Exclusion, Amelioration, Tolerance:

An investigation of the physiological basis for tolerance in serpentine *Mimulus guttatus*

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Abstract

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As sessile organisms, plants must cope with a variety of abiotic stress factors, including the mineral composition of soil. In serpentine soils, the low calcium-to-magnesium ratio has been shown to limit the growth and survival of many plant species. Some species are adapted to serpentine, employing a variety of physiological mechanisms. However the physiological basis for tolerance has not been clearly defined. Populations of *Mimulus guttatus* are found growing on and off of serpentine soil and were used here to identify a potential mechanism conferring tolerance to serpentine-adapted populations. Using a combination of soil and hydroponic culture reciprocal transplants, it was demonstrated that the serpentine and non-serpentine populations were differentially adapted to their native soils and thus ecotypes of *M. guttatus*. Biomass and photosynthetic rates of non-serpentine *M. guttatus* are dramatically reduced in low Ca:Mg conditions relative to the serpentine ecotype. Root and shoot concentrations of calcium (Ca) and magnesium (Mg) reflect external treatment conditions and indicate that tolerance to low Ca:Mg in the serpentine ecotype is not due to exclusion of Mg. Leaf expansion and photosynthetic rates of excised

tissue from serpentine and non-serpentine plants are lower when exposed to low Ca:Mg conditions than in high Ca:Mg conditions. Recovery of the rates of both processes is observed in serpentine plants with continued exposure to low Ca:Mg, indicating that the tolerance to elevated Mg is through gradual acclimation. Uptake rates of Mg by serpentine *M. guttatus* roots are lower and the proportion of Mg contained in the vacuoles of leaf cells is consistent regardless of the treatment Ca:Mg. The results presented here indicate that the Ca:Mg ratio of the soil is the dominant factor affecting growth and that the tolerance of serpentine *M. guttatus* is through gradual acclimation of physiological processes that is likely through a combination of mechanisms of regulating uptake of Mg and the distribution of Mg throughout the tissues of serpentine *Mimulus guttatus*. These results, together with the recently sequenced genome of *Mimulus guttatus*, may provide a valuable framework for studying the transport mechanisms involved in stress response physiology and the genetic basis for tolerance to serpentine soil.

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CHAPTER ONE Introduction

Plants are sessile organisms. As such, they cannot move to a new location to avoid high temperature, low water availability or insufficient nutrients in the soil. Instead, plants must adapt their physiology, growth and/or morphology to cope with conditions that are less than optimal. The effects due to abiotic factors such as climate and water availability are readily seen when one considers the biomes or ecosystems that cover the globe. They range from frigid arctic tundra, to hot dry deserts, to hot humid rainforests and each support a unique set of species. Edaphic factors, those abiotic factors that are specifically related to the soil, are less obvious when one scans the landscape, but have no less of an effect on the plants growing there than drought or extreme heat. And just as with drought or extreme heat, soil properties such as the particle size, pH and nutrient availability may limit the type and distribution of plant species. The soil properties may be anthropogenic in origin or naturally occurring. Both situations provide valuable systems in which to observe and study the physiological limitations of plants to soil conditions as well as the range of phenotypic flexibility that is possible in plant populations.

For growth, metabolism and continuation of a plant population to occur, a suite of macro- and micronutrients are required and must be obtained from the soil. The specific quantities that are required of each nutrient vary among plant species, but the physiological role that each has is fairly conserved. Limited availability of macronutrients such as nitrogen, potassium and phosphorus are common especially in agricultural regions. Excess of micronutrients or heavy metals may be just as detrimental to plants. These conditions may also be due to human activities such as agriculture (increased salts from application of fertilizers) and mining, or they may occur naturally. In both cases, the productivity and colonization of plant communities may be limited.

1.1 The ‘serpentine syndrome’

An example of this may be seen in serpentine soil, a naturally-derived soil type that is often both limited in calcium (Ca) and has extreme concentrations of magnesium (Mg) and heavy metals. The most common and defining characteristic of serpentine soil is the low Ca:Mg ratio. Heavy metals, such as nickel, copper, chromium, and zinc, are often present but vary depending on the parent material of the

soil. Serpentine soils often occupy steep slopes that vulnerable to erosion. As a result, soils are shallow and have low rates of productivity and nutrient cycling. Endemism is extremely common. The vegetation is sparse, and displays xeromorphic growth, closely resembling the morphology of plants growing in dry habitats such as short stature and reduced leaf area. This combination of chemical, physical and biotic factors lead to geologist, Hans Jenny, to coin the term, 'the serpentine syndrome' to describe this remarkable landscape (Jenny 1980).

The distribution of serpentine soil is worldwide, but patchy. Serpentine outcrops can be found in regions of historic volcanic activity, originating from ultramafic rock. The parent material of serpentine soil is serpentinite, a magnesium- and iron-rich mineral, and olivine. The serpentinite weathers as it combines with water over time, releasing a high concentration of magnesium to the forming soil. Relative to the magnesium, the concentration of calcium is very low, and it's these conditions that have a dramatic effect on plant growth.

The plant communities of serpentine soil have fascinated ecologists and plant physiologists for almost two centuries (Kruckeberg 2002). The visual effect alone that the Ca:Mg ratio and heavy metals have on the plant form and distribution is attractive to one interested in understanding the limitations of plant physiology or the pressure exerted by edaphic factors to drive speciation. In attempts to understand the specific effects that Ca:Mg and heavy metals have on plant physiology, a number of hypotheses have been tested, including tolerance to low Ca:Mg, avoidance of Mg toxicity, increased requirement for magnesium, and hyperaccumulation of heavy metals (Brady *et al.* 2005).

Heavy metal tolerance has been studied in many plant species growing on soils rich in heavy metals, including serpentine (Proctor 1970; Baker, 1978; Paliouris 1991; Lombini 2003; Wenzel *et al.,* 2003). Depending on the parent material, there is variation in the type and amount of heavy metal present, but may include copper, chromium, zinc and nickel. Several plant species have been found to exclude the heavy metal from entering the roots, or from moving past the endodermis into the vasculature through a set of ion transporters. Other species are hyperaccumulators, maintaining elevated concentrations of a particular heavy metal, storing it in the vacuole (Gabbrielli *et al.*, 1991; Boyd and Martens, 1998; Küpper *et al.*, 2001). Some species have evolved to become obligate hyperaccumulators, such that their maximal

performance requires the concentration of the heavy metal in the plant tissue (Boyd and Martens 1998). . In all of these cases, the plant species are uniquely adapted to high levels of a particular heavy metal in a way that is outside of the range of what has been identified as normal plant physiology or plant nutrition requirements.

The pressure on plant speciation due to the presence of heavy metals in the soil is strong. Most heavy metals are considered to be micronutrients (needed by the plant in only very small quantities) or toxic to plant growth and function. The other relevant factor in serpentine soils, and the focus of the work presented here, is the calcium to magnesium ratio. Because both calcium and magnesium are macronutrients and essential for plants, the effect of the low Ca:Mg is less clear than with heavy metals like zinc and nickel. Comparisons of serpentine adapted ecotypes have shown that they may be indifferent to elevated concentrations of Mg (Proctor 1970; Marrs and Proctor 1976; Rajakaruna 2003; Rajakaruna *et al.,* 2003; Asemaneh *et al.,* 2007)), excluding the Mg from the plant (Walker *et al.,* 1955; Madhok 1965; Madhok and Walker 1969; Sambatti and Rice 2007), better able to acquire the Ca despite the low availability in the soil (Tibbetts and Smith 1993; Asemaneh *et al.,* 2007) or in some cases, have a higher requirement for Mg (Madhok 1965; Madhok and Walker 1969). When compared with cultivated sunflower, serpentine adapted *Helianthus exilis* showed increased growth with increasing concentrations of Mg and decreasing Ca:Mg (Madhok and Walker 1969**).** While the general mechanism for tolerance has been identified in some species (exclusion, hyperaccumulation, indifference), the internal, physiological impacts of low calcium or high magnesium have yet to be determined. Much is know about the general roles of calcium and magnesium in plants. Identifying the deeper mechanisms occurring in serpentine-adapted ecotypes or species would provide further understanding of the flexibility or tight regulation of these two macronutrients.

1.2 Mineral nutrition of plants

Calcium

Calcium is a divalent cation whose distribution and role in plant physiology is tightly regulated. It is found bound to carboxyl groups in the cell wall, providing structural stability. It may act as a counter-

cation to both inorganic and organic anions in the vacuole. The tight regulation of calcium concentration in the cell is a consequence of its role as a signaling molecule for a variety of processes (Malhó *et al.*, 1998) including red (Shacklock *et al.* 1992) and blue light responses (Baum *et al.,* 1999), stomatal closure due to ABA signaling (McAinsh *et al.,* 1992; Allen *et al.*, 2000; Ng *et al.,* 2001) and heat shock defense mechanisms (Gong *et al.,* 1998). Calcium enters the plant from the soil into roots. As a with most ions, calcium can move through the apoplast, between cells, a process that is heavily driven by the rate of transpiration. To move out of the root cortex and into the vasculature for translocation into the shoot, calcium must cross the casparian strip of the endodermis. The movement of calcium apoplastically directly to the xylem is restricted to the extreme root tips, where the casparian strip is absent or endodermal cells around the stele are unsuberized. Where the casparian strip is present or the endodermis suberized, evidence suggests that calcium enters the endodermal cells through calcium-permeable channels on the cortical side. Whereas the symplastic movement of calcium through the cortex may occur through plasmodesmata, entry into the stele requires active transport by Ca-ATPases or Ca^{2+}/H^{+} antiporters on the stele side of the endodermal cell. In this way, plants may regulate calcium entry into the roots and the concentrations found in the shoot.

Calcium is immobile once it enters a plant cell and is incorporated. It cannot move from older tissues via the phloem to new, developing meristems. While calcium deficiencies of the soil are generally uncommon, they are a common feature of serpentine soil. Calcium deficiency symptoms include cessation of growth at the meristems and reduced expansion and increased curling of young leaves (Walker *et al.,* 1955; White and Broadley 2003). Some plants (classified as calcifuges) are adapted to growing in low calcium soils. Many of these species are indifferent to low calcium, and even exhibit a decrease in shoot dry weight with increased external calcium concentrations (Jefferies and Willis 1964). Similar results have been found in studies comparing the response of serpentine adapted and non-adapted species. Where serpentine plants appear to be indifferent to external calcium concentrations, non-adapted species exhibit marked increases in plant height and biomass with additional calcium (Asemaneh *et al.,* 2007). The mechanism that allows for greater growth rates and higher biomass in serpentine plants despite low calcium availability is unknown.

Magnesium

Excess concentrations of macronutrients are not commonly found in nature, but this is the case in serpentine soil with respect to magnesium, a fact that is known to have significant effects on plant physiology. While much is known about the effects of magnesium deficiency from controlled nutrient soil and hydroponic experiments (Fischer and Bremer 1993; Cakmak *et al.,* 1994; Marschner 1995), there have been few studies regarding the potentially toxic effects of excess magnesium. Mg^{2+} homeostasis within the cytosol and chloroplast is important due to the sensitivity of enzymes to high Mg^{2+} concentrations. The concentration of magnesium within the metabolic pool (the cytoplasm and the chloroplast) is maintained at 2-10 mM (Leigh and Wyn Jones 1986), mainly by the vacuole (Stelzer et al. 1990). As a macronutrient, the divalent magnesium cation is necessary for normal plant function and growth. Magnesium is the central molecule of chlorophyll, making it essential for the photosynthetic process to occur. It also plays an important role in enzymatic function (Sugiyama *et al.,* 1968; Pierce 1986), binding to and assisting in the activation of ATPases (Yazaki *et al.* 1988), phosphatases (Gerhardt *et al.,* 1987), and carboxylases (Pierce 1986; Portis 1992).

Just as with calcium, magnesium is found bound to the cell wall, in the cytosol, in the vacuole, and in the chloroplasts (Marschner 1995). The intracellular distribution of magnesium is important, not for signaling purposes as with calcium, but for a similar reason in that many enzymes are sensitive to small changes in magnesium concentration. Magnesium from the soil enters the plant though the roots, and moves through the cortical cells both apoplastically and symplastically (Yeo et al. 2007). The symplastic pathway is through the plasmodesmata, passing by carboxyl groups in the cell wall that act as cation exchangers. Entry into this pathway, just as with calcium, requires channels or transporters. Neither magnesium-specific channels or transporters have been identified in plant roots (Shaul 2002). However, the *rca* channel, found in wheat roots and defined as a Ca^{2+} channel, is known to be permeable to a wide range of cations, including Mg^{2+} (Pineros and Tester 1997; Pineros and Tester 1995). Selectivity of this channel for calcium is based on having a lower affinity and higher free energy barriers to Mg transport (White *et al.,* 2000). The apoplastic pathway through the cortex is driven by transpiration, but is blocked by the casparian strip from connecting to the vasculature. At this time, little is known about the process of

magnesium entry into the symplast or loading of magnesium into the xylem. There is evidence that flow through the apoplastic pathway is faster than the symplastic pathway for both calcium and magnesium, as shown for measurements of solution $^{25}Mg^{2+}$ and $^{44}Ca^{2+}$ exchanging with apoplasmic calcium and magnesium in the cortex (Kuhn et al. 2000).

While much is known about the effects of magnesium deficiency, there have been few studies regarding the potentially toxic effects of excess magnesium. Though elevated magnesium in the soil is uncommon in nature, it is one of the defining characteristics of serpentine soil and therefore deserves consideration. Mg^{2+} homeostasis within the cytosol and chloroplast is important due to the sensitivity of enzymes to high Mg^{2+} concentrations. The concentration of magnesium within the metabolic pool (the cytoplasm and the chloroplast) is maintained at 2-10 mM (Leigh and Wyn Jones 1986), mainly by the vacuole (Stelzer et al. 1990).

Mg^{2+} homeostasis can be regulated both by selective transport by the roots through the symplastic pathway, or by the buffering capacity of the vacuole. Transport of Mg^{2+} into the vacuole is mediated by Mg^{2+}/H^{+} exchangers. The first evidence of an Mg^{2+}/H^{+} exchanger was found in *Hevea brasiliensis,* specifically in the lutoid membranes (vacuolar-lysosomal like compartments) in latex where Mg accumulates 10 times higher than in the cytosol (Amalou *et al.,* 1992; 1994), and in the roots of *Zea mays* (Pfeiffer and Hager 1993). The AtMHX gene, identified in *Arabidopsis thaliana* encodes a Mg/H transporter that has been localized to the vacuole (Shaul *et al.,* 1999). Transformation of the *AtMHX* gene into tobacco showed accumulation of magnesium when exposed to high external magnesium concentration (Shaul *et al.,* 1999). Furthermore plants overexpressing AtMHX and exposed to increased Mg and Zn showed necrotic lesions in the leaves, while plants with normal expression levels of AtMHX showed none. Internal concentrations were the same in both indicating that AtMHX plays a role not in altering the total content, but in the intracellular distribution of Mg.

1.3 *Mimulus guttatus*

To study the effect of the low Ca:Mg ratio of serpentine soil on plant physiology, *Mimulus guttatus* Fischer DC was used. *M. guttatus*, commonly known as the seep monkeyflower, can be found growing

across the Western United States in a variety of soil and climate conditions. *M. guttatus* has been observed growing in copper- and nickel-rich mine tailings as well as the soils adjacent to thermal vents in Yellowstone Park. A *bodenvag* species (Kruckeberg 1951), populations of *M. guttatus* grow both on and off of serpentine soil. This plant has been studied in regards to its tolerance to low Ca:Mg and heavy metals, specifically copper (Gardner and MacNair 2000; Hughes *et al.,* 2001). *M. guttatus* appears to be tolerant to a wide range of edaphic factors making it a valuable system in which to study phenotypic plasticity and intraspecific genetic variation. *Mimulus nudatus* is a serpentine endemic species considered to be derived from *Mimulus guttatus* suggesting that speciation driven by the edaphic stress of serpentine soil has occurred in the past. The populations used here were collected from serpentine and non-serpentine soils in the McLaughlin Natural Reserve in Lake County, California (Palm *et al.,* 2012). The collection of seeds and soil, as well as the propagation of the experimental populations, is further described in Chapter Two.

Fitter and Hay (2002) described four ways a plant can avoid damage by stressful abiotic factors: 1) escape, in which plant populations are restricted to locations or a season where and when the stress is not present; 2) exclusion, in which survival in the presence of the stressful factor is enabled by an exclusionary mechanism at either the root or shoot level; 3) amelioration; and 4) tolerance, achieved through physiological adjustments that accommodate the stressful factor. Because *M. guttatus* is found growing both on and off of serpentine soil, escape is not a mechanism employed by this species to avoid low Ca:Mg conditions. In Chapter Two, differential adaptation to native soils of serpentine and non-serpentine populations was investigated, as well as the possibility of a magnesium exclusion mechanism. Chapter Three focuses on the direct and indirect effects of elevated magnesium concentration on photosynthesis and leaf cell expansion. Differences in the physiological response of serpentine and non-serpentine ecotypes to elevated magnesium suggested amelioration of increased internal magnesium concentration through differences in magnesium localization. Presented in Chapter Four are uptake rates of calcium and magnesium as well as initial evidence of differences in the intracellular distribution of magnesium between serpentine and non-serpentine ecotypes.

CHAPTER TWO Serpentine Tolerance in *Mimulus guttatus* Does Not Rely on Exclusion of Magnesium

ABSTRACT

The effect of serpentine soil-like low Ca:Mg ratios on growth was investigated in serpentine-adapted and non-adapted populations of *Mimulus guttatus* through both soil and hydroponic reciprocal transplants. Adaptation to Ca:Mg ratios in *M. guttatus* was measured as differences in biomass accumulation, uptake of Ca and Mg and photosynthetic rates. Serpentine-adapted plants persisted on both serpentine and non-serpentine soils, while non-adapted plants survived only on non-serpentine soil. When grown hydroponically, low Ca:Mg ratio decreased the biomass of non-serpentine adapted *M. guttatus* while serpentine-adapted plants increased in biomass relative to their growth on high Ca:Mg. Internal concentrations of Ca and Mg mirrored those of the growth solution in both populations; however serpentine-adapted *M. guttatus* had a higher shoot:root ratio of Mg when grown in low Ca:Mg solutions. Elevated Mg reduced photosynthetic rates in non-adapted plants without changes in chlorophyll concentration or photosystem efficiency. Here, the use of hydroponic culture isolated the Ca:Mg ratio from other soil characteristics as the dominant factor affecting growth. Differences in the growth of plants from these populations of *M. guttatus* in reciprocal transplant experiments indicate a genetic basis for a tolerance mechanism to low Ca:Mg, but one that is not based on the exclusion of Mg.

2.1 INTRODUCTION

Plant populations found on serpentine soils present a natural system to study physiological mechanisms for plant growth in stressful environments due to abiotic factors. Serpentine soils are naturally occurring and are generally low in productivity; the plants growing on them often display xeromorphic growth patterns of small leaves and reduced stature (Kruckeberg 2002). Essential plant nutrients, such as calcium, potassium and phosphorus, are often scarce, while heavy metals such as nickel and chromium are commonly found at elevated concentrations (Proctor and Woodell 1975; Brooks 1987; Brady *et al*. 2005). The unifying characteristic of these landscapes is the low ratio of calcium to magnesium, which is considered to be the driving force for localized adaptation of plant populations to this soil type (Kruckeberg 2002; Brady *et al.* 2005).

Selective uptake of calcium (Ca), and the possible exclusion of magnesium (Mg) and potentially toxic heavy metals has been a frequently tested hypothesis for adaptation of serpentine-adapted plants. Some serpentine natives have been found to absorb more Ca, and less Mg despite the elevated concentration of Mg in the soil, without significant effects on yield (Walker *et al*. 1955; Asemenah *et al.* 2007). In contrast, cultivated species showed a marked increase in yield with the addition of Ca to the soil. An endemic species of sunflower, *Helianthus exilis,* was shown to maintain higher Ca:Mg in its roots and shoots with stable yield in response to decreasing soil Ca:Mg, a sharp contrast to the cultivated sunflower, *H. annuus,* which decreased in yield with decreasing soil Ca:Mg (Madhok &Walker 1969). Serpentine-adapted *H. exilis* excludes more Mg from its leaf tissue than non-adapted *H. exilis* when both populations are grown in low Ca:Mg serpentine soil (Sambatti and Rice 2007). Ca is essential for growth as it is a critical component of cell walls (Marschner 2001). While Mg is also a macronutrient, elevated levels have been shown to decrease the photosynthetic rate of *Phaseolus vulgaris*, especially when coupled with decreased water potential (Rao *et al.* 1987). In addition, high cytosolic Mg concentrations have been shown to alter cytosolic pH and disrupt enzymatic activity, thereby reducing photosynthetic rates (Marschner 2001; Shaul 2002). The ability of some species to adjust internal Ca:Mg ratios that differ from the soil environment suggests a selective uptake mechanism by the plant, and that tolerance to serpentine soil is possible through the regulation of internal Ca:Mg, with increased uptake of Ca coupled with the exclusion of Mg.

Mimulus guttatus, with its recently sequenced genome, has emerged as a genetically-tractable system for studying adaptation (Wu *et al.* 2008). Populations of *M. guttatus* are found naturally growing in the western states of the US in a variety of soil types, both on and off of serpentine. In this study, *Mimulus guttatus* DC (Phrymaceae) seeds were collected from serpentine and non-serpentine soil sites in California and a reciprocal transplant was performed in the greenhouse to determine if the serpentine and non-serpentine populations were adapted to their respective native soils. This was followed by a hydroponics study of the response of *M. guttatus* to solutions mimicking normal, high Ca:Mg soil and serpentine, low Ca:Mg soil (Brooks 1987; Epstein and Bloom, 2005). We hypothesized that *M. guttatus* grown hydroponically in solutions mimicking the calcium to magnesium ratios of non-serpentine and serpentine soils would resemble soil-grown individuals. Further, we predicted that serpentine-adapted *M.*

guttatus would produce more biomass in low Ca:Mg by selectively excluding Mg and would maintain higher internal Ca:Mg than non-adapted populations of *M. guttatus* would on serpentine-like conditions. To evaluate the response to abiotic stress, we used the indices of biomass, photosynthetic rate, and chlorophyll content due to the observed differences in preliminary studies regarding growth and changes in leaf pigmentation.

2.2 MATERIALS and METHODS

Seed collection and development of experimental populations

Serpentine (SP) *M. guttatus* seeds were collected from the Donald and Sylvia McLaughlin Natural Reserve in California. Non-serpentine (NP) *M. guttatus* seeds were collected approximately 100 km south of the serpentine site along Soda Canyon Rd., Napa Co., California to ensure the populations were genetically distinct. At each site, seeds were collected from two plants (SP1, SP2; NP1, NP2) approximately 5 m apart.

The serpentine and non-serpentine seeds collected in the field were sown in the University of Washington's Department of Biology's greenhouse. One offspring from each serpentine (SP1, SP2) and non-serpentine line (NP1, NP2) was selected for crosses. SP and NP lines were generated by reciprocally crossing the two non-serpentine plants (*e.g.*, NP1 x NP2) and the two serpentine plants (*e.g.*, SP1 x SP2). Seeds from reciprocal crosses were pooled.

Soil collection and analysis

For the reciprocal transplant experiment, serpentine soil was collected from the Donald and Sylvia McLaughlin Natural Reserve from a site lacking plants to minimize disturbance; however, a population of *M. guttatus* was located approximately 10-15 m from the soil collection site. The top 15-20 cm of soil was sifted through a metal screen with 1 x 2.5 cm holes to remove large rocks.

For determination of elemental composition, soil samples were collected adjacent to both the serpentine and non-serpentine seed-source plants; samples of serpentine soil collected for the transplant experiment (above) were also included. Soil samples were sieved to less than 2mm. Mineral extractions were performed following the EPA Method 3050B for heavy metals, which includes a digestion protocol

using HNO_3, H_2O_2 and HCl. Digested samples were analyzed using inductively coupled plasma - mass spectrometry (ICP-MS)by Analytic Services in the School of Forest Resources at the University of Washington. Soil pH was measured using a 1:5 ratio of soil to dH_2O. A five gram sample of soil was added to 25 mL of dH_2O, mixed thoroughly and allowed to incubate for 30 min

Reciprocal transplant experiments

Serpentine (SP) and non-serpentine (NP) seeds were germinated on greenhouse soil (hereafter referred to as non-serpentine) (Sunshine No. 4 Professional Growing Mix, Aggregate Plus by Sun Gro Horticulture Canada Ltc.) in plug trays with 2 x 2 x 3 cm openings. Plants were grown in the greenhouse. For the first study, seedlings were transplanted 11 d after germination into serpentine or non-serpentine soil plug trays with 4 x 4 x 5 cm openings, after rinsing the roots in distilled H_2O to remove soil particles. The first experiment included 2 trays total: 1 non-serpentine soil tray and 1 serpentine soil tray with 17 SP and 17 NP plants in each tray. Trays were placed on top of a shallow, watertight tray for sub-irrigation with distilled H_2O. Plants were monitored for survival for five weeks after transplanting.

A second experiment was conducted with slightly larger plants transplanted to treatments 14 d after germination, and consisted of 4 trays total: 2 trays of non-serpentine soil and 2 trays of serpentine soil with 20 SP and 20 NP plants in each tray. Plants were sub-irrigated with either dH_2O or 4mM $Ca(NO_3)_2$. Ten days after transplanting onto the treatment soil, leaf tissue was collected from 10 of the SP and NP plants on each treatment for ion profiling by ICP-MS.

Hydroponic culture

For all experiments conducted with hydroponically grown *Mimulus guttatus*, pooled cuttings were made from 10 parent plants each of SP and NP, maintained year-round in greenhouse conditions in greenhouse soil. Cuttings were placed for five days in dH_2O in a high humidity tent for initiation of roots. Two treatment solutions were used: a control, non-serpentine solution (NS), with a Ca:Mg ratio of 4.0, similar to that of normal soil (Epstein and Bloom 2005), and a serpentine solution (SS) with a Ca:Mg ratio of 0.02, mimicking serpentine soil (Brooks 1987; Epstein and Bloom, 2005). Nutrient content of both solutions was based on 0.25x modified Hoaglands solution (Hoagland and Arnon 1950; Epstein and

Bloom 2005), and varied in both $Ca(NO_3)_2$ and $Mg(NO_3)_2$ to achieve the desired Ca:Mg ratio of experimental treatments without changing the osmolarity or nitrate content of the solution (Table 1). Solution pH was maintained at a value of 6.8 with the addition of KOH. Twelve cuttings of SP and NP each were inserted into the lid of a 54.6 x 40 x 21.6 cm plastic tub which was filled with an aerated nutrient solution; cuttings were held in place with foam plugs. Solutions were replaced once a week over the experimental period. All individuals were started in the non-serpentine (Ca:Mg = 4.0) solution and grown for ten days as cuttings to establish roots and acclimate to greenhouse conditions. After ten days, the roots of the cuttings were rinsed with dH_2O and the 12 SP and 12 NP plants were randomly assigned to one of two treatment groups to be grown for an additional 30 days in either the non-serpentine solution (NS) or serpentine solution (SS). The hydroponics experiment was conducted in three times in the greenhouse in the late spring and summer months between 4/1/2008 and 9/20/2010. The average temperature ranged from 18-24 °C and light intensity from 0 (night) to 800 (midday) μmol m^{-2} s^{-1} over the course of the day.

Gas exchange and fluorescence

Photosynthetic, or CO_2 assimilation, rates, were evaluated using the Li-6400 portable photosynthesis system (Li-Cor Biosciences, Lincoln, Nebraska). Briefly, a 2 cm^2 area of leaf tissue was clamped into the chamber and exposed to a pre-defined set of conditions: CO_2 gas was provided into the chamber at 380 μmol mol^{-1} and relative humidity maintained between 50-60%. The carbon assimilation rate of was measured over a range of fluence rates: 0, 200, 400, 800, 1200, 1600 and 2000 μmol m^{-2} s^{-1}, supplied by a lamp contained within the leaf chamber. Leaf stomatal conductance, transpiration rate and carbon assimilation rate were allowed to reach steady-state values before the next fluence rate was applied, a maximum of ten minutes. All photosynthetic measurements were performed in the greenhouse between early morning and early afternoon.

Photosystem function was evaluated by measurements of fluorescence using a portable fluorometer, Mini PAM (Walz, Effeltrich, Germany). To measure maximum photosystem capacity (F_v/F_m), plants were dark adapted overnight, and measurements taken the following morning in a dark growth room (green light only). Plants were then exposed to 200 μmol m^{-2} s^{-1} ambient light and light-adapted

fluorescence measurements were performed on the same area of leaf. Values of dark-adapted and light-adapted fluorescence were used to calculate photosystem capacity (F_v/F_m) and efficiency (F'_v/F'_m), respectively (Maxwell and Johnson 2000):

Maximum quantum yield of PSII: $F_v/F_m = (F_m - F_o) / F_m$

Quantum yield of PSII: $\Phi PSII = (F'_m - F_t) / F'_m$

Biomass

Destructive sampling for biomass occurred after 30 days in the treatment solutions. Roots and shoots were separated, dried at 70 C for four days, and weighed individually. Dried tissue samples were stored in sealed plastic bags until preparation for ion profile analysis with ICP-MS.

Analysis of mineral content

The procedure of Jones *et al*. (1991) was followed with slight modification. Shoot and root samples were oven-dried for four days at 70° C and finely ground with mortar and pestle. A maximum of 0.5 g of each sample was used for analysis. Ground tissue was digested with concentrated nitric acid at 95°C. Samples were further processed with 30% hydrogen peroxide, heated to evaporation. Each sample was then diluted for analysis with 1:10 HNO_3 and dH_2O. Mineral content was evaluated by the Analytical Lab in the School of Forest Resources at the University of Washington. A 0.5 g sample of tomato leaves from the National Institute of Standards and Technology (NIST, No. 1573a) was digested and analyzed alongside each batch of *M. guttatus* as a calibration standard. The values obtained from ICP-MS were used to calculate Ca and Mg content (ppm) based on the mass and dilution of each sample.

Chlorophyll concentration

Following gas exchange and fluorescence measurements, a 2 cm^2 disc of leaf tissue was collected for analysis of chlorophyll concentration. After obtaining the fresh biomass, each disc was ground with mortar and pestle in one ml 100% acetone, and then diluted with an additional 1 ml of 100% acetone for analysis of chlorophyll a and b concentration. Chlorophyll a was measured at 661 nm and

chlorophyll b at 644 nm. Concentrations were calculated with the measured absorption values, and the corresponding equations and coefficients (Lichtenthaler 1987).

Statistical Analysis

All data were subjected to a two-way analysis of variance to test for differences among soil/solution treatments and plant type, as well as their interactions. *Post-hoc* pairwise comparisons were made using Tukey's Honestly Significant Difference test. Statistical analyses were performed using R Statistical Software (http://www.r-project.org).

2.3 RESULTS

Soil Analyses

Elemental composition of serpentine and non-serpentine soil differ greatly in two fundamental respects (Table 2). First, the Ca:Mg mass ratio is much lower in the serpentine soils (0.008 – 0.012) than in the non-serpentine soils (2.228). Second, Mg and the heavy metals Cr and Ni are found at significantly higher concentrations in the serpentine soil than in the non-serpentine soil.

Reciprocal transplant experiments

Transplants into soil

In the first experiment, five weeks after being transplanted onto serpentine soil all of the serpentine plants (17/17) remained alive, whereas none of the non-serpentine plants (0/17) were alive (Fig. 1a). All serpentine and non-serpentine plants (n=17 for each) transplanted onto non-serpentine soil were alive five weeks after transplanting (Fig. 1a). For the control treatment (dH_2O), all of the serpentine plants (10/10) remained alive, whereas none (0/20) of the non-serpentine plants was alive. All serpentine and non-serpentine plants (n=10 for each) transplanted onto non-serpentine soil were alive six weeks after transplanting.

Transplants into hydroponics

Plant growth response to the Ca:Mg ratio of the hydroponic solutions (Fig. 1b) simulates the survival response of reciprocal transplants onto soil. When grown on serpentine solutions (SS),

serpentine-adapted plants (SP) accumulate more total biomass than non-adapted plants (NP), 3.96 and 1.66 g respectively. The non-adapted *M. guttatus* (NP) developed dramatically less biomass in serpentine (SS) versus non-serpentine (NS) solution (Fig. 1b; 8.13 vs. 1.66 g). This is most clearly seen in the shoot biomass data: when grown on non-serpentine (control) solution (NS) non-adapted plants (NP) have significantly higher shoot biomass (6.96 g) than serpentine plants (1.41 g). This result is reversed when plants are grown on serpentine solution (SS), where shoot biomass of non-serpentine plants (NP) reaches only 1.41 g, whereas serpentine plants accumulate 3.44 g.

Ion Profile Data

When irrigated with dH_2O, serpentine plants grown on serpentine soil contained significantly more Mg in their leaf tissue than serpentine plants grown on greenhouse soil ($P<0.05$) (Fig. 2a). There was significantly less Mg in the leaf tissue of non-serpentine plants than serpentine plants when both were grown on serpentine soil ($P<0.05$) (Fig. 2a). Irrigation of serpentine soil with 4mM $Ca(NO_3)_2$ significantly increased the amount of Mg in the leaf tissue of both serpentine and non-serpentine plants ($P<0.05$) (Fig. 2a). There was no significant difference in the Ca:Mg in the leaf tissue between serpentine and non-serpentine plants grown on serpentine soil on dH_2O (Fig. 2b). Addition of calcium by irrigating serpentine soil with 4mM $Ca(NO_3)_2$ did not significantly change the Ca:Mg in the leaf tissue for either serpentine or non-serpentine plants.

Internal concentrations of Ca and Mg of hydroponically grown *M. guttatus* ecotypes closely reflect those of the growing solution (Fig. 2c,d). In both populations, total Ca content is lower when plants are grown in the serpentine solution as compared to the control (Fig. 2c). Similarly, with more Mg in the serpentine solution, both types of plant show increased total Mg (Fig. 2d). There is a difference in the pattern of internal Mg distribution between root and shoot. The serpentine-adapted *M. guttatus* in the serpentine solution maintains a higher concentration of Mg in the shoots than in non-adapted individuals (Fig. 2e). Indeed, the shoot-to-root ratio in serpentine *M. guttatus* is higher in the serpentine solution, whereas the ratio in non-adapted plants does not differ between the two treatment solutions (Fig. 2e). A similar pattern is found in the shoot-to-root ratio of Ca, in that there is an increase among serpentine plants grown in serpentine solution relative to the non-serpentine solution, and no change in the non-

serpentine plants in response to the solution (data not shown), however not found to be significant.

While the pattern is the same, there is an observable 2-fold difference in the mineral content between soil and hydroponically grown plants. To check whether the degree of leaf expansion and age had an affect on the concentration of Ca and Mg, ICP-MS was performed on cuttings after 10 days in the non-serpentine growth solution. Both Ca and Mg were higher in shoots (approximately 5000 and 1000 mg kg^{-1}, respectively; data not shown).

Photosystem analysis

Carbon assimilation rates, a measurement of photosynthetic rate, were compared over a range of light intensities to evaluate stress responses of both populations to changes in Ca:Mg supplied to the roots. Serpentine plants in the serpentine solution, and the non-serpentine plants in the control solution had the highest carbon assimilation rates over the full range of fluence rates. The assimilation rates of NP in serpentine, and SP in non-serpentine solutions, were significantly lower (Fig. 3a). Focusing on the assimilation rates at 400 µmol m^{-2} s^{-1}, where light saturation occurs in all four treatment groups (Fig. 3a), when grown on serpentine solution (SS) the serpentine-adapted plants (SP) maintained a higher rate of CO_2 assimilation than did the non-adapted plants (NP) (Fig. 3b). In this case, the SP actually exhibited an increased rate from that observed in the non-serpentine solution with significant genotype-treatment interactions. The assimilation light curve indicates that serpentine plants grown in serpentine Ca:Mg ratios achieve the same light saturation point as non-adapted plants in a control solution, but the assimilation rate at which the saturation occurs differs depending on the type of plant and the treatment solution. Fluorescence data revealed no significant difference between the two groups of plants in either solution with respect to maximum light absorption capacity or efficiency (Fig. 3c). There was no significant difference in chlorophyll concentration between the two populations in either solution (Fig. 3d).

2.4 DISCUSSION

Serpentine patches of land are so defined in the overall landscape that the term “serpentine syndrome” was coined to describe the shared characteristics of these plant communities (Jenny 1980). For resistance to toxic ions in general, four strategies have been described: 1) escape through alterations in phenology to avoid seasonally unfavorable conditions, 2) exclusion of toxic ions through recognition by the plant and prevention of uptake, 3) amelioration of toxic internal concentrations through processes of excretion, dilution or sequestration, and 4) tolerance through the development of a metabolic system able to function at abnormally high levels of a particular ion (Fitter and Hay 2002). Analyzing the internal concentrations of minerals in serpentine-adapted plants is a useful starting point to determine the strategy by which they survive in this challenging set of soil characteristics (Lahner *et al*. 2003; Salt 2004).

A number of comparative studies have been performed using *M. guttatus,* and *M. nudatus*, a serpentine endemic species that is considered to be derived from *M. guttatus*. Experiments combining both the low Ca and high Mg of serpentine with drought conditions showed a correlation between tolerance to serpentine and a tolerance to drought in *M. nudatus,* as well as a higher tolerance to well-watered serpentine soil in the serpentine endemic species than in *M. guttatus* populations (Hughes *et al*. 2001). A hydroponic study comparing the growth response of *M. nudatus* with two *M. guttatus* ecotypes (one ecotype from a serpentine soil, and another from non-serpentine soil) in solutions ranging in Ca:Mg from 6:0 to 0:6 showed no significant difference between the *M. guttatus* ecotypes. It was suggested that observed differences in the growth response between species was the result of Ca deficiency and not Mg toxicity (Gardner and MacNair 2000). However, the plants were not evaluated for internal concentrations of Ca and Mg.

We hypothesized that some Californian ecotypes of *M. guttatus* are adapted to serpentine soils, and that this result could be replicated with a hydroponics system mimicking the Ca:Mg ratio of serpentine soil. Greenhouse transplant experiments plainly revealed that serpentine and non-serpentine populations of *M. guttatus* are differentially adapted to serpentine soil (Fig. 1a). This is consistent with other studies that have found localized adaptation to native soils of serpentine and non-serpentine plants within a species complex (Kruckeberg 1951) and with strains of a single species (Kruckeberg 1954; Wright *et al.* 2006; Wright 2007). Hydroponically grown plants showed the same response, with the serpentine

ecotype having a significantly greater biomass in low Ca:Mg than the non-serpentine ecotype (Fig. 1b). To explain this result, we hypothesized that populations of *M. guttatus* found growing on serpentine soil exclude Mg from the roots, enabling the plants to maintain a Ca:Mg ratio closer to that of plants growing on normal soil (Ca:Mg ratio of ~ 1). Surprisingly, our data from the ICP analysis shows that this is not the case (Fig. 2). Both serpentine-adapted and non-adapted plants contained higher total Mg when grown in a high Mg solution. The same pattern was found when tissues were evaluated for the relative composition of Ca: lower available Ca in the solution resulted in lower internal Ca content in both ecotypes. The fact that there was a 2-fold difference in the shoot Ca and Mg between soil and hydroponically grown plants may be due to a difference the degree of leaf expansion. The cuttings used for the hydroponic study were started about the same size as seedlings harvested at 24 days old. As cells expand, water content increases and may dilute the solute concentration within a cell. In addition, the mobility of Ca and Mg will depend on how tightly they are bound in cell walls and to chelators, and remobilization to newer leaf tissue may decrease over time. That both types of plant internally reflect ion composition of the growth solution shows a treatment effect and that there is no genotypic basis for an exclusionary mechanism of Mg from the plant as a whole.

An inversion in biomass was observed among the hydroponically grown plants in that serpentine plants significantly increased in yield while at the same time, non-serpentine plants significantly decreased in yield when grown in serpentine solution. This is consistent with an earlier study that compared the yield of serpentine endemic *Helianthus exilis* and cultivated *Helianthus annuus* over a range of increasing Mg concentrations. The serpentine endemic *H. exilis* increased in yield with increasing Mg until reaching a concentration where no further significant increase occurred (Madhok and Walker 1969). It has been suggested that a possible mechanism for tolerance to serpentine soil is in fact a high Mg requirement (Madhok 1965; Madhok and Walker 1969; Marrs and Proctor 1976; Main 1981). The fact that serpentine grown, serpentine *M. guttatus* in this study did not exceed the yield of non serpentine plants in non-serpentine solution, as well as the leveling off of yield in *H. annuus* (Madhok and Walker 1969), suggests that there may be additional cost to excessively high concentrations that limits growth.

The results for biomass accumulation obtained in this study are contradictory to those found in previous studies comparing growth responses to serpentine or serpentine-like substrates among *M. guttatus* ecotypes. Earlier studies utilized seeds and plants collected from populations growing on Cu-rich serpentine soils (Gardner and MacNair 2000; Hughes *et al*. 2001), whereas the serpentine ecotype used in this study was from soils with elevated Cr and Ni. While not addressed in earlier studies or in this experiment, it is possible that the differential adaptation of some *M. guttatus* ecotypes to serpentine may be partially due to competition among metal ions, and/or the beneficial effect of a fungal association with roots. Hydroponic studies are a pared-down approach designed to elicit an observed growth response; thus they may not fully replicate the cumulative effects exerted by all of the biotic and abiotic factors in the environment to which the plants are actually adapted. However, the similar results found in our reciprocal transplant (soil) and hydroponic experiments reveal that among these Californian populations, Ca:Mg plays a significant role in serpentine adaptation. Furthermore, the addition of Ca to low Ca:Mg substrates, returning them to ratios of 1 or higher, has restored normal yield of non-adapted plants (Vlamis 1949, Walker 1948a, Kruckeberg 1954, Proctor 1970, O'Dell *et al.* 2006), an aspect that was only tested here among soil-grown plants. However, in this case irrigating the plants growing on serpentine soil with 4mM $CaNO_3$ did not restore biomass accumulation among non-adapted plants, and actually increased the concentration of Mg in the leaves of both serpentine tolerant and non-tolerant ecotypes.

Nevertheless, there are similarities between the biomass and carbon assimilation rates collected in this study and earlier studies of the serpentine syndrome: Serpentine-native or -adapted populations show increased biomass with decreasing Ca:Mg; conversely, in non-adapted plants, biomass accumulation slows or even arrests as Ca:Mg decreases (Madhok and Walker 1969; Rajakaruna *et al.* 2003; Asemenah *et al.* 2007). We observed these same responses in our hydroponic experiments.

The standing question from these studies has been what is the physiological cause of such dramatic differences in biomass production. When growth of non-adapted plants is inhibited on serpentine conditions, are photosynthetic rates impaired by an imbalance of Ca and Mg within the plant? Or is meristematic growth inhibited? Here we evaluated the photosynthetic rates of adapted and non-adapted populations of *M. guttatus* in response to high and low Ca:Mg as a possible index for response to abiotic stress. There is a clear relationship between photosynthate supply and growth, and limitations in the

expansion capacity of photosynthetically capable organs may limit the demand on photosynthetic processes. Both populations obtained an assimilation light saturation point at a fluence rate near 400 μmol m^{-2} s^{-1} (Fig. 3a). At this saturation point, the rate of carbon assimilation was higher for serpentine-adapted *M. guttatus* growing on serpentine solution, while non-adapted plants had a significantly lower rate (Fig. 3b). This pattern is reflected in total biomass (Fig. 1b), offering the possibility that differences in biomass accumulation observed for these ecotypes are due to differential inhibition of photosynthesis. It has been shown that genetic variation within a single species can lead to phenotypic differences in leaf size, correlating well with differences in carbon assimilation rates and water-use efficiency (Geber and Dawson 1990; Geber and Dawson 1997).

Assimilation light curves give further indication of possible limitations in the acquisition of carbon (Fig. 3a). When the assimilation rate is graphed over a range of fluence rates, comparisons of light saturation points as well as the maximum assimilation rate (A_{max}) achieved can determine where the limitation exists, whether in the function of the light harvesting photosytems (A_{max} occurs at a low light intensity) or in the regeneration of complexes needed for the fixation of carbon (differences in the absolute value of A_{max}) (Lambers 2006). Here we see that all treatment groups saturate at about the same fluence rate suggesting that photosystems are functioning at the same level. This is confirmed by the fact that there was no significant difference found in the chlorophyll concentration or fluorescence measurements between the treatments. Anthocyanin concentrations should be performed in future studies as the leaves non-serpentine plants do show a significant change in color when grown in serpentine conditions, and turn red (Figure 1A). This change is not due to a decrease in chlorophyll (Figure 3D). Anthocyanins are range in color from blue to red and change depending on the leaf tissue pH and as a protective response to high light intensity, acting as a potential indicator of abiotic stress.

Serpentine-adapted *M. guttatus* shows an increase in the A_{max} from the control to serpentine solution. The opposite is found among non-adapted individuals, suggesting genetic variation in biochemical processes, such as rates of Rubisco activity and ribulose-1,5-bisphosphate regeneration that are involved in the assimilation of carbon and the subsequent biomass accumulation. This was also shown in a study comparing the biochemical limitations to carbon assimilation across 16 lines of *Polygonum arenastrum* (Gerber and Dawson 1997). Differences were found among the lines in stomatal

conductance, Rubisco enzyme activity and electron transport in the regeneration of the substrate for Rubisco. The change in A_{max} (Fig. 3a) reflects a possible target of the increased concentration of Mg in the shoot tissue that may be the enzymes and the transfer of energy that is necessary for the regeneration of RuBP molecules in the carbon fixation cycle. Within species genetic diversity affecting biochemical limitations to carbon assimilation could provide the basis for adaptive evolution in response to severe environmental conditions, such as those presented by serpentine soils.

The mineral content analyses (Fig. 2) show that both serpentine-adapted and non-adapted plants exhibit elevated levels of Mg when grown in a low Ca:Mg, serpentine-like solution. But a potentially interesting pattern arises when the distribution of Mg in the roots and shoots is considered (Fig. 2 d,e): serpentine-adapted plants accumulate more Mg in their shoots when grown in high Mg conditions. Multiple strategies have been identified among a variety of plant systems for avoiding the effects of toxic ions such as excessive Mg, including exclusion and sequestration (Fitter and Hay 2002). For example, serpentine-adapted *H. exilis* was found to exclude high levels of Mg from its shoot tissue, seemingly through the prevention of excessive uptake from the soil (Madhok and Walker 1969; Sambatti and Rice 2007). In contrast, the mineral content analyses in this study (Fig. 2) suggest that serpentine-adapted *M. guttatus* growing on serpentine soil are not excluding Mg and are in fact taking up as much or more than non-adapted populations. In order to avoid the toxic effects of high Mg concentrations, it is possible that serpentine-adapted *M. guttatus* is sequestering Mg in the vacuole. The adapted plants appear to be unaffected by the observed increases in internal Mg concentration, actually increasing their maximum assimilation rate and final yield.

Further investigations into the effects of Mg on the acquisition and assimilation of carbon, as well as the detailed distribution of Mg within the cell, are needed. What remains to be determined is the underlying genetic difference between these two populations of *Mimulus guttatus*, and what process is being affected to produce the differential growth patterns we see in response to Ca and Mg levels in the soil.

	Nutrient concentration (mmol/L)	
	Non-serpentine	Serpentine
$CaNO_3$	1	0.025
$MgNO_3$	0	0.975
$MgSO_4$	0.25	0.25
NH_3PO_4	0.5	0.5
KNO_3	1.5	1.5
Fe-EDTA	0.25	0.25
Micros		
NaCl	0.05	0.05
H_3BO_3	0.025	0.025
$MnCl_2$	0.002	0.002
ZnO	0.002	0.002
$CuCl_2$	0.001	0.001
Na_2MoO_4	0.001	0.001
Ratio Ca:Mg	4	0.02

Table 2.1 Nutrient concentration of non-serpentine and serpentine growth solutions

	Soil Type		
Soil Character	Non-serpentine site (n=1)	Serpentine seed site (n=1)	Serpentine soil site (n=4)
Ca:Mg	2.228	0.008	0.012
Mg	2558	151387	186076
Cr	22	832	1431
Ni	17	1206	1957
pH	6.3	7.1	7.1

Table 2.2 Results of ICP-MS soil analyses on serpentine and non-serpentine soils. Soils collected from serpentine and non-serpentine sites at the McLaughlin Reserve in California. Mean values given. Mineral quantities are in mg kg^{-1}.

	Serpentine Solution		Non-serpentine Solution			
	S Plant	NS Plant	S Plant	NS Plant	Factor	P value
Measurement						
Shoot biomass (grams)	3.4 ± 0.5	1.4 ± 0.3	1.4 ± 0.5	7.0 ± 1.0	Solution	0.02
					Plant	0.02
					Solution x plant	< 0.001
Root biomass (grams)	0.52 ± 0.04	0.25 ± 0.08	0.24 ± 0.04	1.18 ± 0.08	Solution	< 0.001
					Plant	< 0.001
					Solution x plant	< 0.001
Shoot Ca content ($mg\ kg^{-1}$)	125 ± 51	192 ± 38	551 ± 136	464 ± 22	Solution	< 0.001
					Plant	0.90
					Solution x plant	0.32
Root Ca content ($mg\ kg^{-1}$)	35 ± 4	124 ± 29	491 ± 79	235 ± 14	Solution	< 0.001
					Plant	0.05
					Solution x plant	< 0.001
Shoot Mg content ($mg\ kg^{-1}$)	159 ± 30	141 ± 23	61 ± 22	62 ± 3	Solution	< 0.001
					Plant	0.68
					Solution x plant	0.67
Root Mg content ($mg\ kg^{-1}$)	145 ± 25	344 ± 32	89 ± 17	65 ± 5	Solution	< 0.001
					Plant	< 0.001
					Solution x plant	< 0.001
Shoot:root Mg	1.0 ± 0.2	0.48 ± 0.08	0.87 ± 0.39	0.98 ± 0.10	Solution	0.45
					Plant	0.46
					Solution x plant	0.24
Carbon assimilation rate ($\mu mol\ C\ m^{-2}s^{-1}$)	9.0 ± 1.0	1.4 ± 1.3	2.5 ± 0.1	7.3 ± 1.0	Solution	0.61
					Plant	0.02
					Solution x plant	< 0.001
Max. quantum yield	0.81 ± 0.01	0.78 ±0.02	0.79 ± 0.01	0.78 ± 0.01	Solution	0.57
					Plant	0.10
					Solution x plant	0.33
ΦPSII	0.77 ± 0.01	0.74 ± 0.02	0.77 ± 0.01	0.76 ± 0.02	Solution	0.60
					Plant	0.24
					Solution x plant	0.66
Total chlorophyll (mg/g leaf)	0.40 ± 0.03	0.42 ± 0.13	0.35 ± 0.02	0.34 ± 0.03	Solution	0.50
					Plant	0.22
					Solution x plant	0.17
	Serpentine soil		Serpentine soil			
Leaf Ca:Mg	w/ dH_2O			w/ $CaNO_3$	Soil	0.18
	0.40 ± 0.05		0.43 ± 0.05	0.44 ± 0.04	0.28 ± 0.03 Plant	0.16
					Soil x plant	0.03

Table 2.3 Mean values with SE and summary of two-way analysis of variance of all growth and photosynthetic parameters. *P* values are provided for the effects due to the solution and plant type, and their interaction.

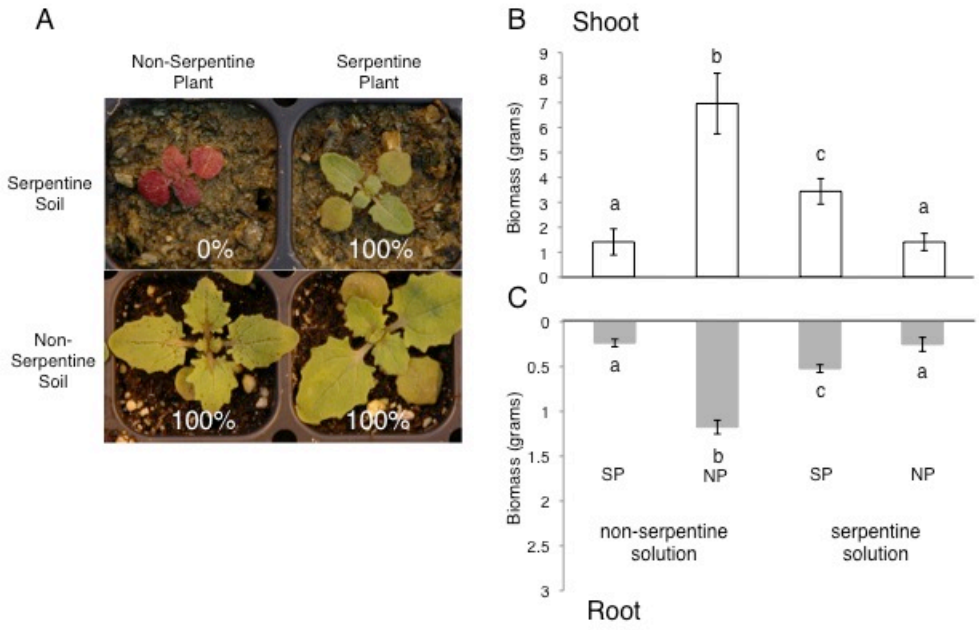

Figure 2.1 The effect of Ca:Mg ratio on survival and growth. (a) Percent survival of soil grown plants. Percentages indicate number of individuals surviving after five weeks. Photos taken 6 days after transplanting onto treatment soil (n=17). (b) Shoot biomass and (c) root biomass of hydroponically grown plants after 40 d (mean ± SE, n= 6). Varying letters among treatment groups indicates significant difference ($P < 0.05$) based on Tukey HSD *post-hoc* pairwise comparison.

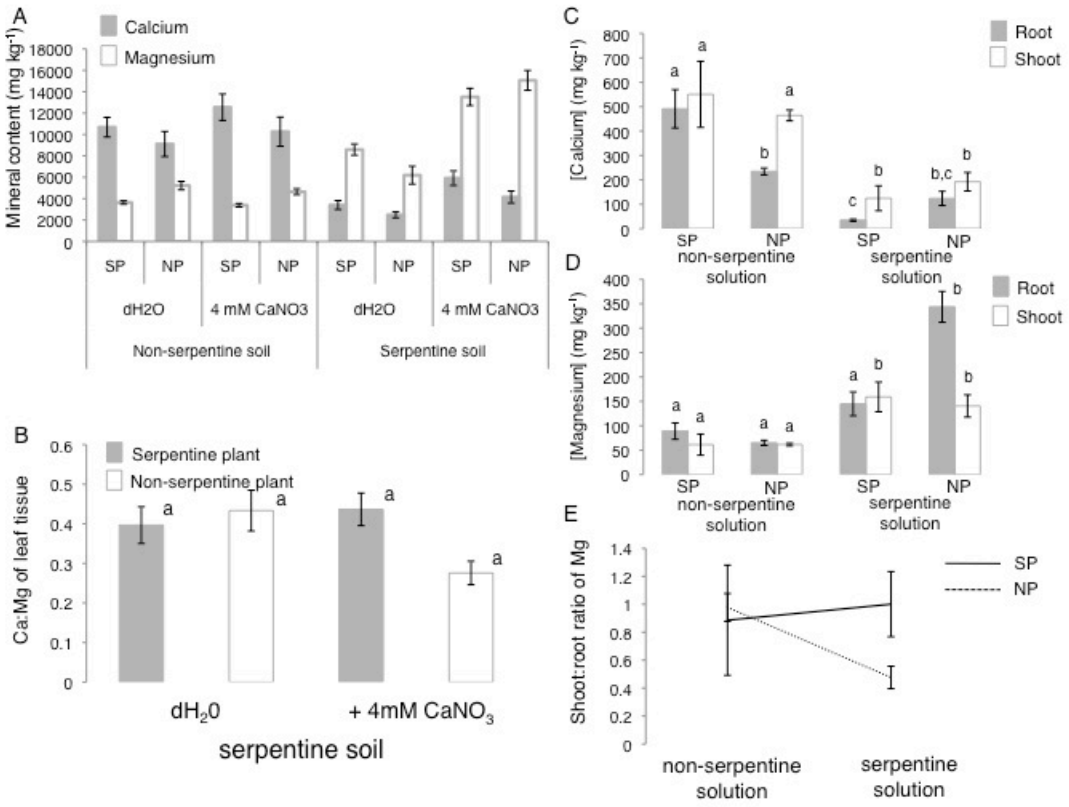

Figure 2.2 Mineral content of *M. guttatus* plants in serpentine and non-serpentine soil and hydroponic reciprocal transplant experiments. (A) Calcium (filled bars) and magnesium (empty bars) content of soil grown leaf tissue (mean ± SE, n = 10). (B) Ca:Mg ratio of leaf tissue of SP and NP plants on serpentine soil with dH_2O or with 4mM $CaNO_3$ (mean ± SE, n = 10). Differences between SP and NP grown in either solution is NS. (C) Calcium content and (D) magnesium content of hydroponically grown SP and NP roots (filled bars) and shoots (empty bars) (mean ± SE, n=6). (E) Shoot to root ratio of Mg content of SP (solid line) and NP (dotted line) (mean ± SE, n=6). Varying letters among treatment groups indicates significant difference ($P < 0.05$) based on Tukey HSD *post-hoc* pairwise comparison, and are tissue specific as in panels C and D.

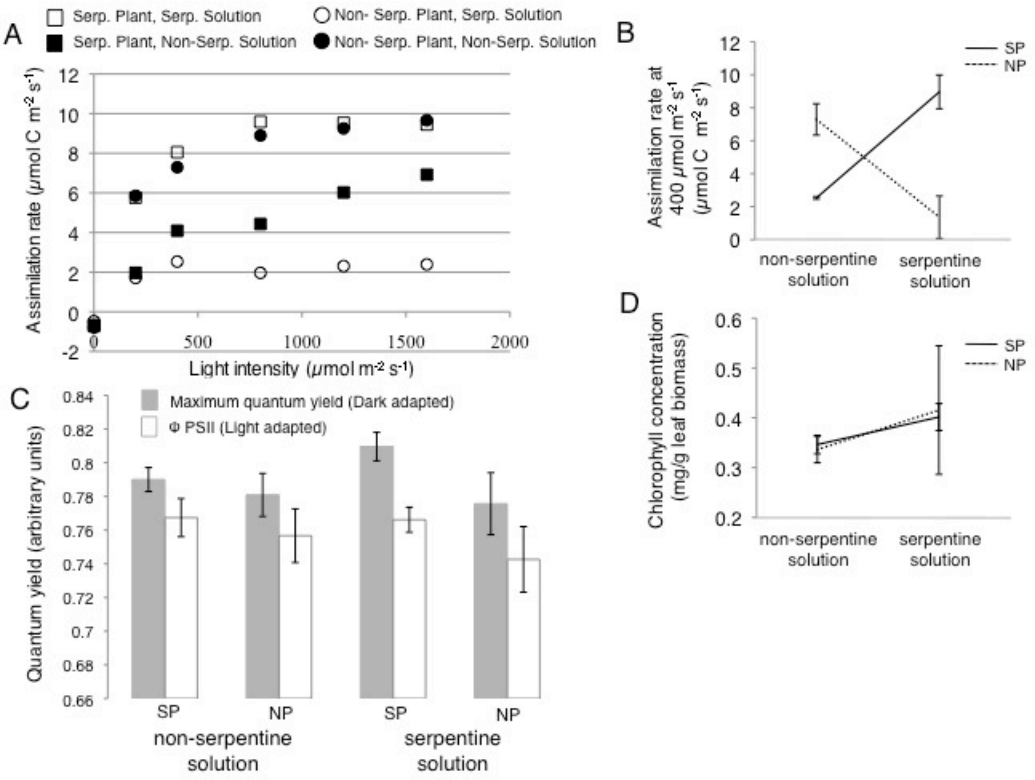

Figure 2.3 Effect of Ca:Mg provided to roots on photosynthetic parameters. (a) Carbon assimilation rates over a range of fluence rates. (n=6 for each data point). (b) Assimilation rates of SP (solid line) and NP (dotted line) at a light intensity of 400 µmol m^{-2} s^{-1} (mean ± SE, n=6). (c) Photosynthetic efficiency (Fv/Fm) of dark-adapted leaves (filled bars) and light adapted leaves (open bars) (n=6 for each bar, mean ± SE). Differences are NS. (d) Total chlorophyll a and b concentrations (mg/g of fresh leaf biomass) (mean ± SE, n = 4).

CHAPTER THREE Serpentine tolerance in *Mimulus guttatus:* gradual acclimation to low Ca:Mg

ABSTRACT

In serpentine soils, the low calcium-to-magnesium ratio is detrimental to the growth and persistence of most plant species. Little is known about the tolerance mechanisms of the plant species that are found growing in low Ca:Mg conditions. Ecotypes of *Mimulus guttatus* adapted to serpentine soils maintain high photosynthetic rates and biomass despite elevated internal concentrations of magnesium. The mechanism providing physiological tolerance to low Ca:Mg in serpentine plants was investigated here by measuring photosynthetic rates, photosynthetic efficiency, and leaf expansion rates on excised leaf tissue directly exposed to low Ca:Mg media. For tissue taken from plants grown for 1-2 weeks in low Ca:Mg in the rooting medium, low Ca:Mg in the disc assays inhibited both photosynthesis and leaf disc expansion. However, after three weeks of exposure to low Ca:Mg conditions, tissue excised from serpentine plants begins to show an increase in both physiological processes while non-serpentine plants remain low. Leaf disc experiments using a photosynthesis-inhibiting herbicide indicate that a source of photosynthates is essential for leaf cell expansion. These results show that low Ca:Mg inhibits photosynthetic rates both indirectly and directly in non-serpentine *M. guttatus.* Serpentine *M. guttatus* displays an inducible tolerance to low Ca:Mg through a mechanism that maintains photosynthetic production of photosynthates necessary for growth either through compartmentalization of Mg or indifference of this process to elevated concentrations of Mg.

3.1 INTRODUCTION

The low calcium:magnesium (Ca:Mg) ratio characteristic of serpentine soils is an abiotic factor that is inhibitory to the growth and persistence of many plant species (Madhok and Walker, 1969; Sambatti and Rice, 2007; Kazakou *et al.,* 2008). Serpentine soils are commonly defined as having low productivity, overall poor nutrient quality, and high levels of endemism (Kruckeberg, 2002; Brady *et al.*, 2005). As discussed by Fitter and Hay (2002) there are a number of ways that a plant can avoid damage by extreme abiotic factors, including escape, exclusion, amelioration and tolerance. The distribution of plant populations may be restricted to growing only where the Ca:Mg ratio is closer to one or higher,

thereby escaping the abiotic stress. In the case of *Mimulus guttatus,* populations were found to be differentially adapted to their native soils, such that when non-adapted plants were grown in serpentine – like conditions, their growth was dramatically reduced (Palm *et al.,* 2012). Plants may avoid damage by excluding the abiotic stress from tissues entirely, especially if it is an ion present in the soil. This was not shown to be the case in *M. guttatus,* where the possibility was tested that the survival of the serpentine population was due to exclusion of Mg (Palm *et al.,* 2012). Instead serpentine adapted populations may employ one of two mechanisms that allow for their distribution onto low Ca:Mg soils: 1) amelioration or adjustment of internal concentrations of Ca and Mg; or 2) tolerance to either elevated or suboptimal levels of these two macronutrients such that they are indifferent.

It is not clear whether survival in low Ca:Mg is achieved in the serpentine ecotype of *M. guttatus* via physiological adjustments or tolerance. In serpentine conditions, high photosynthetic rates and increased biomass are found among serpentine plants, but are dramatically reduced in non-serpentine plants (Palm *et al*., 2012). It remains to be determined which physiological process is primarily affected by the inhibitory Ca:Mg ratio. Is growth rate (the demand for assimilates) inhibited, leading to a reduction in the amount of photosynthetically competent leaf area, or is photosynthesis (the supply of assimilates) inhibited, leading to a final decrease in overall biomass. The answer may lie in the source-sink dynamics occurring in response to Ca:Mg.

Experiments were conducted on excised tissues to identify direct effects of Ca:Mg on either photosynthesis or leaf cell growth rate. It is hypothesized that high internal concentrations of Mg in non-serpentine plants will have a deleterious effect on photosynthetic and growth rates of excised leaf discs. We predict that a decrease in photosynthetic rate will be the cause of a decrease in growth rate, indicating that growth rate is reduced due to a decrease in available assimilates.

3.2 MATERIALS AND METHODS

Experimental hydroponic culture of plant material

Plants of *Mimulus guttatus* from serpentine and non-serpentine populations were used for all measurements described here. Seeds were originally collected in Lake County, CA from the Donald and

Sylvia McLaughlin Reserve and cultivated under greenhouse conditions as previously described (Palm *et al.,* 2012). Cuttings were made from parent plants grown in greenhouse potting soil. After five days in high humidity conditions, cuttings were placed in large tubs containing 0.25x Hoagland's solution with a high Ca:Mg to be grown hydroponically. After an additional five days and the establishment of roots, the plants were randomly transferred to tubs with either a normal, high Ca:Mg (Epstein and Bloom, 2005) or low Ca:Mg serpentine-like solution (Brooks, 1987). The nutrient composition of both the high and low Ca:Mg solutions was based on 0.25x Hoaglands with changes in the concentrations of the $CaNO_3$ and $MgNO_3$ to achieve the desired ratios without altering the osmotic balance (Hoagland and Arnon, 1950) (Table 1). Measurements described below were performed on excised leaf tissue sampled 0, 1, 2, and 3 weeks 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 300 μmol m^{-2} s^{-1}.

Oxygen electrode measurements of photosynthetic rates

Photosynthetic properties were measured on leaf discs excised from intact plants 0, 1, 2, and 3 weeks after exposure of plants to treatment solutions (high and low Ca:Mg). Discs were placed into high or low Ca:Mg incubation solutions modified as noted. Incubation solutions (discs) matched the treatment solutions (intact plants), e.g. discs sampled from plants growing in high Ca:Mg were incubated in high Ca:Mg. 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. Experimental solutions contained 2% $NaHCO_3$ with either 1 mM $CaCl_2$ and 0.25 mM $MgCl_2$ for the high Ca:Mg treatment or 0.02 mM $CaCl_2$ and 1.25 mM $MgCl_2$ for the low Ca:Mg treatment. Following a protocol described by González *et al*., 2001, leaf disc samples were collected from fully mature leaves from each replicate using a cork borer with a 12 mm diameter**,** and incubated in the dark in the treatment solution for 30 min prior to the start of the measurements. The leaf disc was placed in the electrode chamber with 3 mL of fresh incubation solution. The amount of oxygen (μmol O_2) consumed after 15 min in the dark (respiration rate) and produced (assimilation rate) after 6 min in the light at each fluence rate was recorded. Data were

evaluated for assimilation rate maxima, respiration rates and photosynthetic efficiency (quantum yield). Rates of O_2 production (as a proxy for assimilation or photosynthetic rates) collected over a range of fluence rates allowed for the construction of assimilation-light curves that describe net leaf photosynthesis using the following model (Pasian and Leith 1989)**:**

$$A = [(\alpha I + Amax - \sqrt{((\alpha I + Amax)^2 - 4\alpha I Amax\theta))}/ 2\theta] - R_d$$

with the following parameters: net photosynthesis (A), quantum efficiency (α), PAR (I), photosynthetic capacity (Amax), the curvature factor (θ) and dark respiration rate (R_d).

Leaf expansion assay

To assess the effect of Ca:Mg on the expansion of leaves, a leaf disc assay was performed based on protocols previously described (Blum *et al.*, 1992; Stahlberg and Van Volkenburgh 1999). Tissue was collected from young, expanding leaves at 1/3 full leaf area using a cork borer with a 6 mm diameter. Leaf discs were collected toward the later part of the light cycle, placed in a petri dish containing one of solutions listed (Table 2) and briefly described below, and incubated for 24 hours in an environmentally controlled growth chamber with a dark:light cycle and the same conditions as previously described for intact plants. Three leaf discs were collected from the same leaf. For leaves excised from intact plants growing in high Ca:Mg treatment, one of the three leaf discs was placed in each of the following incubation solutions: high Ca:Mg with 40 mM sorbitol, high Ca:Mg with 400 μM DCMU and 20 mM sucrose, and high Ca:Mg with 400 μM DCMU only. The same protocol was followed for plants growing in low Ca:Mg treatment solution, except that the excised leaf discs were incubated in low Ca:Mg with either 40 mM sorbitol, 400 μM DCMU and 20 mM sucrose, or 400 μM DCMU 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. 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. After 24 hours, the leaf discs were removed from the solutions, flattened and photographed; disc area was measured using ImageJ software

(Schneider *et al.,* 2012; http://rsb.info.nih.gov/ij/). The change in leaf disc area after 24 hours was compared to the initial leaf disc area at the start of the 24-hour incubation period.

Starch content

To evaluate the amount of sugars produced and stored that could be utilized for leaf growth, leaf discs were collected at the end of the 14-hour light period using a cork borer with an 18 mm diameter. Samples were weighed to obtain fresh weight (FW) and stored in a -70 °C freezer until analysis could be performed. Samples were processed as described in Sun *et al*., 2011. Briefly, leaf discs were incubated in 80% ethanol at 70 °C several times (4x20min) until colorless. To each sample, 0.2 mL of 0.5 M KOH was added, and incubated for 30 min at 95 °C. The pH was adjusted to 5.5 with 0.2 mL acetic acid, and 10 units of amyloglucosidase were added to a sample volume of 0.4 mL. Samples were diluted to 1 mL with dH_2O and incubated for 2 hours at 55 °C. Following the enzymatic release of sugars, the glucose content of each sample was then analyzed according to manufacturer instructions with a glucose hexokinase kit (Sigma-Aldrich Co, LLC). Absorbance was measured with a spectrophotometer at 340 nm and used to calculate starch content (mg glucose eq.) per gram of leaf tissue.

Statistical analysis

All data were subjected to a two-way ANOVA to test for differences among soil and solution treatments and plant type, as well as their interactions. *Post hoc* pairwise comparisons were made using Tukey's Honestly Significant Difference test. Statistical analyses were performed using R Statistical Software (http://www.r-project.org)

3.3 RESULTS

Photosynthetic rates increased with fluence rates similarly for all leaf discs sampled at week 0 (Fig. 1A) regardless of the treatment or incubation solution. At this time point, all plants had been growing hydroponically on high Ca:Mg treatment solution, and the initial incubation of discs on low Ca:Mg had no direct effect on photosynthetic rate, either for non-serpentine or serpentine plants. After one week of exposure to low Ca:Mg hydroponic treatment, discs excised from both non-serpentine and serpentine plants showed a decrease in photosynthetic rate when discs were likewise incubated on low Ca:Mg (Fig.

1B). This pattern continued for measurements conducted after two weeks of hydroponic treatment to low Ca:Mg (Fig. 1C). After three weeks, serpentine and non-serpentine plants continued to respond in a similar fashion in the high Ca:Mg solution with no significant difference ($p = 0.99$), but discs from non-serpentine plants in the low Ca:Mg incubation solution declined further in maximum photosynthetic rate. In contrast discs from serpentine plants exposed to low Ca:Mg solution for three weeks maintained the same rate as one and two weeks after exposure (Fig. 1D).

A maximum photosynthetic rate was obtained at 800 μmol m^{-2} s^{-1} for all treatment groups. Comparing the maximum photosynthetic rates at this fluence rate, serpentine and non-serpentine plants treated and incubated in high Ca:Mg solution maintain an average photosynthetic rate of about 9 μmol O_2 min-1g $(DW)^{-1}$ over the duration of the experimental period (Fig. 2A). Non-serpentine plants treated and incubated in the low Ca:Mg solution show a precipitous decline over the four weeks, from 9 μmol O_2 min-1g $(DW)^{-1}$ to 4 μmol, while serpentine plants in the same solution showed initial decline from 9 μmol to 6 μmol after two weeks, but maintain this photosynthetic rate for the remainder of the observation period.

The photosynthetic efficiency (quantum yield), evaluated from the initial slope of the light curves, indicates the amount of O_2 produced per unit of light. The pattern for quantum yield is the same as for maximum photosynthetic rate: serpentine and non-serpentine plants in high Ca:Mg solution show no significant difference over the experimental time period, while non-serpentine plants in low Ca:Mg continually decline, and serpentine plants initially decline but then stabilize at week 2 (Fig. 2B). The response to the Ca:Mg was significant ($p < 0.001$). By Week 3, there was a significant difference ($p = 0.03$) in the response to low Ca:Mg in that serpentine plants stabilized while non-serpentine plants declined further. Respiration rates, measured as O_2 consumption in the dark, show no significant difference between plants or solutions over time (Fig. 2C).

Leaf discs excised from either non-serpentine or serpentine plants that had been growing initially on high Ca:Mg treatment solution (week 0) showed equivalent, high growth responses (12 mm^2 increase in disc area over 24h incubation, Fig. 3A). As found for photosynthetic responses, the growth of excised tissue was not directly inhibited in low Ca:Mg (Fig. 3A, week 0). During the two weeks following treatment of intact plants to high or low Ca:Mg (week 1, 2), excised leaf discs of serpentine and non-serpentine

plants grew more in high Ca:Mg than in low Ca:Mg (Fig. 3A). After two weeks, serpentine discs in low Ca:Mg increased in area more than non-serpentine discs but still less than either disc type in high Ca:Mg, and by week three, serpentine discs have recovered more growth capacity. The same pattern was observed in leaf discs grown in the absence of photosynthesis (+DCMU) with the addition of sucrose (Fig. 3B). In the absence of both photosynthesis and a sugar source (+DCMU only), all treatment groups showed a significant decrease in disc area expansion but serpentine and non-serpentine discs in high Ca:Mg had a greater degree of expansion than either in low Ca:Mg. In addition, there was no observed 'recovery' in expansion in serpentine discs in low Ca:Mg (Fig. 3C), as found in the presence of either photosynthetic activity (Fig. 3A) or a sucrose source (Fig. 3B).

By week 3 there was significant increase in the starch content of mature leaves of both serpentine and non-serpentine in response to low Ca:Mg. The starch content of non-serpentine leaves was higher than serpentine leaves, but the difference was not significant ($p = 0.61$) (Fig. 3D).

3.4 DISCUSSION

The Ca:Mg ratio found in serpentine soil can have a dramatic effect on the growth and persistence of plant populations. Ecotypes of *Mimulus guttatus* collected from both serpentine and normal soil sites differentially adapt to their native soils through changes in shoot and root biomass, whole plant photosynthetic rates, and accumulation patterns of Ca and Mg (Palm *et al*., 2012). These results held for plants grown hydroponically with solely altered Ca:Mg ratios, effectively isolating the Ca:Mg as the soil factor responsible for determining the distribution of differentially adapted populations of *M. guttatus*. Here the physiological processes primarily responsible for decreased growth in non-serpentine populations were investigated, focusing on leaf expansion and photosynthetic rates. When incubated in a high Ca:Mg solution, excised tissue from serpentine and non-serpentine plants maintain similarly high rates of photosynthesis and leaf area expansion over an extended period of time in treatment conditions. When grown in a low Ca:Mg treatment, both serpentine and non-serpentine plants exhibit a decline in both photosynthetic rates and leaf expansion, but these processes recover somewhat in serpentine plants after several weeks 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 serpentine and non-serpentine plants, indicating that a photosynthate (sugar) supply enhances growth of leaf cells.

In this study, the effect of low Ca:Mg on photosynthetic 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. In the whole plant, mature "source" leaves photosynthesize, supplying themselves and young expanding "sink" leaves with the assimilates (sucrose and starch) needed for growth (Krapp *et al.*, 1991). Young leaves present a demand on mature leaves for photoassimilates. 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).

Both deficient and elevated concentrations of Mg are known to reduce photosynthetic rates as was shown here with non-serpentine *M. guttatus* plants exposed to low Ca:Mg growing conditions. In high Ca:Mg both serpentine and non-serpentine plants maintain similar responses to fluence rate (Fig. 1A-D) and a consistent maximum assimilation rate over four weeks at a fluence rate of 800 μmol m^{-2} s^{-1}. After one week of exposure to low Ca:Mg hydroponic treatment, discs from both serpentine and non-serpentine plants experience a decrease in response to fluence rate (Fig. 1A-D), maximum assimilation rate (Fig.2A) and quantum yield (Fig. 2B). Non-serpentine tissue continues to decrease in all parameters while serpentine tissue experiences a slight increase in maximum assimilation rate and in quantum yield after two weeks in low Ca:Mg. This increase in quantum yield suggests that at lower fluence rates (0-200 μmol m^{-2} s^{-1}) similar to that of their growing conditions (300 μmol m^{-2} s^{-1}), serpentine plants are more efficient than non-serpentine plants at fixing carbon. Efficiency increases with continued exposure of intact plants to low Ca:Mg (over three weeks). This increased efficiency, without a significant increase in maximum assimilation rate may explain the difference in final biomass between serpentine and non-serpentine plants previously found (Palm *et al.*, 2012). This acclimation would require a gradual change in internal concentrations of Mg, or an adjustment in chloroplast physiology that could accommodate the increase in Mg without the observed inhibition of photosynthetic rates and enzyme function (Berkowitz and Wu, 1993).

Little is know about the effects of elevated concentration of Mg, but the evidence suggests that high concentrations of Mg may also disrupt the rate of photosynthesis (Marshner, 2001; Shaul 2002). Concentrations of Mg in the cytosol, external to the chloroplasts, regulate the movement of potassium ions (K^+) into the stroma (Berkowitz and Wu, 1993). Free Mg binds to the chloroplast membrane, preventing K^+ influx into the stroma, leading to a decrease in proton efflux from the stroma (Gupta and Berkowitz 1989). As a result, the stroma remains acidic and photosynthesis is inhibited. Clearly both deficient and elevated concentrations of Mg are detrimental to chloroplast physiology, and maintaining Mg concentrations in the metabolic pool (the cytosol and the chloroplasts) within an optimal range (2 -10 mM) (Leigh and Wyn Jones, 1986) is important.

As macronutrients, both calcium and magnesium are required for plant growth (Marschner 2001). Calcium binds to pectates in the cell wall, strengthening the microfibillar matrix and enhancing cell wall formation. Magnesium is the central molecule of chlorophyll and is fundamental for the function of many enzymes needed for the fixation for carbon and the production of sugar needed for growth. Calcium deficiencies often lead to decreased inhibition of polygalacturonase, the enzyme that mediates the breakdown of pectates, resulting in cell wall degradation (Konno *et al.*, 1984). High Mg concentrations in the cytosol lead to reduction in photosynthetic rate. Alone or in combination, these factors lead to reduction in overall biomass. In this study the growth of serpentine and non-serpentine leaf discs was measured weekly after exposure of whole plants to high and low Ca:Mg solutions. Initially, at week 0 when all plants had been growing in high Ca:Mg, all leaf discs grow the same amount, regardless of the incubation medium – that is, disc growth is not directly inhibited by low Ca:Mg. With exposure of whole plants to low Ca:Mg (weeks 1, 2 and 3), the ability of discs to grow in low Ca:Mg declines. In contrast, serpentine plants exposed to low Ca:Mg provided leaf discs with the ability to expand on low Ca:Mg. This recovery was only evident when discs were able to photosynthesize or were provided sucrose. These results indicate that leaf cell expansion is not necessarily limited by low Ca:Mg (as serpentine discs can grow in low Ca:Mg) but that growth is limited by a photosynthetically supplied carbohydrate.

A reduction in sink activity is not the sole factor that limits the demand on source activity. Photosynthetic rates have been shown in a number of studies to be reduced prior to a reduction in sink

activity (Fischer and Bremer, 1993; Cakmak *et al.,* 1994; Araya *et al.,* 2006) Excised leaf discs from expanding *M. guttatus* leaves are active sinks that (when supplied with sucrose) are capable of increasing in leaf area in low Ca:Mg. Starch concentrations in source leaves of plants in low Ca:Mg are slightly lower in serpentine plants than in non-serpentine plants by week three, despite also having a higher maximum assimilation rate, indicating that assimilates are being exported not stored by the source tissues. This result coincides with other studies that found accumulation of starch and other assimilates occurring with a decrease in photosynthetic rates in Mg deficient plants through a negative feedback inhibition (Fischer and Bremer 1993; Cakmak *et al*., 1994; Hariadi and Shabala 2004; Herman *et al.,* 2004; Hermans and Verbruggen 2005). The processes identified as possible causes of this negative feedback response are a reduction in phloem uploading by source tissues (Cakmak *et al*., 1994), a reduction in the recycling of P_i from photosynthetic end-products (Pieters *et al*., 2001), carbohydrate (starch) accumulation (Paul and Foyer, 2001) and manipulations in the source/sink ratio (Kasai 2008).

The recovery of the maximum photosynthetic rate seen in the serpentine plants in low Ca:Mg after three weeks suggests that neither the photosynthetic machinery nor enzymes are being permanently inhibited by the elevated concentrations of Mg that have been previously found in these two ecotypes (Palm *et al*., 2012). Shoot concentrations of Mg were elevated in both serpentine and non-serpentine plants when grown in low Ca:Mg. These measurements were conducted on leaf tissue and represent total concentration within the leaf. For serpentine plants to recover a photosynthetic rate that is close to the rate found in a high Ca:Mg growth solution, despite the fact that there is a high total Mg concentration in the leaf, Mg may be compartmentalized somewhere in the leaf cells so as not to impact chloroplast physiology negatively. A likely candidate is the vacuole, a common location of storage when concentrations of supplied ions are high. The vacuole concentrations of Mg were not evaluated in this study. In *Arabidopsis thaliana*, a Mg^{2+}/H^{+} exchanger was identified and localized to the tonoplast (Shaul *et al*., 1999). The transporter regulates the efflux of Mg from the cytosol into the vacuole, and maintains cytosolic Mg concentrations within the optimal range (2-10 mM). In serpentine adapted *M. guttatus*, the activity level or number of an AtMHX-like exchanger may be higher than in non-serpentine *M. guttatus.* There may be plasticity in the expression level or number of exchangers in response to abiotic conditions, allowing for serpentine *M. guttatus* plants to exist over a wider range of Mg concentrations in the soil.

CONCLUSION

It is clear from the results of this study that the acclimation of serpentine-adapted *Mimulus guttatus* ensues over several weeks. Initially both serpentine and non-serpentine plants experienced decreased photosynthetic rates, photosynthetic efficiency and growth rates in response to low Ca:Mg treatment conditions. With continued exposure, serpentine plants did show a recovery in all of these processes that was not seen among non-serpentine plants.

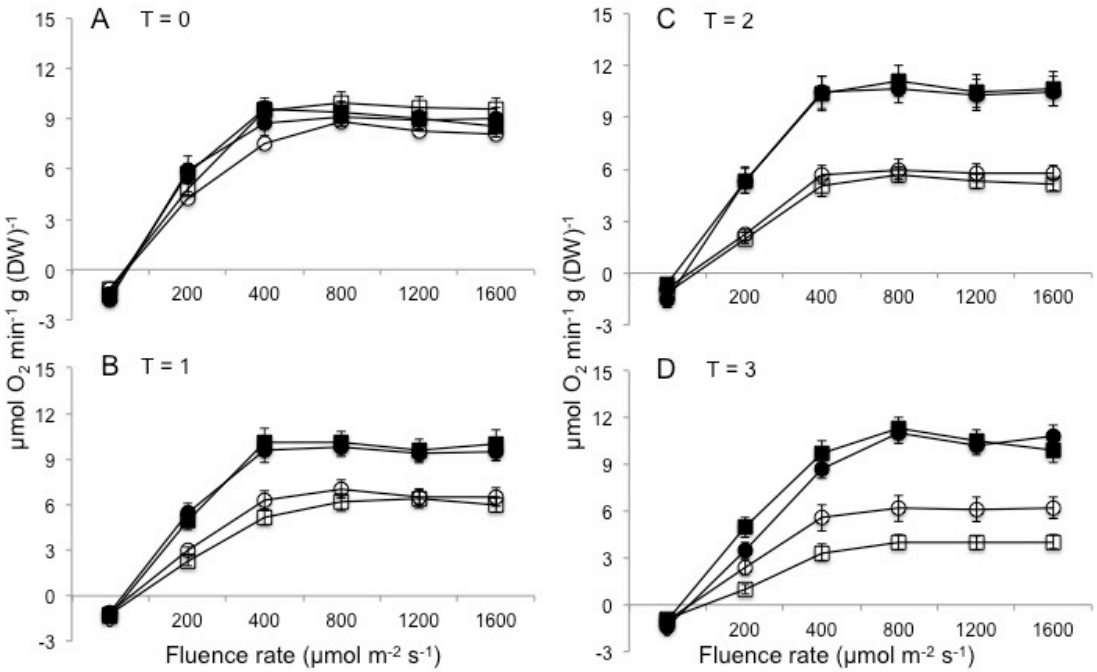

Figure 3.1 The effect of Ca:Mg on net photosynthesis in response to fluence rate. Non-serpentine plant in high Ca:Mg (filled square) (n=36); non-serpentine plant in low Ca:Mg (open square) (n=30); serpentine plant in high Ca:Mg (filled circle) (n=36); serpentine plant in low Ca:Mg (open circle) (n=30). (A) Week 0; (B) Week 1; (C) Week 2; (D) Week 3. Each data point is mean ± s.e.

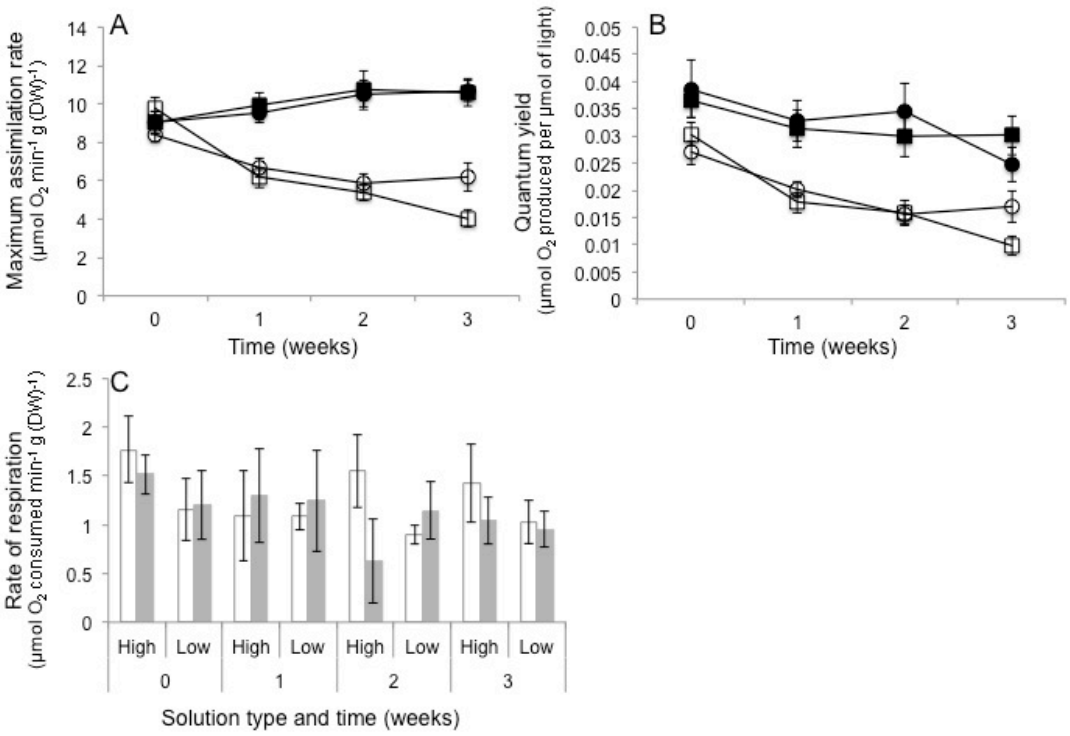

Figure 3.2 The effect of Ca:Mg on photosynthetic parameters: maximum assimilation, quantum yield and respiration rates. (A) Maximum assimilation rates at fluence rates above 800 µmol m^{-2} s^{-1}. (B) Quantum yield between fluence rates of 0-200 µmol m^{-2} s^{-1}. For (A) and (B): Non-serpentine plant in high Ca:Mg (filled square) (n=36); non-serpentine plant in low Ca:Mg (open square) (n=30); serpentine plant in high Ca:Mg (filled circle) (n=36); serpentine plant in low Ca:Mg (open circle) (n=30). (C) Dark respiration rates (fluence rate = 0 µmol m^{-2} s^{-1}). High and low represent solution Ca:Mg. Open bars represent serpentine and filled bars represent non-serpentine.

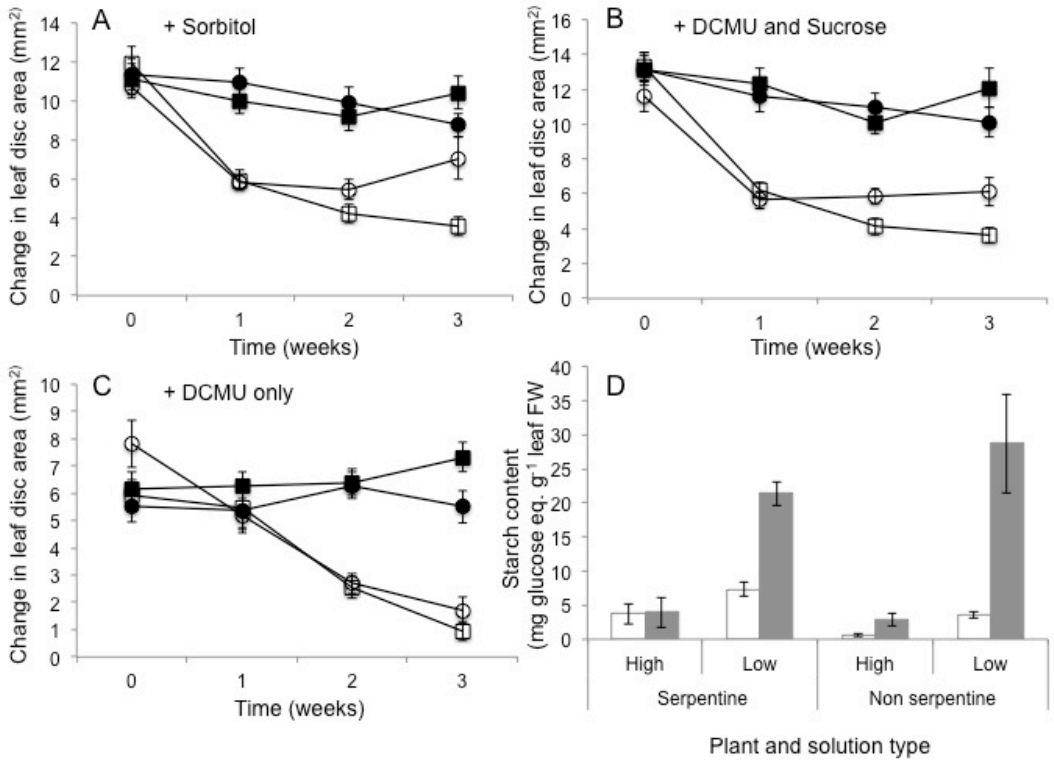

Figure 3.3 Growth rates of serpentine and non-serpentine leaf discs in response to Ca:Mg and with or without the inhibition of photosynthesis or the addition of sucrose. (A) High or low Ca:Mg with 20 mM sorbitol. (B) High or low Ca:Mg with inhibition of photosynthesis (+400 μM DCMU) and 20 mM sucrose. (C) High or low Ca:Mg with inhibition of photosynthesis (+400μM DCMU). For (A), (B) and (C): Non-serpentine plant in high Ca:Mg (filled square) (n=36); non-serpentine plant in low Ca:Mg (open square) (n=30); serpentine plant in high Ca:Mg (filled circle) (n=36); serpentine plant in low Ca:Mg (open circle) (n=30). (D) Starch content of mature leaf tissue. High and low represent solution Ca:Mg. Open bars represent one week of exposure and filled bars represent three weeks of exposure. Each data point is mean ± s.e. (n=4).

Nutrients	Non-serpentine	Serpentine
$CaNO_3$	1	0.025
$MgNO_3$	0	0.975
$MgSO_4$	0.25	0.25
NH_3PO_4	0.5	0.5
KNO_3	1.5	1.5
Fe-EDTA	0.25	0.25
Micronutrients		
NaCl	0.05	0.05
H_3BO_3	0.025	0.025
$MnCl_2$	0.002	0.002
ZnO	0.002	0.002
$CuCl_2$	0.001	0.001
Na_2MoO_4	0.001	0.001
Ca : Mg ratio	4	0.02

Table 3.1 Nutrient concentration (mmol L^{-1}) of non-serpentine and serpentine growth solutions, based on 0.25x Hoagland solution

Solution	Sorbitol	DCMU	DCMU only
High solution			
1 mM CaCl			
0.25 mM MgCl	30 mM sorbitol	400 μM DCMU	400 μM DCMU
10 mM KCl		20 mM sucrose	
Low solution			
0.02 mM CaCl			
1.25 mM MgCl	30 mM sorbitol	400 μM DCMU	400 μM DCMU
10 mM KCl		20 mM sucrose	

Table 3.2 Incubation solution for Leaf disc growth assay

CHAPTER FOUR Magnesium sequestration in the vacuole is not the key mechanism for tolerance in serpentine *Mimulus guttatus*

ABSTRACT

While the naturally occurring low Ca:Mg ratio of serpentine soil limits the growth and survival of most plant species, serpentine *Mimulus guttatus* possess a tolerance mechanism that does not solely rely on exclusion of magnesium (Mg). Here we investigated the potential roles of the roots in the regulation of Mg uptake and the vacuole in sequestering excess Mg in serpentine and non-serpentine *M. guttatus*. Uptake rates of Mg by serpentine roots are significantly lower than that of non-serpentine roots after three weeks exposure to low Ca:Mg. Leaf and vacuole concentrations of Mg are elevated in both ecotypes at the end of three weeks in response to low Ca:Mg, but the proportion of Mg contained in the vacuole of serpentine leaves is consistent (0.4) when measured 1 and 3 weeks after exposure. In non-serpentine leaves, the proportion is low (0.28) at week 1 but increases to 0.4 by week 3. By slowing the uptake rate of Mg into the roots and by sequestering a higher proportion of the cellular concentration of Mg into the vacuole at the onset of exposure to low Ca:Mg, serpentine *M. guttatus* may be avoiding the inhibitory effects of elevated concentrations of Mg into the cytosol on photosynthetic rate and growth rate. These results indicate that a combination of physiological mechanisms may be responsible for the tolerance to serpentine soil in *Mimulus guttatus*.

4.1 INTRODUCTION

Deficient or overabundant concentrations of nutrients in the soil are a common abiotic stress that plants are faced with as sessile organisms. An example of this can be seen in serpentine soil where the weathering of Mg-rich parent material (ultramafic serpentinite) results in a soil Ca:Mg ratio that is extremely low (Brooks 1987**)**. The effects that this edaphic factor has on the vegetation are apparent: low rates of productivity, xeromorphic growth and high rates of species endemism (Kruckeberg 2002; Brady *et al.,* 2005). Both calcium (Ca) and magnesium (Mg) are necessary nutrients for plant growth and function (Marschner 2001). Calcium is important for stabilizing cell walls, and acts as a signaling molecule, while magnesium is involved in photosynthesis as the central molecule of chlorophyll and regulates the activity of many enzymes. Elevated concentrations of magnesium are known to disrupt the pH of the

chloroplast stroma, thereby inhibiting photosynthesis and preventing the production of photosynthates required for cell metabolism and plant growth (Gupta and Berkowitz 1989).

Serpentine tolerant *Mimulus guttatus* Fischer ex DC plants do not exclude excess magnesium from entering the plant (Palm *et al*., 2012). To cope with adverse abiotic stress factors such as increased magnesium or a low Ca:Mg ratio, plants may make internal adjustments and ameliorate the increased concentration of magnesium (Fitter and Hay, 2002). There is a temporary decrease in the growth and photosynthetic rate upon exposure to a low Ca:Mg ratio, but over time, both increase and return to values close to those seen in high Ca:Mg conditions. Non-serpentine *M. guttatus* does not exhibit such a recovery response (Palm and Van Volkenburgh, submitted).

There are many examples of plants that are able to prevent the toxic effects of elevated ions by utilizing the vacuole as a location to sequester the additional ions. This has been shown as one component in the mechanism for salt tolerance in *Arabidopsis thaliana* and for many heavy-metal tolerant and hyperaccumulating serpentine plants. In *A. thaliana*, the gene *AtNHX* encodes a Na^+/H^+ antiporter that is localized to the tonoplast and that has been shown to mediate influx of Na^+ into the vacuole in exchange for protons pumped into the cytosol (Apse *et al.,* 1999). Similarily, studies in zinc (Zn) hyperaccumulators *Thlaspi caerulescens* (Lasat *et al.,*1996) and *Arabidopsis halleri* (Zhao *et al.,* 2000) have showed increased uptake of zinc and accumulation in the shoots, as compared to non-accumulators. In *A. halleri*, a tonoplast localized Zn^{2+}/H^+ antiporter was identified, encoded by the gene *MTP1* (Kawachi *et al.,* 2008). Plants expressing this gene have increased zinc concentration in the vacuole, and overexpression of *MTP1* in non-accumulator *A. thaliana* confers Zn tolerance (Gustin *et al.* 2009).

In the case of serpentine tolerant *M. guttatus,* the tolerance to low Ca:Mg (high magnesium) may be due to a mechanism by which the excess magnesium is sequestered into the vacuole. The vacuole is the main organelle responsible for magnesium homeostasis in the plant cell (Marschner 2001; Stelzer *et al.,* 1990). Patch clamp experiments with *AtMHX* overexpression in tobacco have shown that the transporter is activated at physiologically relevant concentrations (2 mM) (Shaul *et al.,* 1999). Beyond the optimal range of 2-10mM magnesium in the cytosol (Leigh and Wyn Jones 1986), free Mg^{2+} is known to bind to the negatively charged surface of the chloroplast, interrupting the influx of K^+ into the chloroplast in

exchange for H^+ efflux to cytosol. A stroma pH between 7.4 and 8.0 activates enzymes involved in carboxylation, fructose 1,6-bisphosphatase and Rubisco, but are inhibited when the stromal pH is close to that of the cytosol, or a pH of 7.0. (Gardemann *et al.*,1986; Portis 1992).

Here we investigated the possible role of the vacuole in sequestering excess magnesium in serpentine and non-serpentine *M. guttatus* in response to low Ca:Mg (high magnesium concentration) conditions. We hypothesized that there will be a difference in the vacuole magnesium concentration between serpentine and non-serpentine plants. We predict that in response to low Ca:Mg, hydroponically grown serpentine plants will sequester a higher amount of magnesium into the vacuole than non-serpentine plants. Mg^{2+} influx rates into the roots of both serpentine and non-serpentine plants should not be different as internal concentrations of magnesium have already been shown to be high in both plants when exposed to high magnesium solutions.

4.2 MATERIALS AND METHODS

Plant material and culture

Plants from both serpentine and non-serpentine *Mimulus guttatus* ecotypes were used for all measurements conducted. Cuttings were made from plants grown from seeds collected from a serpentine soil site within the McLaughlin Reserve in Lake County, California, and non-serpentine soil site outside of the reserve, as previously described (Palm *et al.,* 2012). These cuttings were placed in plastic cups containing a low concentration nutrient solution supplied by the UW Botany Greenhouse (similar to 0.25x strength Hoaglands solution) and kept in a high humidity tent for one week. After one week, all cuttings were transferred to large tubs containing 0.25x Hoaglands solution with a high Ca:Mg and grown in an environmentally controlled growth chamber (Conviron; Winnepeg, Manitoba, Canada) in the following conditions: 14h L, 10 h D; 25 C L, 18.5 C D, 300 μmol m^{-2} s^{-1} maximum light intensity and 40% humidity. After ten days, cuttings were randomly assigned to a high Ca:Mg or a low Ca:Mg solution, the composition based on a 0.25x strength Hoaglands solution. The components and concentrations of both the high and low Ca:Mg solutions are detailed in Table 1. Root and leaf tissue were sampled for the measurements detailed below one and three weeks after transfer of cuttings to treatment solutions.

Measurement of Ca^{2+} and Mg^{2+} flux rates

Steady-state Ca^{2+} and Mg^{2+} fluxes were measured on excised root segments using non-invasive ion selective vibrating microelectrodes (Newman 2001). Microelectrodes were prepared from 50 µL borosilicate capillary tubes and silanized with tributylchlorosilane. The backfill solutions for the Ca and Mg electrodes were 500 mM $CaCl_2$ and 500 mM $MgCl_2$, respectively. The Ca^{2+} and Mg^{2+} electrodes were front-filled with commercially available ion-selective resin (Ca^{2+}, Ionophore II, Cocktail A; Mg^{2+}, Ionophore I, Cocktail A; Sigma-Aldrich, St. Louis, MO). The reference electrode was prepared with a 2% agar, 3 M KCl solution and an Ag-plated-AgCl wire in a borosilicate capillary tube. Prepared electrodes were mounted in the micromanipulator and each calibrated in a set of standard solutions (0.025 – 1.0 mM $CaCl_2$ for Ca^{2+}; 0.05 – 1.5 mM $MgCl_2$ for Mg^{2+}).

Apical root segments (~15 mm in length) were excised from serpentine and non-serpentine plants growing hydroponically 1 and 3 weeks after exposure to high and low Ca:Mg solution. Root tissue was mounted in a plexiglass chamber, held in place between two pieces of 1 mm diameter rubber tubing and allowed to incubate undisturbed for 15 - 20 minutes in 7 mL of bath solution prior to the start of the measurement. Roots were measured in a bath solution of the same Ca:Mg as the treatment solutions in which the intact plants were grown: high Ca:Mg (1.0 mM $CaCl_2$, 0.25 mM $MgCl_2$) and low Ca:Mg (0.025 mM $CaCl_2$, 1.25 mM $MgCl_2$). Electrodes were positioned 10–20 µm from the root surface and 8mm from the root tip. During the measurements, electrodes moved repeatedly back and forth over a distance of 40µm from the root surface by a computer controlled step motor. Ion fluxes were measured for 10–12 minutes and calculated by MIFEFLUX software as described by Newman (2001). To compensate for interference of the Mg^{2+} electrode with Ca^{2+} ions in the bath solution during simultaneous measurements, Mg^{2+} fluxes were corrected based on the equations provided in Knowles and Shabala (2004) and calibration values of the Mg^{2+} electrode in the set of Ca^{2+} calibration solutions. Flux rates were averaged over a period of 6 minutes. The first 3 minutes of each measurement were not included in the average to avoid confounding effects due aberrant fluctuations in the electrical signal from opening and closing of the Faraday cage.

Vacuole sap extraction for ICP and osmolarity

Mature leaf tissue was harvested to evaluate vacuole sap osmolarity and concentration of calcium and magnesium 1 and 3 weeks after exposure to high and low Ca:Mg treatment solutions. Leaves were frozen on dry ice, allowed to thaw briefly and squeezed with finger pressure. 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 minutes on a gently rotating shaker table. After the incubation in SrCl, leaf strips were rinsed in 1 mM KCl for 5 min, 3 times. Leaf strips were quickly dried to remove excess surface moisture only, and the tissue samples frozen immediately in dry ice for a minimum of 15 minutes. After briefly thawing, leaf tissue was squeezed with finger pressure only and the liquid collected in a 1.5 mL epindorf tube. Vacuole sap osmolarity of a 20 µL sample was measured using a freezing point osmometer (Model 3300, Advanced Instruments Inc; Norwood, MA). The remainder of the vacuole sap was measured (0.4 – 1.0mL) and diluted up to 15 mL with dH_2O in a Falcon tube and stored in 40C until analysis of ion concentrations with ICP-MS could be performed.

Leaf tissue preparation for analysis of mineral content

The total leaf tissue concentration of calcium and magnesium was measured using ICP-MS, following the protocol previously described (Palm et al., 2012). Briefly, mature leaves were harvested at weeks 1 and 3, striped of midvein and petiole, and dried for three days at 70C. Up to 0.5g of dried leaf tissue was ground for each replicate and used for analysis. Dried leaf tissue was then digested with HNO_3 heated in a 50mL flask to 90C over three hours until all liquid had evaporated. 3 mL of 30% hydrogen peroxide was repeatedly added to each sample and evaporated until the remaining residue was white in color. The residue was resuspended in a 1:10 HNO_3:dH_2O solution and stored at 40C until ICP-MS

analysis was performed. All ICP analyses of vacuole sap and leaf tissue calcium and magnesium concentrations were conducted by Analytical Services (School of Environmental and Forestry Sciences, University of Washington, Seattle WA).

Results for all measurements are presented as means with standard error. For vacuole sap and leaf tissue concentration, n=6 for each treatment group. For ion flux measurements, samples sizes varied and are indicated on the graph for each treatment group. Student t-tests were performed to determine significant differences.

4.3 RESULTS

The Ca^{2+} and Mg^{2+} ion flux rates of excised roots, as well as vacuole sap and whole leaf calcium and magnesium concentrations were measured after one and three weeks exposure to high and low Ca:Mg growing conditions. After one week in a high Ca:Mg treatment solution, serpentine and non-serpentine roots have equivalent influx rates of Ca^{2+} (Fig. 1A) and Mg^{2+} (Fig. 1B). Ca^{2+} influx is higher (~8 nmol m^{-2} s^{-1}) than Mg influx (< 1 nmol m^{-2} s^{-1}). In low Ca:Mg, both serpentine and non-serpentine are effluxing Ca^{2+}, 9 and 12 nmol m^{-2} s^{-1}, respectively, negative bars indicating efflux from the root (Fig. 1A). However, fluxes of Mg differ by genotype in that serpentine roots in low Ca:Mg efflux Mg^{2+} at a rate of 3 nmol m^{-2} s^{-1}, while non-serpentine roots in the same growing condition take up Mg^{2+} at a significantly higher rate (13 nmol m^{-2} s^{-1}, $p = 0.003$) (Fig. 1B). After three weeks of continuous exposure to high Ca:Mg, both ecotypes show a similar pattern as in week 1 of Ca^{2+} and Mg^{2+} influx. There is a slight decrease in the Ca^{2+} influx in serpentine roots (6.2 nmol m^{-2} s^{-1}) (Fig. 1A) relative to the influx rate observed during the first week (9.6 nmol m^{-2} s^{-1}). Non-serpentine roots increased Ca^{2+} influx rate in week 3 (16.9 nmol m^{-2} s^{-1}) relative to week 1 (10.8 nmol m^{-2} s^{-1}). In the low Ca:Mg solution, after three weeks, both ecotypes continue to efflux Ca^{2+}, though the rate is significantly higher ($p = 0.04$) in non-serpentine roots. Serpentine roots reverse Mg^{2+} efflux (-2.6, week 1) (Fig. 1B) to influx (2.44, week 3), and non-serpentine roots reverse Mg^{2+} influx (14.7, week 1) to efflux (-1.9, week 3).

Over a wide range of Ca:Mg ratios (4.0 to 0.001), non-serpentine roots maintain higher rates of Ca^{2+} influx than serpentine roots down to Ca:Mg ratio of 0.01 during week 1 (Fig. 1C). Tolerant plants begin to efflux Ca^{2+} at Ca:Mg ratio of 0.01 and both ecotypes are effluxing Ca^{2+} at a Ca:Mg ratio of 0.001.

Week 1 vacuole sap concentrations of calcium and magnesium in both ecotypes mirrored the concentration pattern of calcium and magnesium in the treatment solutions (Fig. 2A). Elevated concentrations of calcium (high Ca:Mg) and high magnesium (low Ca:Mg) in the growing conditions resulted in high calcium and high magnesium vacuole concentrations, respectively. Among plants grown in low Ca:Mg, the magnesium concentration in the vacuole is significantly higher in serpentine and non-serpentine ecotypes after three weeks than after one week ($p = 0.001$ and 0.01 respectively). After 3 weeks, the vacuole concentration of magnesium is significantly higher ($p=0.05$) in non-serpentine leaves (175 ppm) than in serpentine leaves (104 ppm). Total leaf concentrations of calcium and magnesium did not differ between ecotypes after week one (Fig. 2B). Calcium concentrations were higher than magnesium concentrations when both ecotypes were grown in high Ca:Mg. There were equivalent magnesium concentrations in both ecotypes in low Ca:Mg, with significant decrease in calcium ($p << 0.001$) and a significant increase in Mg ($p << 0.001$), as compared to the values in high Ca:Mg. In high Ca:Mg, there is significantly more calcium in leaf vacuoles than magnesium ($p<<0.001$).

Concentrations of potassium in the vacuole (Fig. 2A) and in the whole leaf (Fig. 2B) are significantly higher than both calcium and magnesium. While potassium concentrations do not significantly differ between the ecotypes grown in high Ca:Mg, non-serpentine leaves do have a higher vacuole concentration in low Ca:Mg after one week, and again after three weeks (Fig. 2A). Whole leaf concentrations of potassium are higher after one week in the high Ca:Mg than low, in both ecotypes, suggesting that a higher proportion of potassium in the leaves is found in the vacuole in low Ca:Mg growing conditions (Fig. 2B).

To determine the proportion of the total leaf calcium, magnesium and potassium held in the vacuole, leaf tissue concentrations from previously published experiments (Week 3; Palm *et al.,* 2012) and both the vacuole sap and leaf tissue concentrations from experiments presented here (Week 1, Fig. 2) were used. Values of both total leaf and vacuole sap concentration for each ion were converted to

molarity. To calculate the proportion, it was assumed that the vacuole contains 90% of the cell's liquid volume, and the cell wall and the cytosol, 5% of the volume each. The vacuole proportion of calcium is higher in the first week of exposure to high Ca:Mg but decreased after three weeks for both ecotypes (Fig. 3A). The vacuole proportion is lower in plants exposed to low Ca:Mg for one week, but for serpentine leaves, the vacuole proportion of calcium increases from 0.17 to 0.37 by week 3. There is no change in non-serpentine leaves over time. No significant difference was found in the vacuole proportion of magnesium in serpentine leaves between high and low Ca:Mg treatment or over time (Fig. 3B). After one week in high Ca:Mg, both serpentine and non-serpentine leaves have equivalent proportion of magnesium in the vacuole (~0.4). After three weeks, this decreases to about 0.2 in the non-serpentine leaves. For non-serpentine plants grown in low Ca:Mg the vacuole proportion of magnesium starts off lower (~0.28) and increases to 0.42 by week three. The vacuole proportion of potassium in both ecotypes varies significantly in response to the treatment Ca:Mg, but not over time (Fig. 3C). The proportion is higher for plants grown in high Ca:Mg than for those grown in low Ca:Mg.

There was a similar response to the treatment solutions, by both populations over time, in the osmolarity of the vacuole sap (Fig. 3D). In high Ca:Mg, both ecotypes had an osmolarity of 150 mOsm at weeks one and three. The osmolarity was slightly higher (~200 mOsm) at week one for both ecotypes, and significantly higher (~300 mOsm for tolerant; 325 mOsm for non-tolerant) in low Ca:Mg at week three.

4.4 DISCUSSION

In serpentine soil, the most common abiotic stress to which plants are exposed to is a low Ca:Mg (<1). Most plants are adapted to growing in soil with a Ca:Mg ratio >1. Unless they possess mechanisms for tolerance, growth of individual plants and the distribution of populations are significantly reduced. In the case of *Mimulus guttatus*, populations are found growing in both serpentine and non-serpentine soils and are differentially adapted to their native soil type (Palm *et al.,* 2012). Non-adapted plants show reduced biomass, photosynthetic rates and elevated internal concentrations of magnesium when grown in low Ca:Mg conditions. In contrast to this, the growth and photosynthetic rates of serpentine populations of *M. guttatus* are not negatively impacted by the low Ca:Mg of the soil, despite also exhibiting elevated internal magnesium concentrations. Exclusion of magnesium is not the mechanism for tolerance in these

serpentine-adapted populations of *M. guttatus*. Here we investigated the possible role of the vacuole in the storage of magnesium to prevent disruptions of the ion balance in the three main compartments of the cell (vacuole, cytosol/organelles, and cell wall).

The first point of contact that a plant has with the soil is with its roots. It is also here where selectivity or exclusion of ions may occur, through regulation of ion uptake into the root cortex and into the vasculature for transport into the shoot (Shaul 2002; White and Broadley 2003). In low Ca:Mg treatment solutions, mimicking serpentine soil conditions, serpentine and non-serpentine *M. guttatus* plants show no difference in shoot magnesium concentrations. Therefore, we predicted that Mg^{2+} flux rates into the root would be higher in both serpentine and non-serpentine plants when grown in low Ca:Mg than in high Ca:Mg. Early after exposure to low Ca:Mg (one week) this was found to be the case in non-serpentine plants (Fig. 2B), relative to the influx rate of Mg^{2+} in high Ca:Mg. Influx was not observed after one week for serpentine plants which were instead found to have a low rate of Mg^{2+} efflux. With continued exposure to low Ca:Mg, serpentine plants after three weeks show influx for Mg^{2+}, but at a rate that is still lower than that observed early in non-serpentine plants. In contrast, non-serpentine plants exhibit a low rate of Mg^{2+} efflux after three weeks.

It was previously shown that non-serpentine plants have a higher proportion of Mg in the roots as compared to serpentine plants after 30 days of exposure to low Ca:Mg (Palm *et al.,* 2012). Taken together with the observed flux rates, it would appear that serpentine plants slowly take up the elevated Mg, transferring it more quickly to the shoot tissue than non-serpentine plants and effluxing the excess until further development of photosynthetic tissues of the shoot increases the demand for Mg. Non-serpentine plants have a significantly higher Mg uptake rate initially, some of which moves into the stele and into the shoot, while a majority of it remains in the root. With continued exposure of non-serpentine roots to low Ca:Mg, internal concentrations of Mg may increase until influx of Mg changes to efflux. If concentrations in the apoplastic space of the root cortex exceed that of the external solution, Mg that is not incorporated into the root tissue or translocated to the shoot may be exchanged with the external solution, motivated by the change in the direction of the concentration gradient. While little is known about the mechanism for magnesium uptake into the roots and translocation to the shoot, there is evidence that

a Mg^{2+}/H^{+} exchanger is involved in the loading and unloading of Mg^{2+} into the xylem sap, and the intracellular distribution of magnesium (Shaul 1999). *AtMHX*, the gene encoding a Mg^{2+}/H^{+} exchanger, is localized to the vacuole of xylem parenchyma of all plant organs. It was also shown that differences in expression level of *AtMHX* do not result in changes in the total internal concentration of magnesium, suggesting that the transporter does not play a role in determining uptake rate of Mg^{2+} or total concentration. A similar result was found in serpentine and non-serpentine *M. guttatus* plants grown in low Ca:Mg in that there was no difference in the total concentration of magnesium. A higher concentration of magnesium was found in non-serpentine roots, suggesting that there may be a difference in the rate of translocation of magnesium to the shoot (Palm *et al.*, 2012).

In serpentine soil, calcium is often limiting to plant growth. Calcium moves into the roots and the cortical cells either apoplastically or symplastically, but just as with Mg, Ca must enter the symplast in order to move past the endodermis and into the stele (White and Broadley 2003). In both serpentine and non-serpentine *M. guttatus,* internal calcium concentrations are lower than when either are grown in high Ca:Mg conditions. Here both ecotypes were found to efflux Ca^{2+} in low Ca:Mg. This observation may be the result of the calcium concentration within the apoplast increasing beyond that of the external conditions and the calcium leaving the root downs its concentration gradient before it has the opportunity to move into the symplast and into the vasculature for translocation to the shoot. In serpentine plants this rate of efflux decreases over time, and is unchanged in non-serpentine plants, suggesting that the efficiency of Ca^{2+} movements into the symplast may increase over time in serpentine plants in a way that does not occur in non-serpentine plants or as quickly.

The vacuole plays an important role in the storage of excess ions for many plants in response to elevated salt and heavy metal concentrations (Apse *et al.,* 1999; Kawachi *et al.,* 2008), and was considered here as a possible mechanism for tolerance to elevated magnesium in a serpentine *M. guttatus* ecotype. Elevated internal magnesium concentrations, coupled with the recovery in photosynthetic and growth rates suggested that buffering of cytosolic chloroplast functions by the vacuole was possible (Palm and Van Volkenburgh, *in prep*). Here we compared the leaf tissue and vacuole sap concentrations of magnesium and found that a higher proportion of leaf magnesium is found in the

vacuoles of serpentine plants than non-serpentine plants initially. Over time, this proportion is equivalent. This suggests that serpentine plants are actually sequestering more magnesium in the vacuole than non-serpentine when first exposed to low Ca:Mg conditions, preventing the long-term inhibition of photosynthetic and growth rate found in non-serpentine plants.

If not contained in the vacuole, magnesium may be found in the cytosol where it is associated with many enzymatically-activated processes, and in the chloroplasts, bound in the chlorophyll molecule and various enzymes involved in photosynthesis. Elevated cytosolic free Mg^{2+} concentrations have been shown to disrupt pH of the chloroplast stroma by altering the rate of proton movement across the chloroplast membrane. For serpentine *M. guttatus* plants to recover photosynthetic rate near the level of rates found in high Ca:Mg-grown plants, it is necessary for there to be a mechanism for ameliorating the effects of elevated magnesium not sequestered in the vacuole. Chlorophyll concentration increase in response to low Ca:Mg in both ecotypes, but not significantly. Chelation of magnesium in the cytosol may alter the binding capacity, thereby preventing the disruption of chloroplast stroma pH and the subsequent decrease in photosynthetic rate.

CONCLUSION

It appears that the vacuole may be a playing a role in conferring tolerance in serpentine adapted ecotypes of *M. guttatus*, in combination with slower uptake rates of Mg by the roots. By slowing the rate at which Mg enters the plant and its translocation into the shoot by the vasculature, serpentine *M. guttatus* may be able to make more gradual adjustments to the distribution of magnesium in the leaves. This may then lead to changes in the capacity of magnesium to interact with proteins, enzymes and organelle membranes in the cytosol may be the key to ameliorating the effects of elevated internal concentrations of magnesium. Discerning between free and chelated Mg^{2+} in the cytosol, chloroplast and possibly the vacuole, may explain the ability of serpentine adapted *M. guttatus* to persist in low Ca:Mg conditions when non-serpentine ecotypes are unable.

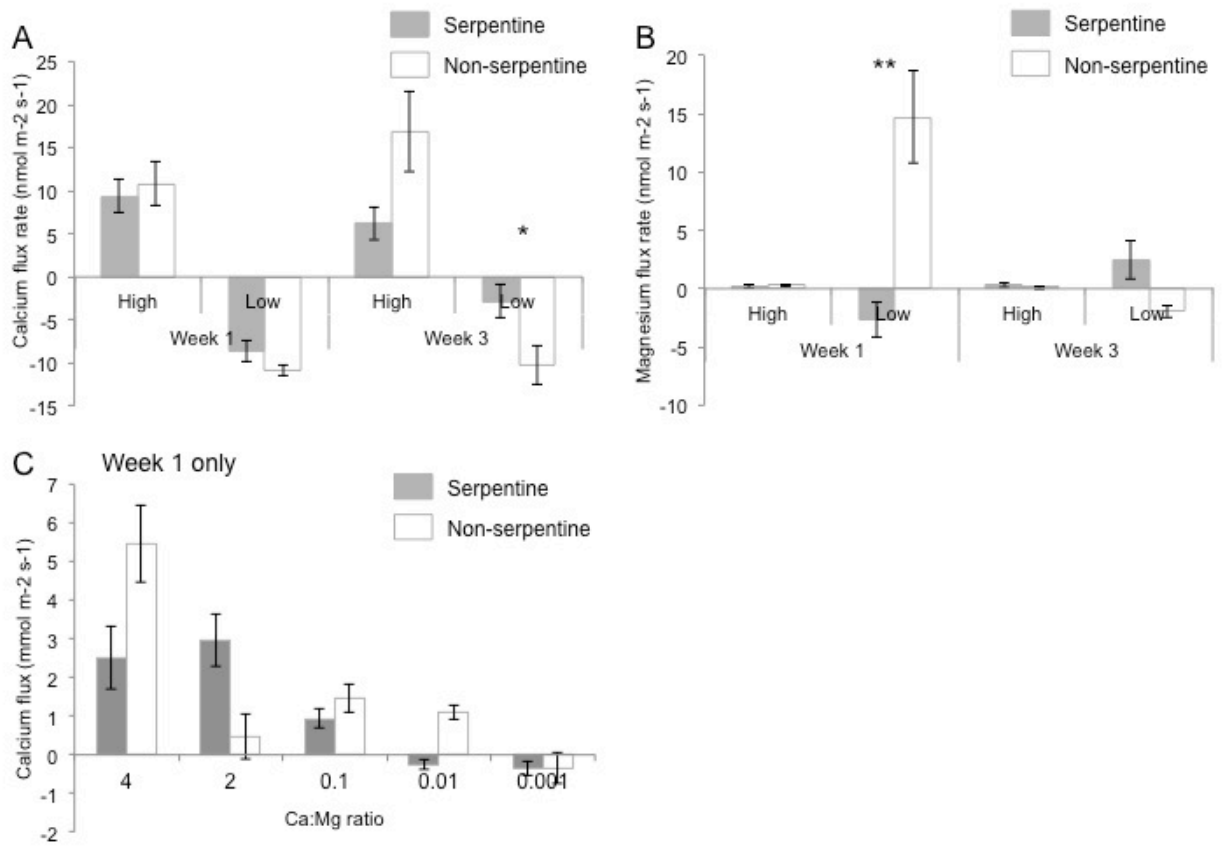

Figure 4.1 The effect of Ca:Mg ratio on Ca^{2+} and Mg^{2+} uptake rates on excised roots. Serpentine roots (filled bars) and non-serpentine roots (open bars); n=5 for panels (A) and (B). High and low refer to the solution Ca:Mg ratio. (A) Calcium flux rate; (B) magnesium flux rate. (C) Ca^{2+} flux only, week 1; serpentine roots (filled bars); non-serpentine roots (open bars); n=17. Each data point is mean ± s.e; * = p value less than 0.05; ** = p value less than 0.01.

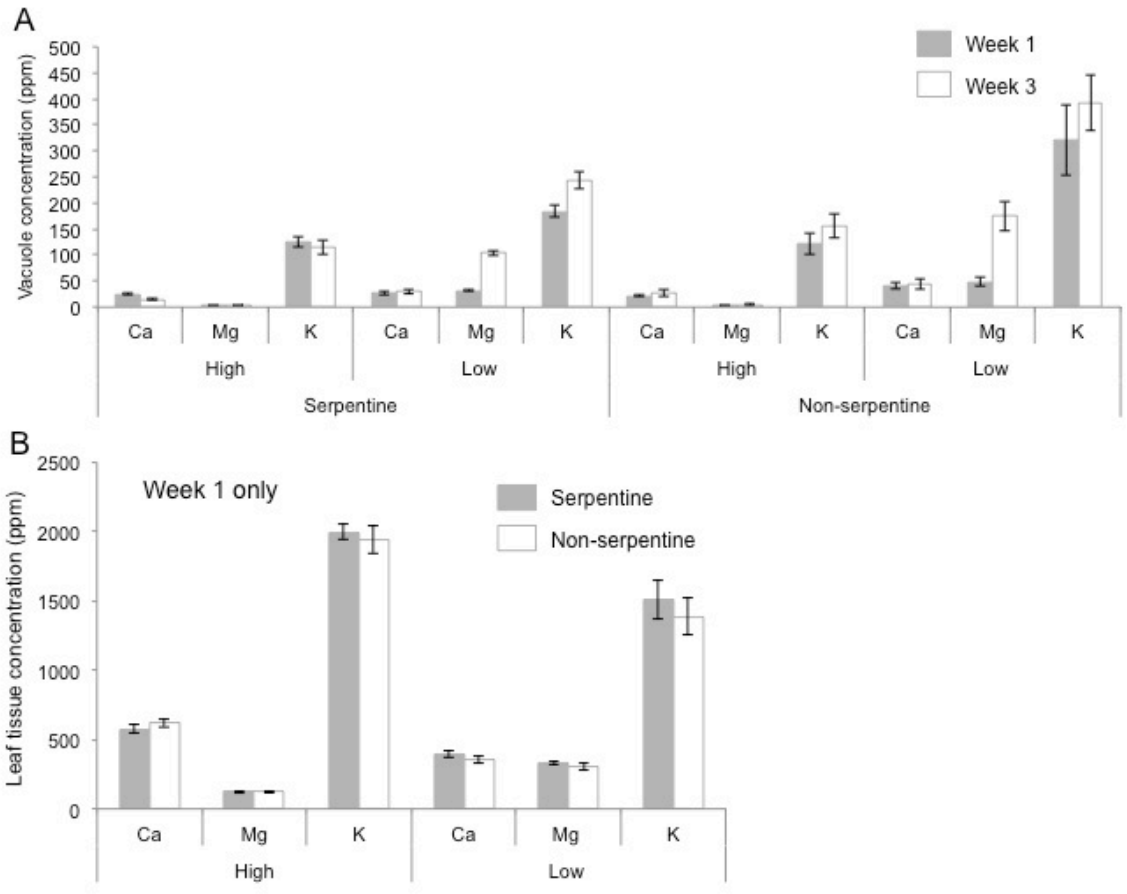

Figure 4.2 Vacuole sap and leaf tissue concentrations of calcium, magnesium and potassium, one and three weeks after growing in high or low Ca:Mg treatment solution. (A) Vacuole sap concentrations (ppm); Week 1 (filled bars); Week 3 (open bars); n=6 (B) Leaf tissue concentration (ppm); serpentine tolerant (filled bars); non-serpentine (open bars), n=6. Each data point is mean ± s.e.

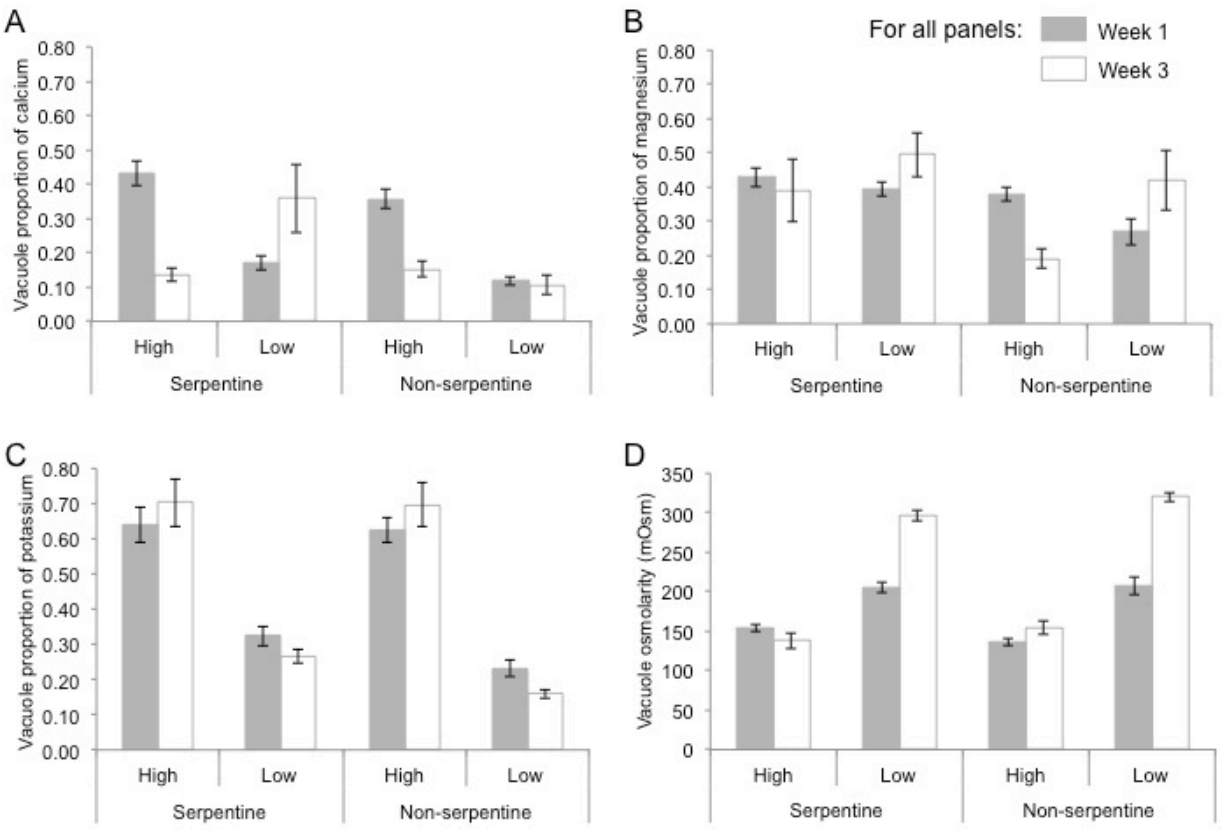

Figure 4.3 The effect of Ca:Mg ratio on vacuole proportion of total leaf calcium (A), magnesium (B), potassium (C) and on vacuole sap osmolarity (D). For all panels, filled bars represent Week 1 and open bars, Week 3. Each data point is mean ± s.e; n=6 for each treatment group.

Nutrients	Non-serpentine	Serpentine
$CaNO_3$	1	0.025
$MgNO_3$	0	0.975
$MgSO_4$	0.25	0.25
NH_3PO_4	0.5	0.5
KNO_3	1.5	1.5
Fe-EDTA	0.25	0.25
Micronutrients		
NaCl	0.05	0.05
H_3BO_3	0.025	0.025
$MnCl_2$	0.002	0.002
ZnO	0.002	0.002
$CuCl_2$	0.001	0.001
Na_2MoO_4	0.001	0.001
Ca : Mg ratio	4	0.02

Table 4.1 Nutrient concentration (mmol L^{-1}) of non-serpentine and serpentine growth solutions, based on 0.25× Hoagland solution.

CHAPTER FIVE Conclusions

Serpentine soil presents a unique set of chemical, physical and biotic conditions that challenge the plant communities colonizing it (Jenny 1980). The low productivity and sparse vegetation indicate that even the plants that are adapted to this landscape have developed tolerance mechanisms that are costly. The unifying characteristic of all serpentine soils is a low Ca:Mg ratio, driven by excessively high concentrations of magnesium. (Whittaker 1954; Proctor and Woodell 1976). There are four strategies described for plants to resist the effects of toxic ions: escape or avoidance by colonizing other non soils or limited, seasonal presence; exclusion of the toxic ion; amelioration of the toxic ion internally through sequestration or chelation; tolerance or indifference to the toxic ion (Fitter and Hay 2002).

The work presented here investigated the possibility of serpentine tolerant ecotypes in *Mimulus guttatus* and the tolerance mechanisms allowing for their distribution and persistence in serpentine soil on both an inter- and intracellular level.

5.1 Differential adaptation of *Mimulus guttatus* ecotypes to serpentine soil

Populations of *Mimulus guttatus* were observed growing on and off of known serpentine and non-serpentine soil sites in the McLaughlin Reserve in Lake County, California. Whether these populations were actually ecotypes and restricted to growing in their native soils was not clear until a reciprocal transplant was performed. Seedlings from the serpentine soil site, grown in both 'normal' (high Ca:Mg) and serpentine (low Ca:Mg) soil developed new, green leaves over the six weeks of observations. In contrast, the seedlings from non-serpentine soil developed new leaves only when grown in the high Ca:Mg soil. All non serpentine seedlings grown in low Ca:Mg were dead by the end of the six weeks. Hydroponic experiments comparing the growth and photosynthetic rates of these two populations again confirmed that they were differentially adapted to their native soils. Furthermore, the differences in the biomass in response to Ca:Mg ratio of the treatment solutions showed conclusively that the Ca:Mg ratio of the soil is the driving factor in the phenotypic response of the two ecotypes. The results in the first part of Chapter 2 are consistent with those found in studies comparing intraspecific variability and local adaptation in response to serpentine soil (Kruckeberg 1951; Wright *et al.,* 2006; Wright 2007). The

distinct differences in survival between the ecotypes in response to low Ca:Mg in the soil experiment and the biomass of plants grown hydroponically suggests a genetically-based tolerance mechanism.

5.2 Serpentine *Mimulus guttatus* does not exclude magnesium

The exclusion of magnesium has been a tolerance mechanism tested in a number of serpentine adapted species (Walker *et al.,*1955; Madhok 1965; Madhok and Walker 1969; Rajakaruna *et al.,* 2003; Sambatti and Rice 2007), and here in *Mimulus guttatus*. As presented in Chapter 1, hydroponically grown serpentine and non-serpentine ecotypes of *M. guttatus* had similar internal concentrations of calcium and magnesium, and closely reflected the Ca:Mg of the treatment solutions. In the low Ca:Mg, serpentine-like treatment solution, both serpentine and non-serpentine ecotypes had increased magnesium concentrations as compared to plants grown in high Ca:Mg. No difference was found in the total plant concentration of magnesium between the ecotypes, indicating that the serpentine ecotype is not excluding magnesium from entering the plant. What did differ between the ecotypes growing in low Ca:Mg was the root:shoot ratio of magnesium, which was higher in the non-serpentine than the serpentine plants. Differences in the distribution of magnesium between the root and the shoot may have an effect on root-and shoot-specific processes including the uptake of other solutes by the roots and photosynthesis and expansion of shoot tissue.

The lack of a magnesium exclusion mechanism in serpentine *M. guttatus* may have interesting implications for the photosynthetic rates and the biomass that were observed in hydroponically-grown plants. The root and shoot biomass of serpentine *M. guttatus,* as well as the photosynthetic rates, increased significantly when the plants were grown in a low Ca:Mg experimental treatment as compared to those grown in a high Ca:Mg treatment. The opposite response was found among non-serpentine plants grown in high and low Ca:Mg. Low photosynthetic rates in non serpentine plants in low Ca:Mg corresponded with a low final root and shoot biomass.

From these results two important points should be considered. The first is that though the serpentine plants had increased photosynthetic rates, close to the values seen in non-serpentine plants in their native, high Ca:Mg conditions, the final biomass of the plants did not increase to the same degree.

The process of photosynthesis produces photosynthates, the sugars and starches that are used by the plant for both growth and metabolism. The metabolic needs of the current tissues need to be met before new leaves, roots and shoots can begin to develop and expand. The difference in biomass between the ecotypes each growing in their native conditions and their similar photosynthetic rate suggests that the serpentine ecotype has a higher energy cost that comes with growing in low Ca:Mg that is not found in non-serpentine ecotypes growing in 'normal' high Ca:Mg conditions. This higher energy cost may be due to higher need for serpentine tolerant plants to sequester or buffer the additional concentrations of Mg. In general, intraspecies differences in leaf size and gas exchanges parameters (stomatal conductance, rates of enzyme activity) may be due to genetic variation in response to gradients in environmental stress (Geber and Dawson 1990; Geber and Dawson 1997). In studies comparing ecotypes of *Polygonum arenastrum* in response to seasonal differences in environmental conditions, Geber and Dawson found that small leaf area despite higher photosynthetic rates in some ecotypes were connected with earlier and increased reproductive output. While reproduction was not evaluated in *M. guttatus*, there is evidence the results presented here of a trade-off occurring to tolerate an environmental stress.

Second, both photosynthetic rates and biomass were impacted in non-serpentine plants in the low Ca:Mg treatment. In order for leaves to expand and root mass to increase, photosynthesis must occur in order to provide the sugars needed for growth. On the other hand, mature expanded leaf area is important for the capture of light that is the first step fueling carbon-fixation in photosynthesis. In the case of non-serpentine plants in low Ca:Mg, it is not clear whether the reduction in growth is due to an initial reduction in leaf area or a reduction in photosynthesis that leads to decreased growth. It is not possible from these data to determine which process is affected first. Calcium and magnesium are intergral to both processes. Calcium deficiency affects growth rates and leads to reductions in cell wall stability and the development of the meristems. Elevated concentrations of magnesium inhibit photosynthesis. This second point was addressed more specifically in Chapter 2, to determine the mechanism for inhibition of growth and photosynthesis in non-serpentine plants in low Ca:Mg conditions.

5.3 Serpentine *Mimulus guttatus* shows gradual recovery of photosynthesis and leaf cell expansion in response to low Ca:Mg

The low Ca:Mg ratio found in serpentine soil is a result of both low calcium availability and an excessively high concentration of magnesium. As macronutrients, both calcium and magnesium are integral and necessary for the normal growth and function of plants. Calcium deficiencies lift the inhibition that adequate calcium has on the activity of polygalacturonase, the enzymes responsible for the degradation of cell wall. The result often is the loss of cell wall stability and cessation of cell expansion and meristematic growth. Magnesium is essential to photosynthesis, as the central molecule of chlorophyll and the activation of enzymes involved directly in carbon fixation. Elevated magnesium concentrations in both the cytosol and the chloroplast can inhibit photosynthesis by altering changes in stromal pH and disrupting RuBP carboxylase activity. The results in Chapter 2 showed that both photosynthesis and the accumulation of biomass are affected in non-serpentine ecotypes of *Mimulus guttatus*. Determining which process is affected first is important for understanding where the limitation due to Ca:Mg is occurring in terms of the physiological response. To isolate the direct effects of the Ca:Mg treatments, excised leaf tissue was used for measurements of photosynthetic rates and growth rates presented in Chapter 3.

Leaf discs were sampled repeatedly from intact serpentine and non-serpentine plants grown in either low or high Ca:Mg over a period of three weeks. A slight recovery in the photosynthetic rate was observed in the serpentine ecotype in low Ca:Mg after three weeks that was not seen in non-serpentine leaf discs. Growth rates also recovered over time in serpentine discs exposed to low Ca:Mg, but the recovery was more apparent in leaf discs in which photosynthesis was not inhibited by DCMU. Leaf discs of both serpentine and non-serpentine plants that were exposed to high and low Ca:Mg and DCMU showed reduced growth rates and no recovery. These results indicate that low Ca:Mg inhibits photosynthesis and that the growth rate is fueled by the products of photosynthesis.

The use of excised tissue was key to isolating the effect of the Ca:Mg ratio of the treatment solutions on the photosynthetic rate and growth rates from the source-sink dynamics that occur in whole, intact plants. Mature leaves photosynthesize, producing photosynthates (sugars and starch) that are used

for mature leaf metabolism and the development and expansion of young, sink leaves. Magnesium plays a key role in photosynthetic metabolism and the partitioning of carbohydrates. Both magnesium deficiency and overabundance have been shown to inhibit photosynthesis. When carbon assimilates, produced by the carbon fixation of photosynthesis, accumulate in source tissues, they create a negative feedback inhibition of photosynthesis (Fischer and Bremer 1993; Hariadi and Shabala 2004). This accumulation of assimilates has been correlated with magnesium deficiency and may be caused by a reduction in phloem uploading by source tissues (Cakmak *et al*., 1994), a reduction in the recycling of Pi from photosynthetic end-products (Pieters *et al*., 2001), carbohydrate (starch) accumulation (Paul and Foyer, 2001) and manipulations in the source/sink ratio (Kasai 2008). Cytosolic concentrations of magnesium are maintained between 2-10 mM. Magnesium concentrations outside of this range can have significant impacts on enzymatic activity, leading to reductions in photosynthetic rate and growth. Illumination of chloroplasts causes an increase in stromal magnesium concentration and acidification of the thylakoid lumen. Changes in thylakoid stacking and thus the efficiency of energy transfer between the photosystems, as well as the activity rate of thylakoid ATPase complexes, are sensitive to pH and magnesium concentrations. The combined protonation and deprotonation of the thylakoid membranes is important for the activity of an ATPase coupling factor, CF1. This same coupling factor may be inhibited by Mg^{2+}, which competes for binding sites with protons.

High cytosolic magnesium concentrations have been shown to affect the pH of the stroma. In order to maintain the activity of carbon dioxide assimilating enzymes, it is necessary for the stroma pH to be at or very close to 8. Lowering the pH below 8 inhibits photosynthesis. Simultaneous proton efflux to the cytosol and the thylakoid lumen maintains this optimal stromal pH. The transport of protons to the cytosol is mediated by a proton pump, fueled by ATP generated by the light reactions, coupled with a K^+ channel that rectifies the change in membrane potential due to H^+ efflux with K^+ influx. The free external cytosolic Mg does not penetrate the chloroplast membrane. Instead the free Mg in the cytosol binds to the negatively charged surface of the chloroplast membrane, disrupting the voltage-dependent K^+ influx to the stroma and consequently the H^+ efflux to the cytosol. As a result the pH of the stroma decreases and the CO_2 assimilating enzymes of photosynthesis are inhibited.

For recovery of photosynthetic rate in serpentine plants and the continued photosynthesis inhibition in non-serpentine plants to occur in response to low Ca:Mg, the homeostasis of magnesium between the cytosol and the vacuole may play an important role. The amelioration of a toxic ion concentration is another potential strategy for coping with elevated ion concentrations in the environment. The vacuole is known to play a part in regulating the intracellular distribution of magnesium and buffering the capacity for magnesium. In Chapter 4 the rate of calcium and magnesium uptake as well as the role of the vacuole in serpentine tolerance by sequestering Mg was investigated.

5.4 Uptake rates of calcium and magnesium in serpentine and non-serpentine ecotypes suggests differences in ion permeability and translocation

Both calcium and magnesium are taken up by the roots and moved through the root cortex apoplastically and symplastically. The apoplastic route is driven by the transpiration stream, and as a result is a faster pathway for ions to reach the endodermis surrounding the vasculature. The symplastic route is through the plasmodesmata, the connections between the cells. It is selective initially, requiring ions to cross the plasma membrane and enter the symplasm through ion channels and transporters. Ions transported through the apoplasm must enter the symplasm in order to move past the casparian strip, the waxy barrier located in the endodermis. Here again the uptake of ions is selective.

The calcium and magnesium uptake rates were measured in serpentine and non-serpentine roots in response to Ca:Mg using ion selective electrodes. Because differences were seen in the photosynthetic and leaf cell expansion rates after one and three weeks of exposure to low or high Ca:Mg, root tissue was sampled at these two time points. Ca^{2+} influx was observed in both ecotypes in response to high Ca:Mg at both time points. In serpentine roots, influx decreased slightly, and increased in non-serpentine roots by week 3. This suggests that the demand for calcium is higher in non-serpentine plants and the Ca^{2+} requirement for serpentine plants is lower. The higher efflux of Ca^{2+} relative to Mg^{2+} in serpentine roots after one week in low Ca:Mg suggests that serpentine roots have a higher permeability to Ca^{2+} than Mg^{2+}. The concentration of calcium in the bath solution is very low, and it's likely that calcium leaves the roots of both serpentine and non-serpentine plants moving down its concentration gradient. The influx of Mg^{2+} into the roots of non-serpentine roots after one week in low Ca:Mg indicates that there

is little selectivity by non-serpentine roots occurring. It is also possible that the magnesium is being translocated to the shoot more quickly in non-serpentine plants than in serpentine plants, creating a higher demand for the uptake of Mg by the roots. After three weeks, non-serpentine roots begin to efflux magnesium, suggesting that the concentration is high in the root tissue and that exchange with the bath solution is now occurring.

The uptake rates of Mg^{2+} in serpentine roots are much lower than those observed in non-serpentine roots, both in terms of influx and efflux rates. The uptake and translocation of Mg^{2+} to the shoot may be more highly regulated in serpentine plants in response to high magnesium conditions. These results contradict the original conclusion described in Chapter 2 that there is no mechanism for selectivity by the roots of serpentine plants in response to elevated Mg (Palm *et al*., 2012). The low magnitude of Mg transport into the roots when initially exposed to the low Ca:Mg conditions, followed by a low rate of Mg flux into the root with continued exposure suggests that there may actually be some regulation of Mg uptake that is occurring by the roots themselves. Because ions move from the soil solution into the roots predominately by bulk flow (through the apoplastic pathway), the differences in flux rates observed here between serpentine and non-serpentine roots may be determined by the concentration gradient that exists between the root cortex and the external solution, as well as the loading of Mg from the cortex into the xylem by Mg-specific transporters. The non-serpentine ecotype has demonstrated a higher root to shoot ratio of Mg concentration in response to low Ca:Mg, which does suggest that there is some difference between the two ecotypes in terms of the regulation of Mg loading into the xylem tissue for translocation to the shoot.

There is little known currently about the transporters that regulate the uptake of Mg into the symplast of roots and its ability to move past the endodermis into the stele. Two candidate transporters have been studied with regard to their permeability and specificity for Mg, as well as their localization and function relative to relevant cell types. The Ca^{2+} channel *rca* is known to transport Ca across the plasma membrane of root cell and is permeable to other cations, including Mg^{2+} (Pineros and Tester 1995; Pineros and Tester 1997; White *et al.,* 2000). The specificity of this channel is based on its lower affinity and higher free energy bar for Mg^{2+}, but in the absence of Ca^{2+}, this channel becomes permeable to a

wide range of monovalent and divalent cations, including Mg^{2+} (White 2000). The *rca* channel has been characterized and studied wheat roots (Pineros and Tester 1995), but homologs of this channel may be involved in regulating the uptake of Mg^{2+} in of *M. guttatus* ecotypes in response to varying concentrations of Ca and Mg in the soil. Differences in the expression of the *rca* channel proteins as well as changes in affinity and permeability of the channel for Mg may exist between the serpentine and non-serpentine ecotypes. Whether or not this channel aides in the uptake of Mg from the soil solution has yet to be determined.

A second potential candidate for Mg^{2+} transport in roots is the Mg^{2+}/H^{+} exchanger that is localized to the tonoplast. In *Arabidopsis thaliana,* the gene that encodes for this exchanger (*AtMHX*) is expressed in all plant tissues. After transformation into tobacco, AtMHX has been shown in patch clamp studies to exchange protons with divalent cations including Mg^{2+}, Zn^{2+} and Fe^{2+}, at physiologically relevant concentrations (Shaul *et al.,* 1999), and elevated expression of *AhMHX* was observed in *Arabidopsis halleri*, a zinc hyperaccumulator (Elbaz *et al.,* 2006). While found throughout the plant tissues, the largest proportion of AtMHX mRNA is localized to the xylem parenchyma, the living cells adjacent to the xylem. Together with observations of the general function of xylem parenchyma, it is thought the Mg^{2+}/H^{+} transporter aids in buffering magnesium and zinc concentrations by xylem loading and unloading, and thus the Mg concentration of the xylem sap. Differences in the activity level of a Mg^{2+}/H^{+} antiporter, like the one identified in *Arabidopsis thaliana*, may explain the observed rates of Mg^{2+} influx and efflux in serpentine and non-serpentine ecotypes. Here, concentrations of Mg in the vacuoles specifically in the roots and in the vascular tissues were not evaluated. Due to the differences observed in the photosynthetic rates and growth rates of serpentine and non-serpentine ecotypes, the focus was on the photosynthetically relevant tissues, the leaves. The flux rate measurements performed suggest that there is a more complex mechanism regulating the distribution throughout the plant, and that the loading and unloading of Mg into the xylem may be involved. A comparison of the expression levels of AtMHX homologs should be performed in both serpentine and non-serpentine *M. guttatus* ecotypes to determine if this exchanger plays a role in conferring tolerance to low Ca:Mg in serpentine plants.

5.5 Serpentine *Mimulus guttatus* have consistent vacuole proportions of Mg in response to Ca:Mg

To cope with the elevated concentrations of magnesium found in both serpentine and non-serpentine ecotypes in response to low Ca:Mg, it was hypothesized that the recovery in photosynthesis and leaf cell expansion rates after three weeks was due to sequestration of Mg into the vacuole. The vacuole is known to regulate magnesium homeostasis, and high cytosolic magnesium concentrations can inhibit photosynthesis. The vacuole sap of serpentine and non-serpentine plants exposed to high and low Ca:Mg treatments for one and three weeks was extracted and the concentrations of calcium, magnesium and potassium measured with ICP-MS. Leaf tissue concentrations of the three ions were also evaluated with ICP-MS. As presented in Chapter 4, there was no significant difference in the calcium, magnesium and potassium concentrations in plants grown in high Ca:Mg at both time points. The concentration of calcium was higher than that of magnesium, and potassium concentrations were higher than both calcium and magnesium. After one week in low Ca:Mg, the concentrations of magnesium in the vacuole sap were already elevated, relative to the concentrations found in high Ca:Mg samples. By week three, the concentration of Mg in the vacuole of non-serpentine leaves was higher than that found in serpentine leaves. A similar pattern was seen in the whole leaf tissue measurements of calcium, magnesium and potassium.

While the total leaf concentrations of Mg were elevated for both ecotypes after one week of exposure to low Ca:Mg and again after three weeks, what did vary between the ecotypes was the proportion of the Mg that was in the vacuole. The proportion of total Mg in the vacuole was consistent in serpentine leaves over time, but in the leaves of non-serpentine plants exposed to low Ca:Mg for one week, the proportion was lower, but increased to the same proportion by week 3. The magnesium that is not sequestered in the vacuole of any plant cell may be found bound to the cell wall and in the metabolic pool (the cytosol and the chloroplasts). The timing of this change in the distribution of Mg in cells of non-serpentine plants may be related to the differences observed in the recovery of photosynthetic rates and growth rates presented here in Chapter 3 between serpentine and non-serpentine ecotypes.

While total concentrations of Mg can be measured using ion couple plasma mass spectrometry, it is not possible to determine the difference between concentrations of free Mg^{2+} and Mg that is bound to anionic compounds (Stelzer *et al.,* 1990) or carboxylates (Tibbetts and Smith 1993) using the ICP-MS method. This distinction has been previously addressed in a serpentine-adapted Crassulacean species, *Sedum anglicum,* that grows naturally on Mg-rich serpentine soil (Tibbetts and Smith 1993). Using substrate-specific enzyme assays, the concentrations of free Mg was determined separately from that bound to malate, citrate and isocitrate in plants grown over a range of Ca:Mg ratios. Several important conclusions were reached: there is a preference for Ca uptake over Mg in this species, there is an order of preference in terms of which carboxylate was chelated, and as the amount of Mg in the treatment increased, the amounts of chelated Mg and free Mg^{2+} increased by a constant proportion. In *Sedum anglicum*, chelation of free Mg^{2+} is a possible mechanism for tolerance to serpentine soil. To prevent the inhibition of photosynthesis that is the result of elevated cytosolic concentrations of magnesium, chelation of free magnesium in the cytosol may alter the binding capacity of Mg to the negatively-charged chloroplast membrane and prevent the disruption of RuBP carboxylase activity in serpentine *M. guttatus.* A higher concentration of free Mg^{2+} in the cytosol of non-serpentine *M. guttatus* may be the reason for the reduction in photosynthetic rate observed over time in response to low Ca:Mg treatments.

The difference observed between the flux rates as well as the variation in cellular distribution of Mg in serpentine and non-serpentine ecotypes in response to low Ca:Mg indicates that there may be more than one mechanism involved in conferring tolerance to low Ca:Mg in serpentine *Mimulus guttatus*. Flux rate measurements show that there may actually be some form of Mg exclusion occurring in the roots. While this exclusion is not complete, as demonstrated by elevated concentrations of Mg in both the roots and shoots of serpentine *M. guttatus*, the vacuole may be playing a valuable role in preventing the inhibitory effects of Mg on photosynthesis and growth. What remains to be investigated is the regulation of Mg movement from the growing media into the roots and into the leaves of serpentine adapted plants. The short-term decline in photosynthetic rate of serpentine *M. guttatus* in response to low Ca:Mg indicates that there may be some inhibitory effect due to Mg that is not localized to the vacuole. The exact that are involved are unknown, but they may be the key to identifying the direct mechanisms that are responsible for tolerance to low Ca:Mg in serpentine *Mimulus guttatus.*

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