<0.0001	<0.0001	ns
Time x Ecotype	<0.0001	ns	ns	ns	0.0374	ns
Solution x Ecotype	ns	ns	ns	ns	0.0006	0.0023
Time x Solution x Ecotype	ns	ns	0.0034	0.0259	ns	ns

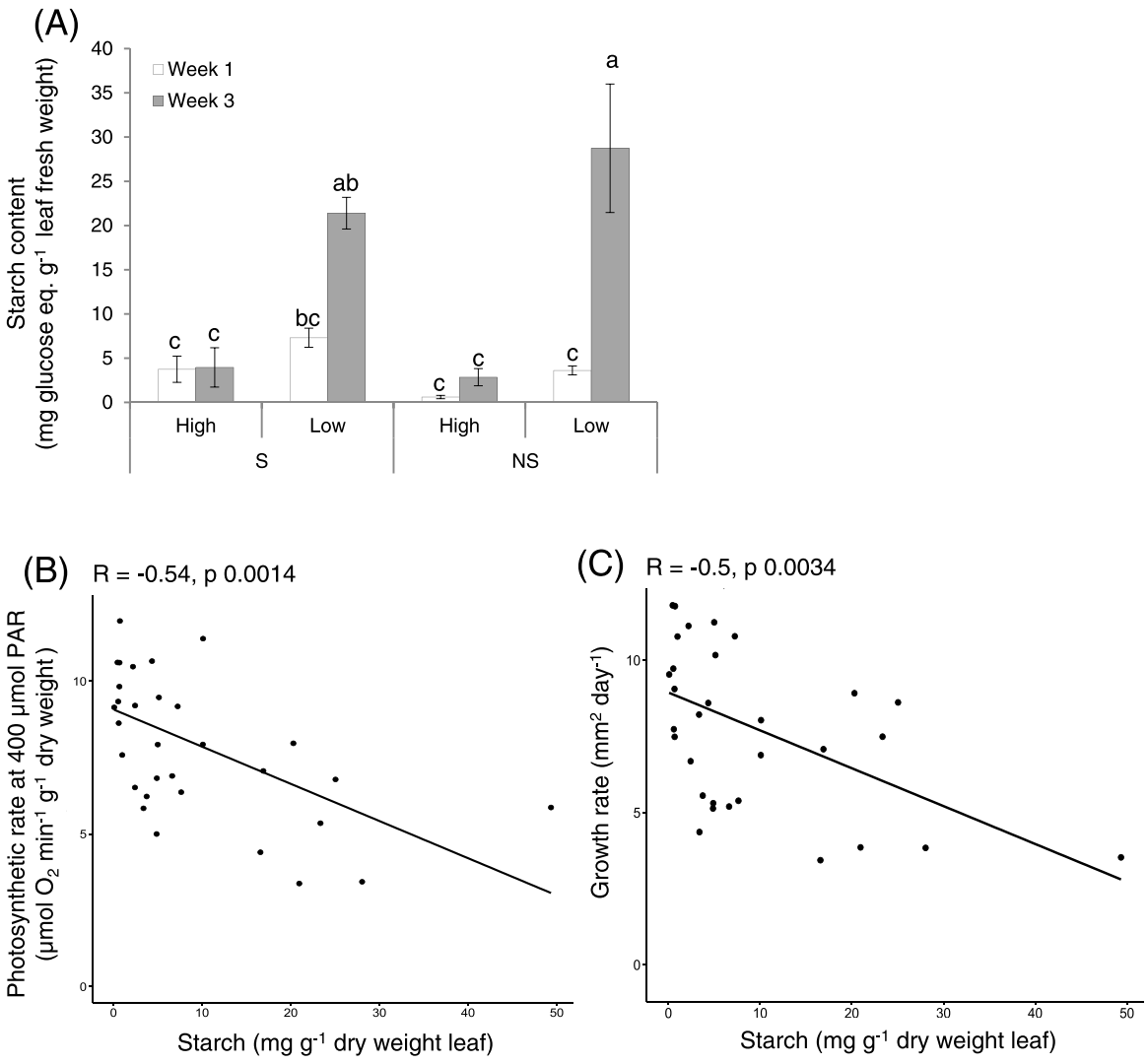


Fig. 4. Starch content and correlation analyses between starch, photosynthetic and growth rates. (A) Starch contents at weeks 1 and 3. (B) Correlational analysis between starch content and photosynthetic rate at 400 µmol m^{-2} s^{-1}. (C) Correlational analysis between starch content and growth rate of discs in high and low Ca:Mg + 40 mM sorbitol. PAR: photosynthetically active radiation. For the starch content analysis, different small letters indicate significant differences for each time point based on *posthoc* Tukey Test with a threshold of $p < 0.05$ following a 3-way ANOVA.

An ecotype and solution-dependent increase in leaf Ca:Mg was found only among low Ca:Mg grown NS leaves.

3.5. Correlation analyses

Both photosynthetic rates at 400 µmol PAR (Fig. 2E) and growth rates in sorbitol (Fig. 3A) differ significantly between the solutions at Week 1 and both ecotypes and solutions at Week 3. At Week 1, photosynthesis was significantly correlated with leaf Ca^{2+} concentrations ($p = 0.029$), the proportion of Ca^{2+} in the vacuole ($p = 0.006$), and the osmolarity of the vacuole ($p = 0.011$). Growth rate was highly affected by starch content ($p = <0.001$), the vacuole concentration and proportion of Ca ($p < 0.001$), the vacuole proportion of Mg^{2+} ($p = <0.001$), the vacuole Ca:Mg ($p = <0.001$) and vacuole osmolarity ($p = 0.004$) (Table 3). By week 3 of the trial, the number of parameters explaining the response of the ecotypes to the solutions is reduced. Correlations were found between the photosynthetic rate and leaf Ca^{2+} concentration ($p = 0.009$) and vacuole osmolarity ($p = 0.004$), and between the growth rate and vacuole osmolarity ($p = 0.008$).

4. Discussion

4.1. E. guttata recovers gradually from Ca deficiency and Mg toxicity to adapt to low Ca:Mg

The Ca:Mg ratio found in serpentine soil can have a dramatic effect on the growth and persistence of plant populations. Plants from seeds collected from both serpentine and non-serpentine soil sites differentially adapt to their native soils, with the Ca:Mg ratio isolated as the soil factor responsible for determining the distribution of differentially adapted ecotypes of *E. guttata* (Palm et al., 2012). Here the physiological processes primarily responsible for decreased growth in the NS ecotype were investigated, focusing on photosynthetic and leaf expansion rates. When grown in a high Ca:Mg solution, excised tissues from S and NS plants maintain similarly high rates of photosynthesis and leaf area expansion. Conversely, when grown in a low Ca:Mg treatment, both S and NS plants exhibit a decline in both photosynthetic rates and leaf expansion, but these processes begin to recover in S leaves after 14 days of exposure of intact plants to low Ca:Mg. In the absence of a functioning photosynthetic apparatus, growth of excised leaf discs is reduced in both S and NS plants, but supplemental carbon resources (sucrose) restore

Table 2
Summary of biochemical analyses for Week 1 and Week 3 samples: whole leaf and vacuole concentrations of calcium (Ca) and magnesium (Mg), and relative vacuole concentrations for calcium (Ca) and magnesium (Mg), starch content (mg glucose eq.) and vacuole osmolarity (mOsm). Treatment groups: S – serpentine; NS – non serpentine; high Ca:Mg – 4.0; low Ca:Mg – 0.025. Main effects (Eco – ecotype; Sol – solution) and interactions (ecotype x solution) from the 2-way ANOVA are indicated. Different small letters indicate significant differences among the treatment combinations based on Tukey Test with a threshold of $p < 0.05$. ns indicates p-values above 0.05.

Week 1								
		S high	S low	NS high	NS low	Eco	Sol	Eco x Sol
Ca (mM)	[Leaf]	18.0 ± 0.9a	12.4 ± 0.8b	19.4 ± 0.9a	11.0 ± 0.9b	ns	<0.001	ns
	[Vacuole]	8.2 ± 0.5a	2.2 ± 0.2c	7.3 ± 0.5b	1.4 ± 0.1c	0.002	<0.001	ns
	Rel. conc vacuole	0.46 ± 0.04a	0.18 ± 0.02b	0.38 ± 0.03a	0.13 ± 0.01b	0.027	<0.001	ns
Mg (mM)	[Leaf]	3.8 ± 0.3b	10.4 ± 0.4a	3.9 ± 0.2b	9.6 ± 0.8a	ns	<0.001	ns
	[Vacuole]	1.7 ± 0.1c	4.3 ± 0.2a	1.5 ± 0.1c	2.6 ± 0.2b	<0.001	<0.001	<0.001
	Rel. conc vacuole	0.47 ± 0.03a	0.42 ± 0.02a	0.40 ± 0.02a	0.29 ± 0.04b	0.005	0.016	ns
Ca:Mg	Leaf	4.78 ± 0.16a	1.19 ± 0.05b	5.03 ± 0.17a	1.18 ± 0.09b	ns	<0.001	ns
	Vacuole	4.82 ± 0.33	0.52 ± 0.06	4.72 ± 0.23	0.54 ± 0.07	ns	<0.001	ns
Starch content		3.74 ± 1.48 ab	7.31 ± 1.08a	0.60 ± 0.19b	3.61 ± 0.05 ab	0.004	0.005	ns
Vacuole osmolarity		154 ± 5b	206 ± 7a	136 ± 5b	207 ± 11a	ns	<0.001	ns
Week 3								
		S high	S low	NS high	NS low	Eco	Sol	Eco x Sol
Ca (mM)	[Leaf]	24.6 ± 0.5a	7.8 ± 0.3b	28.9 ± 0.8a	10.1 ± 0.3b	ns	<0.001	ns
	[Vacuole]	4.2 ± 0.5a	1.5 ± 0.3b	4.7 ± 0.8a	1.0 ± 0.3b	ns	<0.001	ns
	Rel. conc vacuole	0.25 ± 0.11a	0.33 ± 0.10a	0.16 ± 0.02a	0.11 ± 0.03a	ns	ns	ns
Mg (mM)	[Leaf]	3.0 ± 2.3b	18.9 ± 3.1a	6.3 ± 0.2b	16.1 ± 0.7a	ns	<0.001	0.016
	[Vacuole]	1.1 ± 0.1c	9.5 ± 0.9a	1.3 ± 0.2c	6.5 ± 0.7b	ns	<0.001	0.045
	Rel. conc vacuole	0.36 ± 0.10 ab	0.53 ± 0.07a	0.20 ± 0.03b	0.44 ± 0.09 ab	ns	0.009	ns
Ca:Mg	Leaf	10.31 ± 2.13a	0.26 ± 0.07c	4.65 ± 1.42b	0.62 ± 0.03c	0.001	<0.001	<0.001
	Vacuole	3.63 ± 0.26a	0.17 ± 0.02b	3.83 ± 0.41a	0.16 ± 0.04b	ns	<0.001	ns
Starch content		3.95 ± 2.23b	21.39 ± 1.79a	2.84 ± 0.97b	28.73 ± 7.27a	ns	<0.001	ns
Vacuole osmolarity		138 ± 10b	323 ± 26a	155 ± 9b	320 ± 6a	ns	<0.001	ns

Table 3
Results from correlation models for biochemical data at Weeks 1 and 3. Parameters that led to the model explaining the greatest amount of variation between the two ecotypes growing in either high or low Ca-Mg solutions are indicated, with those parameters found to have a significant effect either on photosynthetic rate or growth rate in bold. ns indicates p-values above 0.05. Abbreviations: [Ca] – calcium concentration; [Mg] - magnesium concentration; Vac – vacuole; Vac prop Ca – vacuole proportion of total calcium; Vac prop Mg – vacuole proportion of total magnesium; Vac osm – vacuole osmolarity.

Week 1								
	Photosynthesis					Growth		
	Estimate	Std. Error	t value	Pr (>|t|)		Estimate	Std. Error	t value	Pr (>|t|)
Intercept	−46.422	19.159	−2.423	0.042	Intercept	3.090	2.102	1.47	0.179
Starch	−0.522	0.463	−1.128	0.292	**Starch**	**0.625**	**0.074**	**8.444**	**<0.001**
Leaf [Ca]	**1.394**	**0.525**	**2.654**	**0.029**	Leaf [Ca]	−0.106	0.120	−0.879	0.405
Vac prop Ca	**53.473**	**14.638**	**3.653**	**0.006**	**Vac [Ca]**	**3.032**	**0.466**	**6.504**	**<0.001**
Leaf [Mg]	0.364	0.948	0.384	0.711	**Vac prop Ca**	**−52.632**	**8.631**	**−6.098**	**<0.001**
Vac [Mg]	1.223	2.559	0.478	0.646	**Vac prop Mg**	**−13.679**	**2.303**	**−5.940**	**<0.001**
Vac prop Mg	−49.617	23.252	−2.134	0.065	**Vac Ca:Mg**	**1.694**	**0.278**	**6.089**	**<0.001**
Vac osm	**0.192**	**0.059**	**3.240**	**0.012**	**Vac osm**	**0.034**	**0.008**	**4.052**	**0.004**
Multiple R-squared 0.7607, p value: 0.0454					Multiple R-squared 0.9897, p value: <0.001				
Week 3								
	Photosynthesis					Growth		
	Estimate	Std. Error	t value	Pr (>|t|)		Estimate	Std. Error	t value	Pr (>|t|)
Intercept	33.706	6.112	5.515	<0.001	Intercept	7.479	5.516	1.356	0.205
Leaf [Ca]	**−0.662**	**0.192**	**−3.449**	**0.009**	Leaf [Ca]	0.254	0.152	1.674	0.125
Vac prop Ca	−9.595	9.194	−1.044	0.327	Leaf [Mg]	0.392	0.199	1.959	0.078
Leaf [Mg]	0.298	0.161	1.856	0.101	Vac [Mg]	0.751	0.361	2.081	0.064
Vac [Mg]	−0.719	0.371	−1.94	0.088	**Vac osm**	**−0.056**	**0.017**	**−3.285**	**0.008**
Vac prop Mg	−3.234	2.821	−1.146	0.285					
Vac Ca:Mg	1.455	0.863	1.686	0.130					
Vac osm	**−0.067**	**0.017**	**−3.963**	**0.004**					
Multiple R-squared 0.8924, p value: 0.0025					Multiple R-squared 0.6967, p value: 0.0115				

serpentine leaf expansion even in low Ca:Mg. These two main findings, together with the negative correlations with starch content, suggest that processes connected to carbon fixation and metabolism are more sensitive to the low Ca:Mg ratio of serpentine soil in these ecotypes of *E. guttata* than processes directly involved in growth. Furthermore, the lack of a gradual recovery in either photosynthetic rate or growth rate in NS leaves in low Ca:Mg indicate that this ecotype is experiencing both continued Ca^{2+} deficiency and Mg^{2+} toxicity while the S ecotype overcomes Ca^{2+} deficiency symptoms more quickly to resume growth, followed by a recovery in Mg^{2+}-toxicity induced inhibition of photosynthetic function.

4.2. Low Ca:Mg has a temporary negative effect on photosynthetic rates of serpentine leaves

In the present study, the excised leaf tissues of S and NS plants grown in low (0.02) Ca:Mg solutions exhibited initial dramatic reductions in photosynthetic rates relative to plants grown in high (4.0) Ca:Mg conditions but only S leaves began to show increased photosynthetic rates and quantum efficiency by 21 days after the start of the trial. These results coincide with the photosynthetic data of our previous study, measured on the intact leaves of plant grown in both high and low Ca:Mg conditions Palm et al., 2012). Though it has been established that for these two ecotypes the Ca:Mg ratio is an important factor, it is not immediately clear whether the NS plants are experiencing Ca^{2+} deficiency or Mg^{2+} toxicity. However, here, the timing of the recovery observed in S leaves and the general roles of Ca^{2+} and Mg^{2+} point to both Ca^{2+} deficiency and Mg^{2+} toxicity and their effect on growth and photosynthesis as the determining long term stress inducing factor in NS plants. Together, excess Mg^{2+} and limited Ca^{2+} may be directly and indirectly inhibiting photosynthetic productivity in these two ecotypes upon initial exposure to low Ca:Mg. The tissue concentrations at three weeks indicate elevated levels of Mg and reduced Ca in the leaves of both S and NS plants grown in low Ca:Mg relative to those grown in high Ca:Mg conditions. Leaf tissue concentrations in both ecotypes are high (18.9 and 16.1 mM for S and NS, respectively), potentially exceeding the optimal range of 2–10 mM Mg^{2+} in the metabolic pool (Leigh and Wyn Jones, 1986). In contrast, Ca^{2+} concentrations of S and NS leaves, 7.8 and 10.1 mM, respectively in low Ca:Mg are in the range considered Ca deficiency. Though little is known about the effects of elevated concentrations of Mg^{2+}, evidence suggests that they may disrupt the rate of photosynthesis (Marschner, 2012; Shaul, 2002). Concentrations of Mg^{2+} in the cytosol, external to the chloroplasts, regulate the movement of K^+ into the stroma, which is electrochemically balanced by proton efflux from the stroma to the cytoplasm (Berkowitz and Wu, 1993), simultaneously and indirectly creating a pH that is more favorable to the activity of carbon reduction cycle enzymes of photosynthesis (Werdam et al., 1975). When concentrations of Mg^{2+} are high, Mg^{2+} may bind to the negatively charged chloroplast envelope, inhibiting K^+ influx and H^+ efflux and leading to reduced photosynthetic rates. While Ca^{2+} deficiency directly inhibits growth due to its relative immobility in plant tissues (White and Broadley, 2003), it may also indirectly affect photosynthetic rates by reducing the Ca^{2+} signaling that induces response to ionic stress, such as Mg^{2+} stress in the present study, and consequently result in reduced photosynthetic rates due to unmitigated Mg^{2+} toxicity.

4.3. Leaf expansion recovers more quickly than photosynthesis in S leaves under low Ca:Mg

The more rapid recovery of leaf expansion rates in S leaves under low Ca:Mg (14 days) relative to photosynthetic rates (21 days) is another factor that suggests that the serpentine ecotype is less sensitive to Ca^{2+} deficiency than the NS ecotype and its recovery is further aided by a more resilient rate of photosynthetic activity. In the growth assays conducted in the present study, the availability of components fundamental to growth (Ca^{2+} and photosynthates) was altered in the solutions: Ca^{2+} - high or low Ca:Mg; photosynthates (photosynthetically derived: ± DCMU or supplied: ± sucrose). The recovery of S leaf expansion in low Ca:Mg in the presence of functioning photosystem energy transport (-DCMU, + sorbitol; Fig. 3A) began between 7 and 14 days, and was accelerated by the addition of sucrose (Fig. 3D), providing further evidence that photosynthetic activity is likely key to the serpentine ecotype's adaptation to low Ca:Mg. Calcium availability plays an important role in growth, as a key component of cell walls and as a signaling molecule for growth regulating signals such as light and hormones (White and Broadley, 2003). As such, one of the earliest symptoms of Ca^{2+} deficiency observed is the inhibition of new growth. Here, despite lower Ca^{2+} tissue concentrations in S leaves than in NS leaves at day 21, expansion is higher in this ecotype in low Ca:Mg. This suggests that growth of the S ecotype is less sensitive to deficient levels of Ca^{2+} than the NS ecotype, and that the gradual recovery of growth rates of S plants not previously exposed to low Ca:Mg is further aided by providing additional carbon resources. To our knowledge no other studies investigating the effect of low Ca:Mg on growth in serpentine systems have attempted to identify a distinct mechanism regulating growth. In our previous study, a significant reduction in both shoot and root biomass was observed in the NS ecotype relative to the S ecotype after three weeks in low Ca:Mg. Both ecotypes had deficient levels of Ca^{2+} in root and leaf tissues, suggesting that some other factor was limiting growth of the NS ecotype. The results of the present study indicate that growth is further limited by photosynthate supply driven by a differential response to Mg^{2+} toxicity in these two ecotypes.

4.4. Elevated starch concentrations in NS leaves in low Ca:Mg suggest negative assimilate feedback

In this study, the effect of low Ca:Mg on photosynthesis and growth rates was investigated using excised leaf tissue. This method isolated the individual sample from the source/sink dynamics that occur in a whole plant measurement (Paul and Foyer, 2001; Glans-Idan et al., 2020). In the whole plant, mature source leaves photosynthesize, supplying themselves and young expanding sink leaves with the assimilates needed for growth (Krapp et al., 1991). Sink strength may affect the rate at which source tissues produce assimilates, or store them (photosynthetic rate and starch accumulation, respectively) (Fischer and Bremer, 1993; Hermans et al., 2004; Hermans and Verbruggen, 2005; Araya et al., 2006). This dynamic may explain the differences in magnitude observed in our study between intact leaves (Palm et al., 2012) and excised tissues used here. Low Ca^{2+} and high Mg^{2+} may reduce sink and source activity simultaneously as was observed here in both ecotypes immediately following exposure to low Ca:Mg. When sink strength is reduced as would happen when growth rates decline, an accumulation of starch can occur that acts as a negative inhibitor of photosynthate production (Smith and Stitt, 2007). Higher concentrations of starch were observed 7 days after the start of the low Ca:Mg treatment, relative to high Ca:Mg grown plants. By day 21, these values had increased in both ecotypes, but more so in NS leaves. However, the gradual recovery in S leaf growth rate indicates that sink activity is resuming and reduces the degree to which starch is accumulated. Other studies have found that accumulation of starch and other assimilates occurs with a decrease in photosynthetic rates in Mg^{2+} deficient plants (Fischer and Bremer, 1993; Cakmak et al., 1994; Hermans et al., 2004; Hermans and Verbruggen, 2005). However, here, the negative effect of elevated Mg^{2+} on photosynthetic rate may be further compounded by inhibitory feedback of accumulated photosynthates in NS leaves. In contrast, sink strength is less limited in S leaves in low Ca:Mg as demonstrated by the more rapid rebound in growth rates relative to photosynthetic rates. Supplying sucrose in the absence of photosynthetic activity in the growth assay accelerated growth, suggesting that growth processes are less inhibited by low Ca:Mg than photosynthesis. For S leaves in low Ca:Mg, it is possible that the induced tolerance to Ca deficiency restores faster

growth rates and thus sink activity, relieving the repression of photosynthetic activity produced by an accumulation of starch in source tissues.

4.5. Ca^{2+} and Mg^{2+} homeostasis, and decrease sensitivity to Ca^{2+} deficiency may be key to the adaptation of the serpentine ecotype to low Ca:Mg

For low Ca:Mg grown S plants to recover photosynthetic rates and growth rates that are close to those found in a high Ca:Mg growth solution despite low Ca^{2+} and high Mg^{2+} concentrations in their leaves, the localization of Ca^{2+} and Mg^{2+} to specific compartments within the cell is likely better regulated in S than in NS *E. guttata* plants. This acclimation would require a gradual change in internal concentrations of Ca^{2+} and Mg^{2+}, or an adjustment in chloroplast physiology that could accommodate the increase in Mg^{2+} without the observed inhibition of photosynthetic rates and enzyme function (Berkowitz and Wu, 1993). The dynamics between Ca^{2+} and Mg^{2+} within the main compartments of the plant cell are complex. Both are divalent cations, and nonselective cation channels (NSCCs) localized to the plasma membrane and tonoplast can transport these ions in and out of the cytoplasm (Demidchik and Maathuis, 2007), affecting their relative concentrations and activities. Furthermore, the cation exchange capacity of the cell wall for both Ca^{2+} and Mg^{2+}, creating another source of variability in their distribution within the cell (White et al., 2018; Ray and George, 2011). Among the cellular components that may participate in regulating Ca^{2+} and Mg^{2+} homeostasis, the vacuole is a likely candidate. It is a common storage location when ion concentrations in the cell are high (Tang et al., 2022) and is actively involved in maintaining the low cytosolic concentrations of Ca^{2+} needed for Ca^{2+} signaling pathways (Cheng et al., 2005), gas exchange and growth (Yan et al., 2018; Conn et al., 2011b). One study, in which *Arabidopsis thaliana* (L.) Heynh. mutants were screened for tolerance to the low Ca:Mg of serpentine soils, identified the potential role of CAX1, a Ca^{2+}/H^{+} antiporter localized to the tonoplast (Brady et al., 2005). Loss-of-function mutations in *cax1* produced plants with greater tolerance to low Ca^{2+} and high Mg^{2+}, a high Mg^{2+} requirement and lower Mg^{2+} concentrations in leaves – three principal characteristics identified as traits of serpentine adaptation (Tyndall and Hull, 1999) The role of CAX1 in maintaining low cytosolic Ca^{2+} concentrations for Ca^{2+}-based signaling events is disrupted by the low Ca^{2+} and high Mg^{2+} of serpentine soil. To compensate, it was hypothesized that an NSCC attempts to rectify this Ca^{2+} deficiency by opening but instead allows for the passage of Mg^{2+} into the cytoplasm, resulting in rapid increases in Mg^{2+} concentration that become toxic in non-tolerant plants. Magnesium ions may play a greater role in regulating stomatal aperture than was previously understood. A recent study by Inoue et al. (2022) found that the Mg^{2+} sequestered into the vacuole of guard cells by the magnesium transporter protein MGR1/CSR2-1 (Tang et al., 2022) functions in an analogous way to K^{+} in regulating stomatal aperture and stomatal conductance rates by altering the water potential of the guard cells to favor the influx of water. Furthermore, overexpression of this protein inhibited stomatal closure induced by high Mg^{2+} and improved tolerance to high Mg^{2+} conditions. In the S ecotype, both photosynthetic rates and growth rates of plants exposed to low Ca:Mg conditions (high Mg) for three weeks were higher than those of the NS ecotype, both in the present study and in our previous work (Palm et al., 2012). Given that elevated concentrations of Mg^{2+} in the vacuoles of leaf tissues of S plants in low Ca:Mg conditions were also observed suggests that at least a portion of the excess Mg^{2+} may have been sequestered specifically in the guard cells. MGR1/CSR2-1 is localized to the tonoplast of guard cell vacuoles (Inoue et al., 2022). Where the Mg^{2+} accumulations occurred in terms of cell type was not assessed in the present study. It would be worthwhile to determine if the S ecotype benefits from the increased availability of Mg^{2+} in serpentine conditions to further stimulate stomatal conductance, and whether this is regulated by the expression levels of MGR1/CSR2-1, together with the MRS2 genes previously described.

Both *E. guttata* ecotypes used in the present study exhibit increased leaf tissue concentrations of Mg^{2+}, demonstrating that neither are Mg^{2+} excluders and that maintaining Mg^{2+} homeostasis within its leaf cells is likely a key factor in adaptation to serpentine soil conditions. A family of Mg^{2+} transporters, MRS2, identified in bacteria with homologs in *A. thaliana,* have been implicated in regulating cytosolic Mg^{2+} concentrations. In particular, MRS2-2, MRS2-6, and MRS2-7 were shown to respond to serpentine conditions by improving Mg^{2+} partitioning in the face of Ca^{2+} deficiency (Conn et al., 2011a). More specifically, AtMRS2-1 and AtMRS205 were both localized to the tonoplast of mesophyll cells and were highly induced after seven days in plants grown in both low Ca^{2+} and high Mg^{2+} conditions mimicking serpentine soils, leading to greater accumulation of Mg^{2+} within the mesophyll vacuole. This was also true in *cax1* and *cax3* mutants, suggesting a complementary role of MRS2-1 and MRS2-5 to regulate Ca^{2+} and Mg^{2+} homeostasis. More recently, increased expression of MRS2 genes was found in tomato leaves grown under elevated Mg^{2+} conditions, including MRS2-2, MRS2-3, MRS2-4 and MRS2-5 (Liu et al., 2023). This study also demonstrated that the expression levels were dependent on leaf age, indicating the developmental stage and timing of stress application are important factors, as found in the present study. CAX 1 and 3 and MRS2 genes are likely involved in the responses observed of the two ecotypes investigated here. At both day 7 and 21, Ca^{2+} and Mg^{2+} concentrations in the vacuole were higher in S leaves than NS leaves for plants grown in low Ca:Mg. The expression levels of these genes should be evaluated in both ecotypes together with vacuole concentrations, growth and photosynthetic rates. If there are indeed differences in the expression levels of homologs of for these genes in *E. guttata* S leaves relative to NS leaves under low Ca:Mg, this could provide a reasonable explanation for improved growth and photosynthesis of this ecotype. Compartmentalization of both Ca^{2+} and Mg^{2+} would prevent inhibition of carbon fixation in the chloroplasts as well as more tightly regulated Ca^{2+} cytoplasmic concentrations to maintain normal growth and Ca^{2+}-based signaling pathways.

Based on the wide-ranging results of various studies concerning the adaptation mechanisms of plants to serpentine soils, it is clear that the response is complex and not all plant species found growing on serpentine soils respond in the same way. Some plant can tolerate the low Ca availability of serpentine soil, such as the serpentine accession of *Achillea millefolium* by translocating a greater amount of Ca from roots to shoots (O'Dell and Claassen, 2006). Other species, such as serpentine endemics *Poa curtifolia* (Main, 1981) and *Helianthus bolanderi* Gray subspecies exilis Heiser (Madhok and Walker, 1969) accommodate the high concentrations of Mg because they have a higher requirement for Mg, displaying symptoms of Mg deficiency when grown at Mg concentrations of non-serpentine soils. The gradual adaptation mechanism described here for two ecotypes of *E. guttata* is more likely to be found in cases where serpentine and non-serpentine ecotypes exist and the presence of serpentine ecotype is not restricted to serpentine soil. For example, two races of *Lasthenia californica*, A and C, were found to grow in ionically challenging soils (serpentine soils, alkaline flats) and ionically moderate soils (oak woodlands, pastures), respectively, with Race C exclusively restricted to ionically moderate soils (Rajakaruna and Bohm, 1999). A further investigation into these two *L. californica* races revealed that the serpentine tolerant Race A was more tolerant to elevated concentrations of Mg in the soil and translocated 95% of the accumulated Mg into its shoot tissues (Rajakaruna et al., 2003). Where the Mg was localized within the shoot tissues was not assessed in the study, but it does suggest that the mechanisms for ion accumulation and sequestration are adaptable to the conditions in which the ecotype is growing.

5. Conclusions

The recovery of both growth and photosynthetic rates seen in the

serpentine plants in low Ca:Mg after 21 days suggests that neither the photosynthetic machinery nor growth enzymes are being permanently inhibited by the elevated concentrations of Mg^{2+} that have been previously found in these two ecotypes (Palm et al., 2012). Shoot concentrations of Mg^{2+} were elevated in both S and NS plants when grown in low Ca:Mg. However, S leaves exhibited compartmentalization of both Ca^{2+} and Mg^{2+} to the vacuole suggesting greater regulation of ion distribution over time in this ecotype. It is clear from the results of this study that the acclimation of serpentine-adapted *E. guttata* takes several weeks, and that NS plants experience both Ca^{2+} deficiency and Mg^{2+} toxicity. Regulation of cytoplasmic concentrations of Ca^{2+} and Mg^{2+} likely differs between these two ecotypes and explains the differences in growth as a consequent variable response to low Ca:Mg. It may be that this mechanism is unique to the ecotypes of *E. guttata* studied here, but it would be worthwhile to further investigate other species with serpentine ecotypes that are not restricted to growing solely on serpentine soil.

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CRediT authorship contribution statement

Emily Palm: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Werther Guidi Nissim:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Giacomo Colasurdo:** Formal analysis, Data curation. **Elizabeth Van Volkenburgh:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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