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Biofortification and subcellular localization of minerals in faba bean as influenced by Mg foliar application

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Summary

Foliar application of Mg is a measure for the correction of Mg deficiency in crop plants. Foliar applied nutrients need to access the symplastic side where majority of physiological processes take place. To achieve an adequate uptake of the Mg ions through the leaf surface, concentrations of 100-200 mM MgSO₄ are usually supplied. This can cause antagonistic perturbations on the subcellular distribution of Ca and K cations. To test for such unintended side effects, we used the infiltration-centrifugation method to extract ions from the apoplastic and symplastic side of *Vicia faba* leaves and quantified concentrations of Mg, Ca and K in dependency to the dose of the foliar fertilized Mg. Results show that a large fraction of Mg accesses the symplast whereas the apoplastic fraction shows a concomitant increase. Symplastic and apoplastic K and Ca relations were only affected under conditions of high exogenous leaf supply of Mg (200 mM) but did not change upon moderate Mg supply (50; 100 mM). Overall, results reveal the suitability of leaf fertilization to biofortify plant-based products with magnesium.

Introduction

In plants, magnesium (Mg) activates a myriad of enzymes and is important for growth and development (EPSTEIN and BLOOM, 2004). Various environmental conditions cause magnesium deficiency and may lead to the appearance of characteristic deficiency symptoms in the form of an interveinal chlorosis that appear at older leaves of dicotyledons plant species (VERBRUGGEN and HERMANS, 2013; HERMANS et al., 2013; NEUHAUS et al., 2014; SENBAYRAM et al., 2016). In humans, Mg has a fundamental role as co-factor for more than 300 catalytic reactions (WACKER and PARISI, 1968) and is especially required for energy generation and nucleic acid synthesis (Institute of Medicine, 1997; FAWCETT et al., 1999). Besides being implicated in human protein synthesis, Mg has also a dedicated role in the activation of the adenylate cyclase system and this system regulates cellular activities and is modulated by hormones and neurotransmitters (MAGUIRE, 1984). For human nutrition, plant-based products represent a main source of dietary magnesium, ultimately highlighting the need for plant nutritional measures to ensure adequate Mg supply to growing plants in order to enrich plant-based foods with Mg (WHITE and BROADLEY, 2009; GERENDÁS and FÜHRS, 2013). Because magnesium is the central atom in chlorophyll, green organs of the plants are rich in magnesium (Institute of Medicine, 1997). The foliar application of Mg represents one strategy to supply Mg-deficient crops rapidly with Mg because, when compared to soil fertilization, the divalent cation is directly added in the vicinity of the Mg-deficient tissues (JEZEK et al., 2015). Foliar application of Mg ions is in particular valuable at peak demand times and under conditions of low soil availability (FERNANDEZ and EICHERT, 2009). To achieve sufficient Mg uptake over the leaves, MgSO₄ * 7 H₂O at concentrations of 40 mM (REAY et al., 1998), 80 mM (DORDAS, 2009), 200 mM (TEKLIĆ et al., 2009; VRATARIC et al., 2006; KRISTEK

et al., 2000), or even higher (WEBSTER and DROWE, 1982) have been tested. However, those studies did not evaluate the apoplastic fractioning of the foliar applied Mg. The amount of Mg that is trapped in the apoplastic compartment is relevant for the fertilizing effect because Mg is quantitatively involved in metabolic processes that run in the cell symplast. Thus, the physiological availability of the foliar applied Mg is a crucial issue in leaf fertilization because it is possible that the applied Mg is immobilized in the apoplastic leaf compartment and, by this means, is not immediately available for metabolic processes. As the apoplast comprises many fixed anions and is therefore supposed to serve as a storage site for cations (GRIGNON and SENTENAC, 1991), the Mg²⁺ supplied to the leaves might be partly bound apoplastically, rather than taken up into the symplast. It was one aim of this study to elucidate this topic by determining the fraction of the applied Mg that accumulates in the apoplast. The second part of this study dealt with the problem associated with nutrient-nutrient interactions. The addition of Mg may have side effects on the distribution of other mineral nutrients (RIOS et al., 2012). As demonstrated in experiments with soil or nutrient solution, the supply of the one cation can lead to a reduced uptake of others cations. This antagonistic effect was frequently reported for potassium (K) which influences the uptake of calcium (Ca) and Mg (e.g., MILLER, 1999; SPEAR et al., 1987; OLOGUNDE and SORENSEN, 1982; TROYANOS et al., 2000). In the other way around, a high supply of Mg can lead to reduced tissue concentrations of K and Ca (GRANT et al., 1988; OLOGUNDE and SORENSEN, 1982; TROYANOS et al., 2000). Based on these antagonistic effects, we hypothesize that foliar application of Mg will affect leaf cation composition. For this reason it was the second aim of this study to evaluate whether foliar applied Mg effects the compartmental distribution of K and Ca. This study was based on *Vicia faba* L. because the field bean has advantageous leaf anatomical features that facilitate to investigate leaf apoplastic ion relations.

Material and methods

Plant material and cultivation

Vicia faba L. plants of the cultivar Scirocco (NPZ, Hohenlieth, Germany) were grown hydroponically in climate chambers (14/10 h day/night; 20 °C/15 °C; 250 μmol m⁻² s⁻¹ light intensity; and 50% relative humidity). Seedlings were placed in 0.5 mM aerated CaSO₄ solution for one day at 25 °C and were subsequently transplanted into sterile quartz sand. After 10 days, the seedlings were transferred to 4 l plastic pots (4 plants per pot) containing one quarter-strength nutrient solution, which was raised to full concentration in a stepwise manner. Nutrient concentrations as given by GEILFUS et al. (2015a). Plants were subjected to six different groups (treatments) with four biological replications each. First group was supplied with 0.5 mM MgSO₄ * 7 H₂O at the roots. Plants from this group represent the positive control since they were sufficiently supplied with Mg over the experiment. The remaining five treatments were grown in a Mg-low nutrient solution containing only 0.05 mM MgSO₄, from which three groups were subjected to MgSO₄ leaf applications receiving 50 mM (2nd group), 100 mM (3rd group), or

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200 mM (4th group) MgSO₄, respectively. A wetting agent (0.1% (v/v) ARMA; Agrolanta, Langenpreising-Zusdorf, Germany) was added to the application solutions to ensure contact with the leaf. The fifth group was not foliar fertilized with MgSO₄ but was sprayed with the same amount of water plus wetting agent. This group guarded the experiment against effects contributable to the water spray and the ARMA. The sixth group was neither foliar applied with Mg nor with the wetting agent because these plants served as negative control representing plants with lack in Mg. Foliar application was conducted three times, starting four weeks after plants were set to the full nutrient concentration. The application was conducted using a handheld sprayer. In order to assure the application of equal amounts of 3.5 g of the fertilizer solution, the pots were placed on a balance. Contamination of the nutrient solution was avoided by enwrapping the plant basis with foam. The plants were harvested 3 days after the last treatment, when plants had developed 15-16 leaves. Samples were collected in a randomized manner.

Extraction of apoplastic and symplastic washing fluids

Cations were extracted by using the infiltration-centrifugation method as described by MÜHLING and SATTELMACHER (1995) and SHAHZAD et al. (2013). Of each plant the 5th to 8th leaves were cut with a razor blade and washed with deionized water. Subsequently, the leaf segments were placed in a 60 ml syringe infiltrated with 50 mM BaCl₂. BaCl₂ extracts apoplastically bound cations together with water soluble apoplastic ions. The solution was infiltrated into the leaves by applying a reduced pressure of ca. 20 kPa by pulling the syringe's plunger. Leaves were centrifuged for 5 minutes at 90 g and 4 °C, and the infiltration solution was collected. For the extraction of symplastic cations, leaves in which BaCl₂-extractable apoplastic cations had been extracted, were subsequently shock-frosted in liquid nitrogen and, after being thawed, were centrifuged again for 5 minutes at 142 g and 4 °C. Samples were stored at -80 °C.

Determination of Mg, Ca and K concentrations

Mg²⁺, Ca²⁺, and K⁺ in the symplastic fluid were determined by atomic absorption spectrometry (AAS 5EA Thermo Electron S, Carl Zeiss, Jena, Germany) whereas ion chromatography (DX 300, Dionex, Idstein, Germany) was used to determine corresponding concentration in the apoplastic washing fluids according to GEILFUS et al. (2015b). The latter analysis required to clear protein and chlorophyll content by means of adding chloroform to a final ratio of 1:2 (water:chloroform). Samples were mixed, centrifuged and the supernatant was then retained and filtered through C-18 columns (Torrance, CA, USA) for further clarification before ion analysis was started. Since the quantified values represent the concentrations in the washing fluids, values were corrected about an empirical factor that estimates the ratio of gaseous volume to aqueous volume in the apoplastic space of field bean leaves (LOHAUS et al., 2001).

Determination of Mg in leaf dry matter

Plant samples were dried for 48 h at 80 °C for dry weight measurements and then ground and ashed for 4 h at 550 °C in an oven. Magnesium was extracted with 4 M nitric acid for 4 h, diluted to 0.8 M nitric acid and filtered for subsequent measurements with the atomic absorption spectrometer (AAS 5EA Thermo Electron S, Carl Zeiss, Jena, Germany).

Statistics

Statistical analysis was carried out by using R (R version 2.11.1). For the comparison of means, multiple t-tests were adjusted according to Bonferroni-Holm. The homogeneity of variances and normal

distribution was evaluated by Boxplots, the Shapiro-Wilk test, and the Levene test.

Results

Whole tissue Mg concentration

The reduction from 0.5 mM MgSO₄ to 0.05 mM MgSO₄ induced a severe Mg-deficiency with the characteristic leaf interveinal chlorosis (Fig. 1). With respect to whole leaf Mg concentrations, a clear effect of the foliar supply became evident. Low MgSO₄ (0.05 mM) in the nutrient solution resulted in a concentrations of only 1.3 mg Mg/g dry matter (Fig. 2, negative control). The foliar application raised the concentration significantly to 15 mg Mg/g dry matter (200 mM MgSO₄ treatment), which was significantly higher than that in the positive control (3 mg/g dry matter) that was supplied with 0.5 mM Mg to the roots (Fig. 2).



Fig. 1: Older leaves of *Vicia faba* plants show Mg deficiency symptoms (left; 0.05 mM MgSO₄). Right side, leaves without deficiency (0.5 mM MgSO₄).

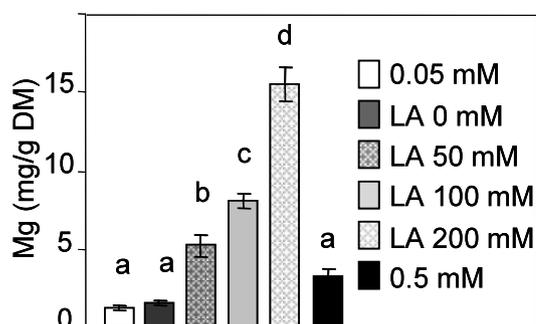


Fig. 2: Effect of magnesium treatments on Mg concentration in whole leaf dry matter. a, b, c, d: significant differences ($p \leq 0.05$) between treatments, LA: leaf application (50 mM, 100 mM or 200 mM MgSO₄), means \pm SE (n=4 independent replicates).

Effect of Mg foliar application on subcellular Mg concentrations

Mg concentrations as quantified in the BaCl₂-containing washing fluid are composed of water soluble plus apoplastically bound Mg and, by this means, ideally reflect the amount of leaf apoplastic Mg. Data clearly indicate that this Mg fraction markedly increased upon Mg leaf fertilization (Fig. 3a) finally exceeding the apoplastic Mg

concentrations of the positive control that received 0.5 mM Mg via the roots. The same pattern was observed with respect to symplastic Mg, however, absolute values were in average 1/3 higher (Fig. 3b) when compared to the apoplastic fraction.

Nutrient-nutrient antagonistic effects between Mg and subcellular Ca and K fractions

The application of 200 mM Mg onto the leaf decreased symplastic K concentrations whereas such an effect did not occur in the apoplast (Fig. 3c-d). Same treatment correlated with a decrease in apoplastic Ca whereas symplastic Ca concentrations remained unaffected (Fig. 3e-f). Concentrations of 50 or 100 mM of foliar applied Mg did not modulate subcellular Ca and K patterns.

Discussion

Mg foliar application fills pools of symplastic and apoplastic Mg

This study aimed to elucidate whether leaf foliar application of Mg re-fills symplastic and apoplastic Mg pools in leaves of *Vicia faba* plants that depleted in Mg. Deficient plants showed pronounced interveinal chlorosis (Fig. 1) and low whole leaf tissue Mg concentrations (Fig. 2). Foliar application of MgSO_4 ameliorated the nutritional status of the Mg-deficient leaves with regard to Mg. The Mg^{2+} concentrations in leaf dry matter (Fig. 2) of plants subjected to Mg^{2+} foliar application reached values that were markedly above the threshold value of 2 mg/g which is needed on average for an optimal Mg supply (KIRKBY and MENGEL, 1976). The fertilization of Mg over the leaf significantly increased Mg in both, the apoplastic (Fig. 3a) and symplastic (Fig. 3b) compartment. Such an apoplastic enrichment in Mg is not the primary aim of an Mg fertilization because the majority of problems that are associated with Mg-deficiency are based on lack in symplastic Mg. For example, Mg is required

for the functionality of many enzymes such as RNA polymerases, carboxylases, phosphatases, glutathione synthases or plasma membrane H^+ -ATPases (MARSCHNER, 1995; HANSTEIN et al., 2011). The apoplast is known to act as an ion exchanger that is thought to function in storage of nutrients (BERNSTEIN and NIEMAN, 1960). For this reason, the fact that a large fraction of the foliar applied Mg is bound to the apoplast must not necessarily be associated with a permanent immobilization. A later release to symplastic sinks is possible. Furthermore, apoplastic located Mg plays a role for cell wall and plasma membrane stability (KEEGSTRA, 2010; PASTERNAK et al., 2010). More importantly, this study clearly showed a remarkable accumulation of Mg in the symplast (Fig. 3b). It appears that the leaf application of 50 mM Mg which was replicated in triplicate represents an efficient means for enriching the Mg-depleted symplast rapidly with Mg. From this we suggest to conduct a Mg foliar application as a rapid measurement to avoid incipient Mg deficiency. Intriguingly, JEZEK et al. (2015) reported that the foliar application of 200 mM Mg increased net assimilation rate already after 2 days. We reason that such an agronomic measure is particularly advantageous at advanced developmental stages during the transition from the vegetative in the generative phase when a soil fertilization would come to late. Based on the results presented here that prove the transfer of foliar applied Mg into the leaf we encourage to adopt this strategy to other crop and horticultural plants with the aim to enrich plant based-foods in Mg by means of agronomic biofortification.

Mg foliar application affects Ca and K leaf concentrations in a dose-dependent manner

Mg deficiency often results in an increment of Ca or K concentrations in the leaves (MERHAUT, 2007). However, with regard to K, other studies have reported a synergistic effect between Mg supply

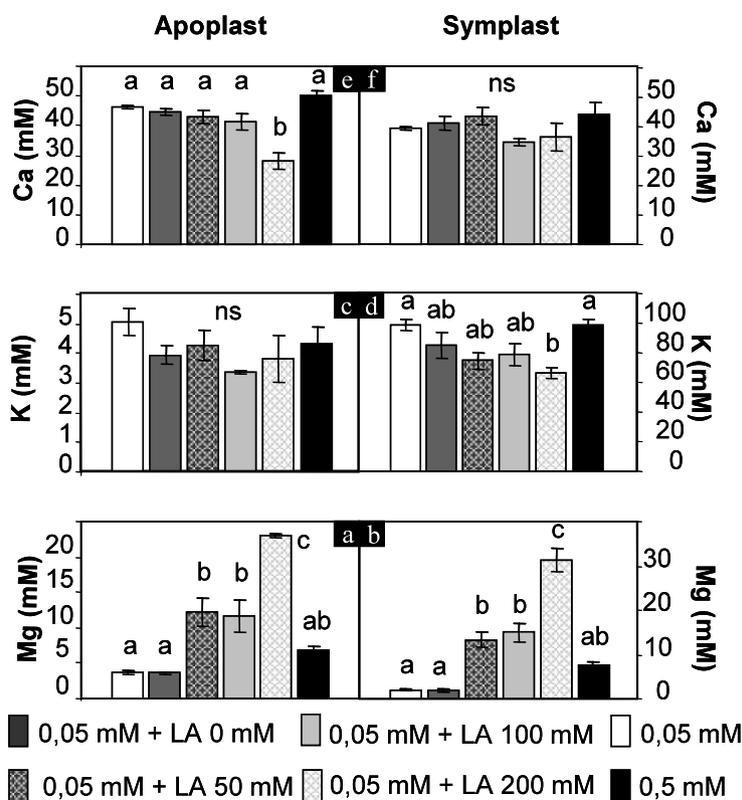


Fig. 3: Effect of magnesium treatment on Mg (a), K (b), and Ca (c) concentrations in the symplastic and apoplastic leaf compartments. a, b, c: significant differences ($p \leq 0.05$) between treatments; means \pm SE ($n=4$ independent replicates).

and K concentration (HARIADI and SHABALA, 2004; DING et al., 2006). In our study, none of those synergistic or antagonistic effects on K^+ or Ca^{2+} concentration were observed under conditions when plants were supplied with sufficient (0.5 mM) or deficient (0.05 mM) Mg over the roots (Fig. 3c-f). The discrepancies between those results may be attributable to differences in the developmental state and Mg supply: HARIADI and SHABALA (2004) reported that effects on Ca and K concentrations in leaves of the broad bean were induced in Mg-deficient plants only at early sampling times. These effects prevailed at later sampling times only in severely deficient plants. Interestingly, we found an effect of $MgSO_4$ leaf application on Ca and K concentrations only in response to the 200 mM $MgSO_4$ foliar treatment. Upon fertilization with 200 mM Mg, the leaf apoplastic fraction of bound plus water soluble Ca significantly dropped in concentration. This decrease was not associated with an concomitant increase in symplastic Ca. This indicates that foliar fertilization with 200 mM Mg can also negatively affect food quality of green plant organs since calcium is of utmost importance for human nutrition (SHERWOOD, 2015). The application of $MgSO_4$ may lead to a displacement of Ca from negative binding sites in the cell wall, constituted by carboxyl groups of pectins (SATTELMACHER et al., 1998). The displacement of Ca by Mg has also been shown by KIRKBY and MENGEL (1976) under Ca-deficiency conditions. Ca in the cell wall is important for both, the stabilizing the matrix pectins (GRIGNON and SENTENAC, 1991) and the integrity of the plasma membrane (HEPLER and WINSHIP, 2010). Thus, a loss of Ca would favor a solubilization of pectins and subsequent leakage of ions across the membrane. Mg has a smaller ionic radius than Ca but has significantly less bond angle flexibility than other cations (KEHRES and MAGUIRES, 2002). Thus, the displacement of Ca by Mg due to foliar application could have negative effects on cell wall structure and plasma membrane permeability.

Results further reveal an effect of the application of 200 mM $MgSO_4$ on symplastic K^+ (Fig. 3d). In general, the measured symplastic K concentrations that range from 66 to 100 mM are in accordance with the ranges of 40-200 mM reported by (BRITTO and KRONZUCKER, 2008) and 10-200 mM (MARSCHNER, 1995). The measured concentrations of apoplastic K ranging from 3.6 to 5.0 mM also fit in the range reported by others (MÜHLING and SATTELMACHER, 1995; MÜHLING and LAÜCHLI, 1999). The concentration of symplastic K^+ significantly decreased following the 200 mM foliar treatment. This decrease in K might be attributable to a K efflux and subsequent vascular transport to other organs in response to raising symplastic Mg concentrations, as fluxes of K^+ serve to counterbalance fluxes of other ions in terms of charge compensation (AMTMANN and BLATT, 2009), an effect that could also be shown by SHABALA and HARIADI (2005) who measured K^+ efflux in high Mg supply treatments of mesophyll segments. Another effect of the high Mg concentration on symplastic K relations might be conferred through non-selective cation channels in the plasma membrane. These have been shown to reduce uptake of monovalent cations, such as K^+ , if the concentration of divalent cations, such as Mg^{2+} , is high (DAVENPORT and TESTER, 2000; SHABALA and HARIADI, 2005). As we see decreases in K concentrations we reason that the fertilization of 200 mM Mg over the leaf is not favorable for the production of plant-based foods – should this fertilization strategy be used in plant production – since K plays key role in a myriad of human metabolic processes (FOX, 1995).

Conclusion

The pressing question regarding leaf nutrient fertilization is whether the sprayed nutrients reach the symplast where majority of physiological processes take place. The data in this paper approve accessibility of foliar applied Mg to the symplast. Nevertheless considerable amount do not overcome the apoplastic site which must not

necessarily be a problem since the leaf apoplastic compartment is thought to function as nutrient store. With respect to nutrient-nutrient interactions only the highest Mg dose exhibited antagonistic effects on apoplastic Ca or symplastic K pools whereas 50 or 100 mM Mg did not interfere with the compartmental distribution of those cations. Overall, our results reveal the functionality of leaf fertilization to enrich plants with magnesium.

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