

SCHRIFTENREIHE

**Institut für Pflanzenernährung und Bodenkunde
Universität Kiel**



Nr. 102 (2013)

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**Development of magnesium deficiency, ionic
composition and yield of faba bean (*Vicia
faba* L.) as affected by foliar MgSO_4
fertilization**

Herausgeber: R. Horn und K. H. Mühling

**Gedruckt mit Genehmigung der Agrar- und Ernährungswissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel**

**Vertrieb: Institut für Pflanzenernährung und Bodenkunde
der Christian-Albrechts-Universität zu Kiel
Hermann Rodewald Str. 2
D - 24118 Kiel
(e-Mail: h.fleige@soils.uni-kiel.de)**

ISSN 0933-680 X

Preis: Eur 15 (incl. Versandkosten)

Gedruckt mit der Genehmigung
der Agrar- und Ernährungswissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

Diese Arbeit kann als pdf-Dokument unter
<http://eldiss.uni-kiel.de/macau/content/below/index.xml>
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Aus dem Institut für Pflanzenernährung und Bodenkunde
der Christian-Albrechts-Universität zu Kiel

**Development of magnesium deficiency, ionic composition and yield
of faba bean (*Vicia faba* L.) as affected by foliar MgSO₄ fertilization**

Dissertation
zur Erlangung des Doktorgrades
der Agrar- und Ernährungswissenschaftlichen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von
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aus Lüdenscheid

Kiel, 2013

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Tag der mündlichen Prüfung: 25. April 2013

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Chapter 1

General Introduction

1. Introduction

As Magnesium fulfills various crucial functions in the plant metabolism a sufficient supply is important to gain high yields and crop quality. Apart from soil amendment magnesium can also be applied by foliar fertilization. The following chapter will give an introduction to the parameters affecting the magnesium supply in the soil and the impact of magnesium deficiency on plant physiology. Furthermore it discusses different aspects of foliar fertilization, such as advantages and disadvantages, mechanisms and parameters that affect foliar nutrient uptake. It closes with information literature provides with respect to foliar magnesium fertilization.

1.1 Magnesium availability in soils

According to the geochemical atlas of Europe (Foregs, www.gtk.fi/publ/foregsatlas), the magnesium concentration in topsoils of Europe are highly variable. Especially low concentrations are found in Poland, the north of Germany, Denmark and the Netherlands. In Germany, the magnesium concentrations in the soil range from 0.6-2.4 g kg⁻¹ in the north to 4.6-11.3 g kg⁻¹ in the south (Fig. 1). Low magnesium concentrations are generally related to the soil origin. Soils with low magnesium content are sandy soils, originating from granites, sandstones and post glacial and alluvial sandy deposits (Grzebisz 2011). According to Scheffer and Schachtschabel (2002), no yield increases could be obtained by magnesium fertilization in soils containing magnesium concentrations higher than 0.2 to 0.5 g kg⁻¹ Mg. Nevertheless, a number of authors claim the increasing problem of suboptimal magnesium provision in agriculture worldwide (Hariadi and Shabala 2004; Hermans et al. 2004; Cakmak and Kirkby 2008). The agricultural chamber in Schleswig-Holstein also classifies only 25% of the light sandy soils and one third of the heavier soils as optimally supplied with magnesium. On the contrary, 50% of the light sandy soils and 40% of the heavy soils are low in magnesium content, and therefore magnesium fertilization is recommended (Lausen and Gosch 2012). Besides low magnesium content in the parent rock material, the concentration of cations such as Ca²⁺, K⁺, NH₄⁺, H⁺ and Al³⁺ has a fundamental influence on the plant availability of magnesium, as they compete for the uptake by roots (Marschner 1995). Soil humidity influences the uptake of magnesium partially due to the transportation to the roots via mass flow and partially due to the removal of magnesium via leaching, which plays an important role as Mg²⁺ sorbs less strongly to soil colloids than other cations (Ding et al. 2006). The use of new cultivars, higher yields and high fertilization with N, P and K have also caused an enhanced occurrence of magnesium deficiency in light textured soils since the

1960's (Ding et al. 2006). Concomitantly the magnesium concentration in vegetable food has declined. Therewith, the human daily intake was reduced below the optimal level (Grzebisz 2011).

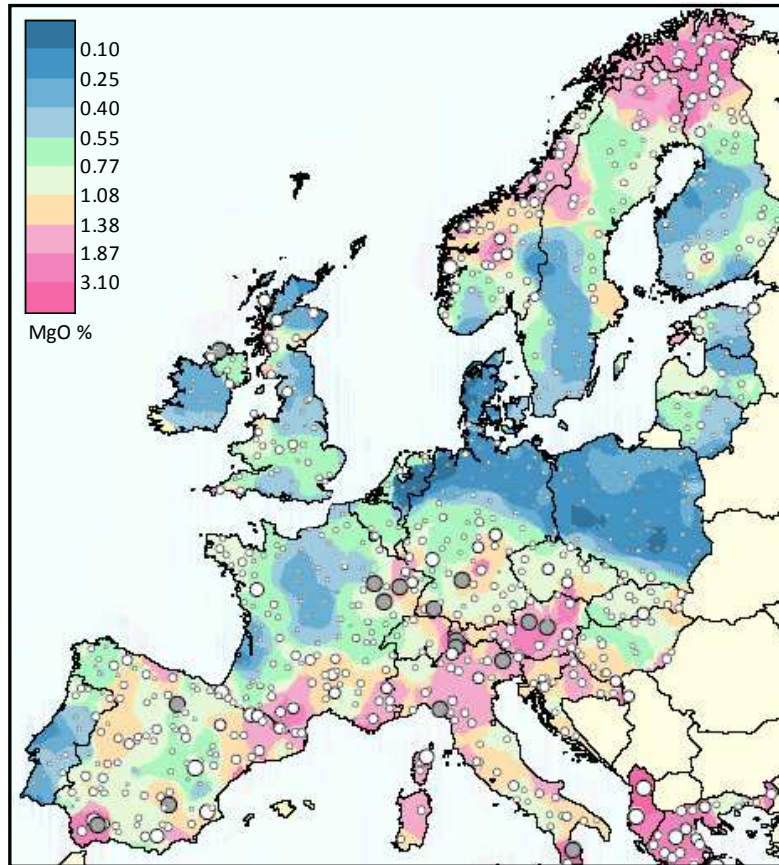


Figure 1: Concentrations of MgO (%) in topsoils of Europe (adapted from http://weppi.gtk.fi/publ/foregsatlas/maps/Topsoil/t_xrf_mgo_edit.pdf, accessed January, 2013)

1.2 Functions of magnesium in the plant and magnesium deficiency symptoms

Magnesium is essential for plant growth, as it is involved in all major cell reactions (Bergmann 1993). These include the protein synthesis, the energy and the fatty acid metabolism. Magnesium affects more than 300 enzyme reactions, often because of its ability to form chelates (Bergmann 1993).

Magnesium is involved in each step of protein formation, starting with the synthesis of purine and pyrimidine precursors, to the formation of DNA and RNA. Therein it has structural functions in stabilizing the configurations of DNA and RNA strands and the aggregation of the ribosomal subunits, also activating enzymes involved in these processes (Günther 2006).

Magnesium also fulfils a structural role for the photoassimilation, as it is the central atom of the chlorophyll molecule and is important for the assembling of the thylakoids. Additionally,

magnesium activates enzymes such as Rubisco and is crucial in counterbalancing the proton gradient built up from lumen to stroma of the thylakoids to promote the formation of ATP (Bergmann 1993; Marschner 1995). It is involved in almost all processes that are important for energy conversion and associated with phosphorylation, dephosphorylation and hydrolysis of compounds (Merhaut 2007). As magnesium activates Acetyl-Coenzyme A it has a central role in the fatty acid metabolism. Furthermore, magnesium is important for the stabilization of the cell membrane, the plant water relations and the phytohormon balance (Bergmann 1993). Due to these various important functions of magnesium, a deficiency of this macronutrient causes specific symptoms.

The most obvious symptoms are the reduction in root and shoot dry matter as well as the characteristic pattern of chlorosis in the older leaves of plants. In dicotyledoneous plants magnesium deficiency causes an interveinal chlorosis (Fig. 2). This can be accompanied by the formation of necrotic spots or reddening of the leaves, and will lead to advanced necrosis and senescence if the deficiency is aggravated (Bergmann 1993; Merhaut 2007). In monocotyledoneous plants symptoms differ; appearing as dark green spots against a yellow background colour of the leaf in grasses, or browning of needle tips in conifers (Kirkby and Mengel 1976; Merhaut 2007; Fig. 2).

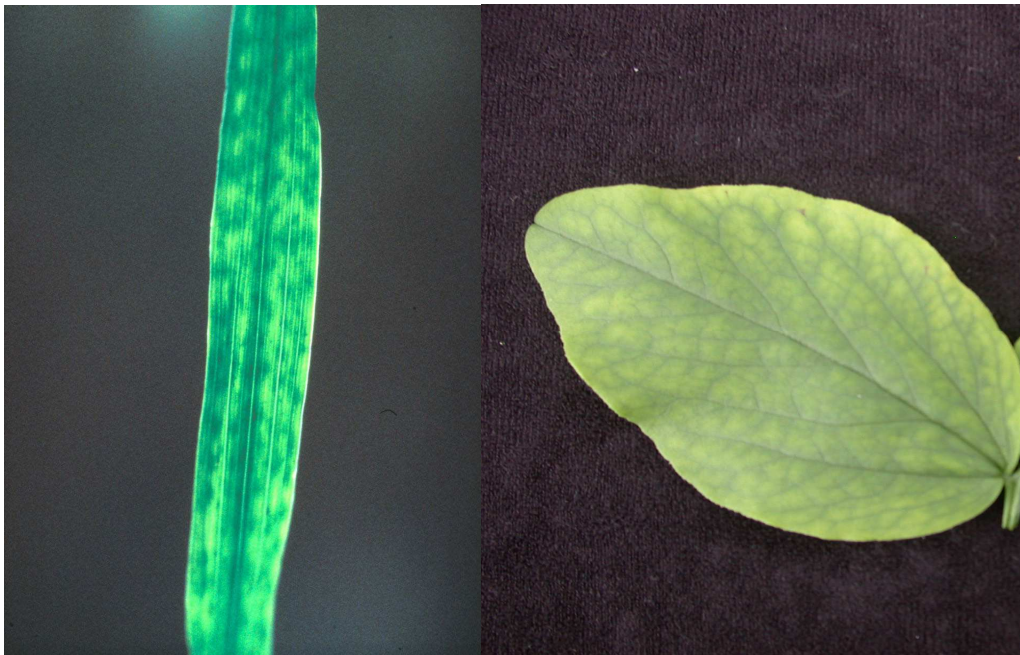


Figure 2: Magnesium deficiency symptoms in an oat leaf (left; Pissarek, H.P., <http://plantnutrition.iserver-online.de/rech.FAU?sid=0746EFFD24&dm=1&aft=1>, accessed January, 2013) and a faba bean leaf (right, Neuhaus, C.)

As an early response to magnesium deficiency, sucrose and starch accumulate in the leaves (Michael and Schilling 1957; Marschner 1995). Two reasons for this accumulation of sugars have been discussed. Fischer et al. (1998) proposed that a reduced utilization of photosynthates in the sink organs leads to an accumulation in source leaves. However, more recent works (Hermans et al. 2006) supported the idea of Cakmak et al. (1994) that the accumulation of sugars is due to the role of magnesium in the phloem loading process. As sucrose is loaded into the phloem via a H⁺/sucrose co-transport, there is a requirement for a proton gradient across the plasma membrane of phloem cells. This is built up by the H⁺-ATPase, which uses Mg-ATP as substrate and is inhibited by Mg free ATP (Hanstein et al. 2011).

As already mentioned, a reduction of the chlorophyll concentration is observable under magnesium deficiency. Moreover, photosynthesis rate is reduced and concomitantly increased activities of antioxidant enzymes are measurable under magnesium deficiency (e.g. Riga et al. 2005; Cakmak and Kirkby 2008; Chou et al. 2011; Yang et al. 2012). The investigations of Hermans et al. (2005, 2010) also revealed an influence of gene transcription on the development of symptoms. In roots and leaves, a number of circadian clock associated genes are differently expressed under magnesium deficiency. This also refers to genes encoding proteins involved in chlorophyll structure and photosynthesis. Their results also hint to an important role of ethylene in the plants response to low magnesium supply.

In order to avoid the above mentioned detrimental effects of magnesium deficiency, an adequate supply of magnesium to crops is needed. Besides the application of nutrients to the roots, these can also be supplied to the leaves. It is known since the 19th century that also the leaves can take up mineral nutrients and since then the benefits of foliar application as well as the mechanisms by which leaves take up nutrients are under discussion (Fernandez and Eichert, 2009).

1.3 Beneficial and adverse aspects of foliar fertilization

Research about the foliar fertilization of crops was conducted since the beginning of the 20th century and it has been proven to be a valuable complement to soil fertilization under certain circumstances (Kannan 1986; Fernandez und Eichert 2009; Oosterhuis and Weir, 2010).

Especially if the uptake of nutrients by the roots is inhibited, the supply of nutrients to the leaves can be beneficial. This may be the case for different reasons, such as reduced soil moisture, diseases, ion antagonism, fixation of ions, but also low root activity because of the development of reproductive organs (Gooding and Davies 1992; Schönherr and Luber 2001;

Wojcik 2004; Oosterhuis and Weir 2010). The foliar supply of nutrients can lead to a rapid improvement of the nutritional status of the plant under deficiency conditions (Fageria et al. 2009), as they are supplied directly to the assimilating organs and do not have to be translocated. Moreover, foliar application possibly leads to reduced environmental pollution e.g. with regards to the supply of N and can save costs and time, because of the possibility to supply other agrochemicals simultaneously (Gooding and Davies 1986).

However, the supply of macronutrients, such as magnesium, is especially difficult as they are needed in high amounts. The concentration of the applied solution must be high enough to meet the plants demand, without causing damage to the leaves (Gooding and Davies 1986; Oosterhuis and Weir 2010; Noack et al. 2010; Murtic et al. 2012).

Publications on foliar application of nutrients show contrasting results, sometimes proving the beneficial impact, sometimes showing no effects and sometimes having detrimental effects (Gooding and Davies 1986; Fageria et al. 2009). This great variety of results is caused by the high number of factors influencing the success of foliar application (Fernandez and Eichert 2009).

1.4 Uptake of nutrients via the leaves

Besides the uptake over trichomes and specialized epidermal cells, there are two pathways by which plants can take up hydrophilic solutes supplied to the leaves (Fernandez and Eichert 2009; Oosterhuis and Weir 2010). Unlike the uptake of ions over the roots, in the leaves the first barrier to encounter is the leaf cuticle, which protects the leaf from the invasion of deleterious substances, and also from the leaching of nutrients or the loss of water. It is generally composed of cutin embedded with waxes, which are also deposited on the surface, but varies between plant species, organs and developmental stages (Fernandez und Eichert 2009). As the cuticle is mainly lipophilic, diffusion of hydrophilic solutes, such as nutrient solutions, is low. They are believed to be taken up by either the “cuticular pathway” or the “stomatal pathway”. The cuticular pathway consists of “polar pores”, which are hydrated polar functional groups in the membrane, such as unesterified carboxyl groups, ester and hydroxylic groups and carboxylic groups (Fernandez and Eichert 2009). Ion movement therein is influenced by a concentration gradient from the applied solution to the concentrations of ions in the leaf and a charge gradient consisting of fixed negative charges of cell wall carbohydrates, which increase from the outer side of the cuticle to the cell wall. These pores are only indirectly proven and are believed to be accessible by molecules with radii of 0.45 nm (Schönherr and Schreiber 2004) to 2.4 nm (Eichert and Goldbach 2008).

Due to the cuticular hydrophobicity, the high surface tension of water and the stomatal morphology, it was formerly thought that the uptake of solutes through the stomata is impaired. However, experiments demonstrated the enhanced uptake of solutes in presence of stomata. Schönherr (2005) denoted this to an accumulation of polar pores at guard cells, but works of Eichert and Goldbach (2008) indicated that ions are able to enter the stomata. According to Burkhardt et al. (2012), the cuticular hydrophobicity and water surface tension is reduced by leaf surface particles originating from atmospheric deposition, agricultural sprays or plant exudations, as well as by microbial colonization. This leads to the creation of a liquid water film which connects the leaf surface and the mesophyll, and in which solutes can diffuse and move along the surfaces of stomatal pores. This pathway is believed to also be accessible to larger molecules, irrespective of charge or molecular weight, and to facilitate higher transport velocities than through the cuticle.

1.5 Parameters affecting the uptake of nutrients via the leaves

The effectiveness of foliar fertilizer application depends on several factors which are related to the environmental conditions, to the composition and the physiological state of the plant and to the physicochemistry of the applied solution. This has been reviewed thoroughly by Fernandez und Eichert (2009).

Environmental conditions such as temperature, light, air humidity and wind, affect the uptake of foliar fertilizers on the one hand by influencing the leaf properties and on the other hand by changing the properties of the applied solution. Weather conditions during plant growth have an impact on e.g. the cuticle thickness, amount and composition of waxes, amount of stomata and the leaf surface roughness. With respect to the solution properties, especially the air humidity is an important factor. As the nutrients can only be taken up due to the transportation in a liquid phase, the uptake of nutrients will be lower if the crystallization, due to low air humidity or wind, is fast. Light, temperature, water status and stress conditions affect physiological properties such as photosynthesis and nutrient transport and thereby also affect nutrient penetration (Wojcik 2004; Fernandez and Eichert 2009; Oosterhuis and Weir 2010).

Important leaf properties affecting the uptake of nutrient solutions are the cuticle thickness, the existence of leaf hairs or specialized epidermal cells, the number of stomata and the composition and integrity of the leaf surface. Leaves of *Vicia faba* have stomata on the adaxial and abaxial leaf surface. The frequency and number of stomata and trichomes of *Vicia faba* leaves is dependent on environmental factors, cultivar and leaf position on the main stem

(Tanzarella et al. 1984). Leaf properties are also affected by the leaf age, the nutritional status of the plant and pathogens.

The composition of the solution also plays an important role. Adjuvants change the wettability of the leaf surface, the penetration of the applied solute and the humectancy, but can also have phytotoxic effects. The concentration of the nutrient, the particle size and the pH of the solution affect the diffusion rate and the type of salt defines the point of deliquescence, which indicates the relative air humidity at which a salt crystallizes. Finally, the droplet size affects the even distribution of the applied solution (Wojcik 2004; Fernandez and Eichert 2009; Oosterhuis and Weir 2010).

1.6 Foliar fertilization with magnesium

Various magnesium salts, e.g. $\text{Mg}(\text{NO}_3)_2$, MgCl_2 , MgO , MgSO_4 and Mg-chelates, are used for the foliar fertilizer application (Allen 1959; Webster 1985; Dekker and Schoofs 2002; Borowski and Michalek 2010). As these salts have differing points of deliquescence, uptake of magnesium can be very different due to varying relative air humidities (Allen 1959). However, Allen (1959) could demonstrate that the uptake of Mg^{2+} was independent of the anion, if the salts remained soluble. Additionally, in two studies conducted on spinach (Borowski and Michalek 2010) and on apple trees (Dekker and Schoofs 2010), fertilization with different magnesium salts did not reveal differences with regards to the fresh weight or the magnesium concentrations, respectively.

Magnesium foliar application has become a common practice in agriculture (Webster and Drowe 1982; Allison et al. 2001; Borowski and Michalek 2010). Especially $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ is often used. Applied concentrations usually vary from 1 to 5% MgSO_4 . Under conditions of low magnesium supply in the soil, the foliar application has often been proven beneficial. In various crops such as oregano, sugar beet and tea, yields were raised in response to foliar magnesium application (Obatolu 1999; Barlog and Grzebisz 2001; Dordas 2009). In soils supplying only moderate magnesium concentrations, yield of sugar beet was also raised (Kristek et al. 2000). Furthermore, Teklic et al. (2009) and Rao and Rajput (2011) report of yield increases in soybean and palmarosa, due to magnesium foliar fertilization, without referring to the provision by the soil. Nevertheless, a strong influence of environmental conditions or genetical background (differences between cultivars) on the effect of the foliar application has been documented (Kristek et al. 2000; Barlog and Grzebisz 2001; Vrataric et al. 2006). No yield response to magnesium foliar application could be observed in a study of Reinbott and Blevins (1995) on soybean and rape (Yang-Yuen et al. 1999).

Foliar application often raises the magnesium levels in leaves (Webster and Drowe 1982; Dordas 2009; Teklic et al 2009) or in fruits (Reay et al. 1998). Though there are also studies in which this effect was only tendencially apparent (Reay et al. 1998; Teklic et al. 2009). Yang-Yuen (1999) and Szulc et al. (2001) did not detect higher magnesium contents in the dry matter due to magnesium foliar application.

If spray concentrations of 1 or 2% MgSO_4 are used, a second application proved to further increase yield in the study of Barlog and Grzebisz (2001), and Dordas (2009) found the application of 2% superior to the application of 1%. On apple it was regarded commercially reasonable to make at least 3 applications of 2% $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ (Boynton 1954). In contrast, repeated applications did not result in yield increases, when a 5% concentrated MgSO_4 solution was used in soybean (Vrataric et al. 2006; Teklic et al. 2009).

Magnesium foliar application has been proven valuable for increasing the chlorophyll concentration in leaves and also in apple fruits (Szulc et al. 2001; Reay et al. 1998; Dordas 2009; Teklic et al. 2009). However, the influence on other traits of quality is less consistent. Dordas (2009) did not assess an influence of foliar magnesium application on the concentration of essential oil yield in oregano, but the content of geraniol in palmarosa was raised. In the seeds of soybean foliar magnesium application resulted in increases of protein and oil contents in the grains (Vrataric et al. 2006), whereas in potato the protein concentration was not influenced (Cieko et al. 2010). In sugar beet, Kristek et al. (2000) could show an increase of sugar concentration due to magnesium foliar application, whereas this was not the case in a study conducted by Barlog and Grzebisz (2001).

In resume, some parameters, such as biomass accumulation and chlorophyll concentration, respond well to magnesium foliar application, whereas others such as yield and quality of harvested plant parts are only partially influenced. Besides yield formation and chlorophyll concentration there is not much information about the impact of foliar fertilization on plant physiology.

1.7 Objectives

Suboptimal soil magnesium concentrations for crop production are a worldwide problem and affect crop yield formation and quality (Bennett 1997; Hermans and Verbruggen 2005). Foliar fertilization is a possibility to ameliorate the nutritional status of deficient plants. It leads to rapid improvement of nutrient availability and soil factors like water availability, ion fixation and cation competition are less influential.

Overall, studies show that magnesium foliar application often has beneficial effects, especially with regards to increasing the biomass yield and the chlorophyll concentration. However, the influence on crop quality as indicated by carbohydrate, protein and oil concentrations are quite contrasting. Apart from agronomic characteristics, the physiological impacts of foliar magnesium fertilization on the plant are not yet well studied.

In the present examination, faba beans (*Vicia faba* L.) growing under conditions of magnesium deficiency were subjected to foliar applications of different MgSO_4 concentrations. The impacts of magnesium foliar fertilization on the following aspects of plant physiology were studied:

- In order to obtain a sufficient uptake of magnesium, high concentrations need to be applied. Does this lead to a displacement of other cations in the leaf compartments apoplast and symplast?
- Leaf applied cations primarily have to encounter the cuticle and the apoplast. Is the uptake of magnesium into the symplast affected by the fixed negative charges in the apoplast which presumably bind a high quantity of the applied magnesium?
- Is the transcript abundance of genes that are related to the development of deficiency symptoms, namely Mg chelatase and plasmalemma H^+ -ATPase, affected by Magnesium deficiency and foliar application?
- Is the supply of magnesium to the leaves sufficient to result in increased yield and to raise the quality of seeds in *Vicia faba* L.?

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Chapter 2

Influence of a magnesium foliar application on subcellular Mg²⁺, Ca²⁺, and K⁺ ion concentrations of *Vicia faba* L. leaves

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Journal of Plant Nutrition and Soil Science (in revision)

2. Influence of a Mg foliar application on subcellular Mg²⁺, Ca²⁺, and K⁺ ion concentrations of *Vicia faba* L. leaves

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2.1 Abstract

Foliar application is a possible method for the correction of Mg deficiency in crop plants. To achieve an adequate uptake of the ion over the leaf surface, high concentrations of MgSO₄ are usually supplied. This might cause perturbations in the ion concentration and distribution of Mg²⁺, Ca²⁺, and K⁺ in the various leaf compartments. As the apoplast binds cations, the extent to which the applied Mg²⁺ can be taken up into the leaf symplast is unknown. Therefore, we used the infiltration-centrifugation method to extract ions residing in the apoplast or symplast separately. *Vicia faba* plants were grown under controlled environmental conditions in nutrient solution. Plants growing in low-magnesium solution were treated with foliar applications of 0 mM, 50 mM, 100 mM, or 200 mM MgSO₄.

Foliar application raised the Mg²⁺ concentration in the apoplast and the symplast significantly. Ca²⁺ and K⁺ concentrations were affected significantly only following the application of 200 mM MgSO₄, which led to a decrease of apoplastic bound Ca²⁺ and symplastic K⁺ concentrations.

We have thus demonstrated that foliar application of MgSO₄ ameliorates the nutritional status of *Vicia faba* with regards to magnesium while leading to the replacement of Ca²⁺ and K⁺ in the apoplast and the symplast, respectively. Mg²⁺ ions are bound in the apoplast, but this effect does not hinder uptake into the symplast.

Keywords

Apoplast, faba bean, foliar fertilization, ion antagonism, magnesium

2.2 Introduction

Mg plays a role in many enzyme activation reactions and it is therefore necessary for a large number of developmental and growth processes in plants (*Epstein and Bloom, 2004*). Furthermore it is the central atom of chlorophyll, making it essential for an efficient photosynthesis and yield formation. Various environmental conditions cause magnesium deficiency in plants and can lead to the appearance of characteristic deficiency symptoms in the form of an interveinal chlorosis of the older leaves in dicotyledons (Fig. 1). One possible method to prevent this and to correct for nutrient deficiencies in crop plants is foliar application, which can be especially valuable at peak demand times and under conditions of low soil availability (*Fernandez and Eichert, 2009*). To achieve sufficient uptake of a nutrient over the leaves, highly concentrated solutions are supplied to the foliage. In the case of magnesium, leaf applications with $\text{MgSO}_4 \cdot (\text{H}_2\text{O})_7$ at concentrations of 40 mM (*Reay et al., 1998*), 80 mM (*Dordas, 2009*), 200 mM (*Teklic et al., 2009; Vrataric et al., 2006; Kristek et al., 2000*), or even higher have been tested (*Webster and Drowe, 1982*).

In addition to the intended fertilizing effect of the foliar-applied nutrient, side effects on the distribution of other mineral nutrients need to be considered. As demonstrated in experiments with soil or nutrient solution, a high supply of specific cations can lead to the reduced uptake of others. This antagonistic effect has often been shown for K^+ , which influences the uptake of Ca^{2+} or Mg^{2+} (e.g., *Miller, 1999; Spear et al., 1987; Ologunde and Sorensen, 1982; Troyanos et al., 2000*). Furthermore, a high supply of Mg^{2+} can lead to reduced tissue concentrations of K^+ and Ca^{2+} (*Grant et al., 1988; Ologunde and Sorensen, 1982; Troyanos et al., 2000*). Bearing these antagonistic effects in mind, we consider it likely that foliar applications of MgSO_4 will affect leaf ion composition and will cause perturbations in the ion concentrations and/or distributions within the various leaf compartments.

Of the leaf compartments, the apoplast is the first to be encountered by the applied solution. As the apoplast comprises many fixed anions and is therefore supposed to serve as a storage site for cations (*Grignon and Sentenac, 1991*), the Mg^{2+} supplied to the leaves might be partly bound apoplastically, rather than taken up into the symplast, and might therefore not be physiologically available.

In this report, we have investigated (1) whether, under Mg deficiency conditions, a foliar application of Mg can ameliorate the Mg nutritional status of the plant, (2) in which leaf compartment the Mg ions applied via the leaves predominately accumulate, and (3) what effects this accumulation exerts on the concentrations and distribution of K^+ and Ca^{2+} .

2.3 Material and Methods

Plant cultivation

Vicia faba L. plants of the cultivar Scirocco (NPZ, Hohenlieth, Germany) were grown hydroponically in climate chambers (14/10 h day/night; 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation). Seedlings were placed in 0.5 mM aerated CaSO_4 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. The full nutrient solution had the following composition: 2.0 mM $\text{Ca}(\text{NO}_3)_2$, 1.0 mM K_2SO_4 , 0.1 mM KH_2PO_4 , 0.01 mM NaCl , 10 μM H_3BO_4 , 2.0 μM MnSO_4 , 0.5 μM ZnSO_4 , 0.2 μM CuSO_4 , 0.05 μM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 60 μM Fe-EDTA. Plants were subjected to six different treatments with four biological replications each. Control plants were supplied with 0.5 mM $\text{MgSO}_4 \cdot (\text{H}_2\text{O})_7$ to the roots. The remaining five treatments were grown in nutrient solution containing only 0.05 mM MgSO_4 , of which four groups were subjected to MgSO_4 leaf applications, with leaf application solutions containing 0 mM, 50 mM, 100 mM, or 200 mM MgSO_4 . To ensure the wettability of the plant surface, a wetting agent (0.1% (v/v) ARMA; Agroplanta, Langenpreising-Zusdorf, Germany) was used. Foliar application was conducted three times, starting after four weeks of growth. The application was conducted using a hand held sprayer. In order to assure the application of equal amounts of ca. 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. Growth conditions were 20°C at day and 15°C at night at 50% relative humidity. The plants were harvested 3 days after the last treatment, when plants had developed 15-16 leaves. The leaves of different treatments 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). 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 filled with either deionized water or 50 mM BaCl_2 . Deionized water was used for the extraction of soluble apoplastic ions, whereas the solution containing BaCl_2 was used to extract the apoplastic-bound cations. As for the determination of soluble and bound ions, individual leaves were used for the extraction; the ions extracted with BaCl_2 comprise bound and soluble ions. The solutions were infiltrated into the leaves by applying a reduced pressure

of ca. 20 kPa by pulling the plunger. Subsequently, the leaves were centrifuged for 5 minutes at 90 g and 4°C, and the infiltration solution was collected. For the extraction of the symplastic cations, the same leaves 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 kept frozen at -80°C, until further use. To calculate the correct ion concentration in the apoplastic leaf space a dilution factor was used (*Lohaus et al.*, 2001). This is needed as the infiltration solution also occupies the air filled apoplastic space, which leads to a dilution of the apoplastic ion concentration. It was determined for *Vicia faba* by *Lohaus et al.* (2001).

Determination of Mg²⁺, Ca²⁺ and K⁺ concentrations

The ions Mg²⁺, Ca²⁺, and K⁺ in the symplastic extraction were determined by using atomic absorption spectrometry (AAS 5EA Thermo Electron S, Carl Zeiss, Jena, Germany), while cation concentrations in the apoplastic washing fluids were determined by using ion chromatography (DX 300, Dionex, Idstein, Germany). This was done because of higher matrix effects in the symplastic samples, whereas the apoplastic samples could not be measured on the AAS because of the BaCl₂. For precipitation of proteins and to get rid of chlorophyll, samples were diluted and mixed in 1:2 water/chloroform. Subsequently, samples were centrifuged and the supernatant was then filtered through C-18 columns (Torrance, CA, USA) for further clarification and then analyzed.

Determination of Mg²⁺ in leaf dry matter

Plant samples were dried for 48 h at 80°C for dry weight measurements and then were ground and ashed for 4 h at 550°C in an oven for cation analysis. Magnesium was extracted with 4 M nitric acid for 4 h, the nitric acid then being diluted to 0.8 M and filtered for measurements with an 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, The R foundation for statistical computing, 2010). 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.

2.4 Results

2.4.1 Dry matter and Mg^{2+} concentration

In comparison with the high Mg-treated control plants, neither the influence of low $MgSO_4$ in the nutrient solution nor the application of $MgSO_4$ to the leaves affected the production of shoot dry matter significantly (data not shown). In all treatments, shoot dry matter ranged between 11-15 g per pot. However, a clear influence of treatments was noted with regard to whole leaf Mg^{2+} concentrations (Fig 2). Low $MgSO_4$ in the nutrient solution led to concentrations of only 1.3 mg Mg/g dry matter. The foliar application raised the concentration significantly to 15 mg Mg/g dry matter (200 mM $MgSO_4$ treatment), which was significantly higher than that in the control (3 mg/g dry matter) grown in adequate $MgSO_4$ nutrient solution.

2.4.2 Concentration of Mg^{2+} , Ca^{2+} , and K^+ in apoplastic washing fluids

Apoplastic concentrations of Mg^{2+} were significantly raised by the application of $MgSO_4$ to the leaves (Fig. 3 a).

The application of 50 mM and 100 mM $MgSO_4$ led to an augmentation up to 12 mM Mg^{2+} , a concentration that was nearly doubled by the application of 200 mM $MgSO_4$. On separation of apoplastic bound and apoplastic soluble ions, we found that both fractions were raised following $MgSO_4$ leaf application. In particular, the amount of soluble apoplastic Mg^{2+} was raised by 750% (Fig. 4).

The concentration of Ca^{2+} behaved in an opposite fashion under the influence of $MgSO_4$ leaf application (Fig. 3 e). No significant change occurred in the concentration of soluble apoplastic Ca^{2+} , but the apoplastic-bound Ca^{2+} declined significantly after 200 mM $MgSO_4$ foliar treatment (Fig. 4).

The apoplastic K^+ concentration was not influenced by the application of $MgSO_4$ to the leaves (Fig. 3 c). With values of 3.6 mM to 4.9 mM K^+ , the extraction with H_2O infiltration solution resulted in higher apoplastic K^+ concentrations than those after extraction with $BaCl_2$ (Supplemental material 1).

2.4.3 Concentration of Mg^{2+} , Ca^{2+} , and K^+ in the symplast

The symplastic concentrations of Mg^{2+} were significantly raised after $MgSO_4$ leaf application (Fig. 3 b). The symplastic Mg^{2+} concentration was 1.9 mM in plants growing under low $MgSO_4$ conditions without $MgSO_4$ leaf application, and 7.7 mM in plants growing under

adequate Mg conditions. Following MgSO₄ leaf application, the Mg²⁺ concentration rose to 13, 15, and 31 mM MgSO₄ after treatment with 50, 100, or 200 mM MgSO₄, respectively.

The symplastic Ca²⁺ concentrations differed between 35 and 44 mM but were not affected significantly by the foliar MgSO₄ treatment or Mg deficiency conditions (Fig. 3 f).

However, an effect of MgSO₄ treatment was detected on the symplastic K⁺ concentrations, which declined. A significant difference of 66 to 100 mM K⁺ was found only between the 200 mM treatment and the controls, respectively (Fig 3 d).

2.5 Discussion

2.5.1 Influence of MgSO₄ foliar application on Mg²⁺ distribution in leaves

Foliar application of MgSO₄ ameliorated the nutritional status of the studied plants with regards to Mg. This was indicated by the Mg²⁺ concentrations in leaf dry matter of plants subjected to Mg²⁺ foliar application (Fig. 2) being higher than the value of 2 mg/g needed on average for an optimal Mg supply (*Kirkby and Mengel, 1976*). As we wanted to investigate the subcellular distribution of the Mg²⁺ ion, we needed to discriminate between the apoplast and symplast; this aim was achieved by means of an extraction procedure. Figures 3 a and b show that foliar MgSO₄ application raised the concentration of Mg²⁺ in both the apoplast and symplast significantly. Interestingly the ratio of Mg²⁺ concentration between apoplast and symplast, when comparing foliar applications and the adequate-Mg control, remained constant (Fig. 5). This shows that in reaction to a rise of apoplastic Mg also the symplastic Mg concentration rose. It appears therefore that if a certain amount of Mg is bound in the apoplast, the further capacity to bind Mg (Fig. 4) does not restrict the uptake into the symplast.

As the function of many enzymes, e.g., RNA polymerases, carboxylases, phosphatases, and glutathione synthase, is dependent on magnesium (*Marschner, 1995*), and as the Mg²⁺ level regulates the activity of enzymes, e.g., the plasma membrane H⁺-ATPase (*Hanstein et al., 2011*), the cytosolic Mg²⁺ concentration is assumed to be strictly regulated (*Gardner, 2003; Shaul, 2002*). Therefore, the rise in symplastic Mg²⁺ concentrations in reaction to the foliar MgSO₄ applications probably reflects a rise of Mg²⁺ in storage sites, such as the vacuole or cell organelles, whereas the cytosolic Mg²⁺ remains constant.

2.5.2 Influence of MgSO₄ foliar application on Ca²⁺ and K⁺ concentrations in leaves

Effect of Mg deficiency

Mg deficiency often results in an increment of Ca²⁺ or K⁺ concentrations in the leaves, as demonstrated in a number of studies (references in *Merhaut*, 2007). However, with regard to K⁺, other studies have reported a synergistic effect of the Mg²⁺ supply on the K⁺ concentration (*Hariadi and Shabala*, 2004; *Ding et al.*, 2006). In our study, none of those synergistic or antagonistic effects of Mg deficiency on the K⁺ and Ca²⁺ concentration have been observed in comparison with the normal MgSO₄ treatment in control plants (Figs 3 c-f). This may be attributable to the inference of only moderate magnesium deficiency and to sampling time. Such a dependency has been demonstrated by *Hariadi and Shabala* (2004). In their study, the effects on Ca²⁺ and K⁺ concentrations in leaves of the broad bean were induced in Mg-deficient plants at early sampling times, but this effect prevailed at later sampling times only in severely deficient plants.

Effect of foliar application

Interestingly, we found an effect of MgSO₄ leaf application on Ca²⁺ and K⁺ concentrations. The apoplastic Ca²⁺ concentration decreased significantly in response to the 200 mM MgSO₄ foliar treatment. The differentiation between soluble and bound apoplastic Ca²⁺ (Fig. 4) shows that this decrease is attributable to the bound apoplastic Ca²⁺, which declines as the apoplastic-bound Mg²⁺ increases. Therefore, the application of MgSO₄ probably leads to a displacement of Ca²⁺ from negative binding sites in the cell wall, constituted by carboxyl groups of pectins (*Sattelmacher et al.*, 1998). The displacement of Ca²⁺ by Mg²⁺ has also been shown by *Kirkby and Mengel* (1976) under Ca-deficiency conditions. Moreover, the high supply of other cations such as Na⁺ can lead to a displacement of Ca²⁺ (*Dayod et al.*, 2010). Ca²⁺ in the cell wall is important for the stabilization of the matrix pectin (*Grignon and Sentenac*, 1991) and the stability of the plasma membrane (*Hepler and Winship*, 2010). Thus, a loss of Ca²⁺ would cause a solubilization of pectins and lead to a leakage of ions across the membrane. Possibly Mg²⁺ could replace Ca²⁺ in this function. However, Mg²⁺ has a smaller ionic radius than Ca, and has significantly less bond angle flexibility than other cations (*Kehres and Maguires*, 2002). Therefore the displacement of Ca²⁺ by Mg²⁺ due to foliar application could have negative effects on cell wall structure and plasma membrane permeability.

The symplastic Ca²⁺ was not affected by foliar MgSO₄ treatment (Fig. 3 f). The measured concentrations of 35 to 44 mM Ca²⁺ are possibly attributable to the storage of Ca²⁺ in

organelles and the vacuole for which great differences in concentrations have been reported dependent on plant species and cell type (Dayod *et al.*, 2010).

The symplastic K^+ was affected by the $MgSO_4$ application (Fig. 3 d). The measured values of 66 to 100 mM agree with the range of K^+ concentrations reported for the cytosol and vacuole of 40-200 mM (Britto and Kronzucker, 2008) and 10-200 mM (Marschner, 1995), respectively. The concentration of symplastic K^+ decreased under $MgSO_4$ foliar treatment, a decrease that became significant following the 200 mM foliar treatment. The efflux of K^+ might be attributable to the higher concentration of Mg^{2+} in the symplast, 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 impact of the high Mg concentration might be the effect on 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). Therefore the uptake of K^+ into the cytosol might also be reduced.

On the other hand, apoplastic K^+ was not influenced by foliar Mg^{2+} application. The measured concentrations of apoplastic K^+ 3.6 of to 5.0 mM lie in the range of concentrations previously reported (Mühling and Sattelmacher, 1995; 1997; Mühling and Läuchli, 1999). As indicated by Mühling und Sattelmacher (1995), the use of the washing fluid containing $BaCl_2$ does not lead to a higher concentration of apoplastic K^+ , a finding that is explainable by the low adsorption of K^+ by the cell wall.

Whereas Mg^{2+} concentrations were raised in the apoplast and symplast, the apoplastic-bound Ca^{2+} and symplastic K^+ concentrations decreased, thereby keeping the sum of charge roughly constant (Fig. 6). This effect has also been observed by Ologunde and Sorensen (1982) who suggested, in accordance with Bedi and Sekhon (1977) and Ding *et al.* (2006), that one cation can partly replace another for some functions in plants. The exchange of Ca^{2+} and K^+ for Mg^{2+} might also be beneficial in order to keep the leaf osmotic pressure constant. This was suggested by Conn *et al.* (2012), who observed that low Mg in the vacuole led to an increased concentration of vacuolar Ca^{2+} and K^+ . Also high ionic concentrations could induce osmotic stress, under foliar Mg application this was possibly avoided by reducing the K^+ concentration in the symplast.

As the decrease of Ca^{2+} and K^+ concentration did not lead to corresponding ion increases in one of the measured fractions, it is more likely that Ca^{2+} and K^+ were distributed via the long-distance transport. As Ca^{2+} is not transported in the phloem it may be possible that it is

transported towards the developing leaves. As for K^+ it is possible that also a translocation to the roots was stimulated.

2.6 Conclusion

We have demonstrated significant influences of Mg leaf application on the ion relationships in the apoplast and symplast. Foliar application could ameliorate the nutritional status of the plant, raising not only the concentration of Mg^{2+} bound to the negative binding sites in the apoplast, but also the concentration of Mg^{2+} in the symplast. The negative binding sites in the apoplast apparently did not lead to a pondage of the positively charged Mg^{2+} in the apoplast, as the ratio between apoplastic and symplastic Mg^{2+} remained constant under a supply of different $MgSO_4$ concentrations.

$MgSO_4$ application changed the concentrations of Ca^{2+} and K^+ in the leaf. The Ca^{2+} concentration was affected in the leaf apoplast in which Mg^{2+} replaces Ca^{2+} at negative binding sites, whereas the K^+ concentration was affected in the symplast in which it probably serves in charge compensation.

Acknowledgements

C. Neuhaus is grateful for the receipt of a Kiel University federal state grant. We thank Ms. A. Thießen and Ms. S. thor Straten for excellent technical assistance.

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Figure legends

Fig. 1: Oldest leaves of *Vicia faba* plants showing Mg deficiency symptoms (left) compared with leaves without deficiency (right).

Fig. 2: Effect of magnesium treatment on Mg^{2+} concentration in whole leaf dry matter. a, b, c, d: significant differences ($p \leq 0.05$) between treatments, LA: leaf application (with the $MgSO_4$ concentration of the solution being 0 mM, 50 mM, 100 mM or 200 mM), error bars represent $\pm SE$ of four independent replications.

Fig. 3: Effect of magnesium treatment on Mg^{2+} (a), K^+ (b), and Ca^{2+} (c) concentrations in the symplastic and apoplastic leaf compartments (extracted with $BaCl_2$). a, b, c: significant differences ($p \leq 0.05$) between treatments; error bars represent $\pm SE$ of four independent replications.

Fig. 4: Percent change of Mg^{2+} and Ca^{2+} concentrations under differential Mg foliar treatment compared with the control supplied with sufficient Mg via the roots (-). a, b, c: significant differences ($p \leq 0.05$) between treatments for apoplastic soluble Mg (small letters) and apoplastic soluble and bound Mg (capital letters), d, e, f: significant differences for apoplastic soluble Ca (small letters) and apoplastically bound and soluble Ca (capital letters); error bars represent $\pm SE$ of four independent replications.

Fig. 5: Ratio of Mg^{2+} concentration in the apoplast (extracted with $BaCl_2$) divided by the symplastic Mg^{2+} concentration. a, b: significant differences ($p \leq 0.05$) between treatments; error bars represent $\pm SE$ of four independent replications.

Fig. 6: Sum of cation charge [$(Mg^{2+}) + (Ca^{2+}) + (K^+ * 2)$]. No significant differences were found between the treatments; error bars represent $\pm SE$ of four independent replications

Supplemental figure legend

S1: Apoplastic K^+ concentration (mM) extracted with H_2O or $BaCl_2$. Error bars represent $\pm SE$ of four independent replications. No significant differences were found.

Figure Files

Figure 1



Figure 2

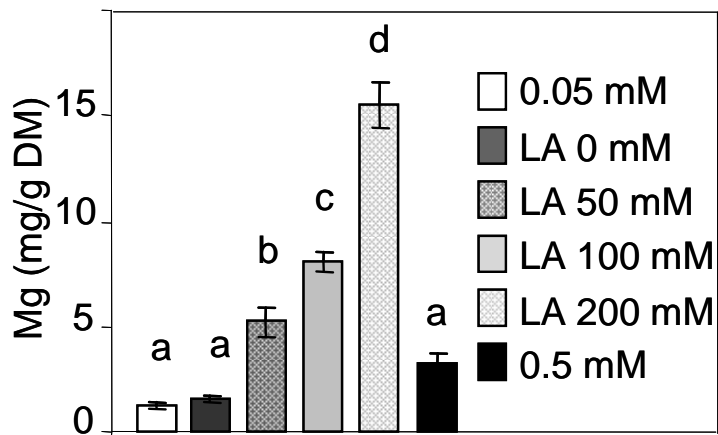


Figure 3

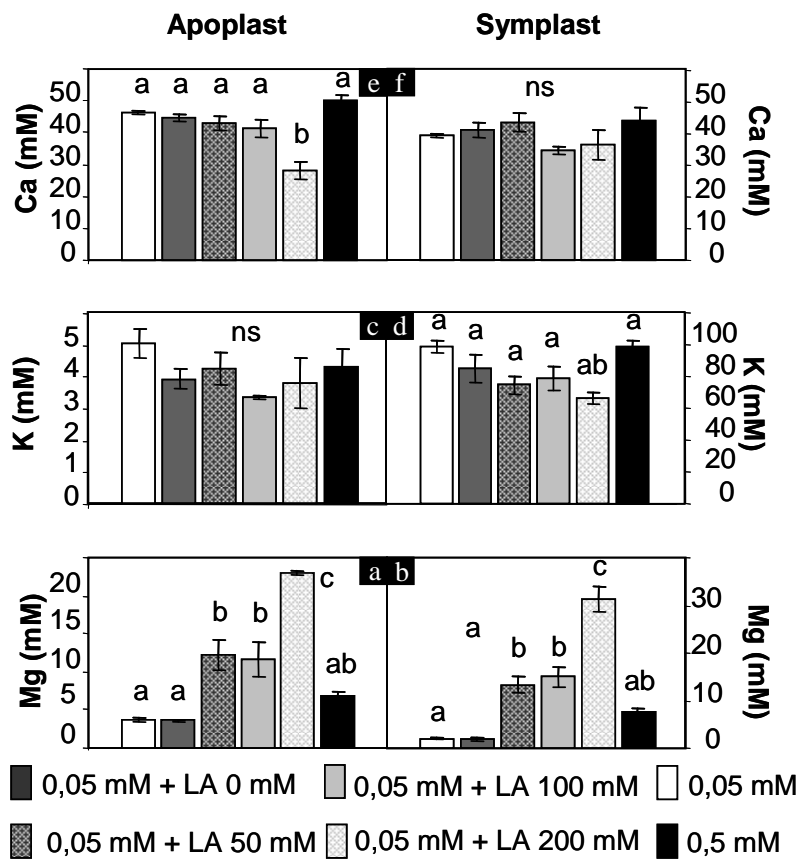


Figure 4

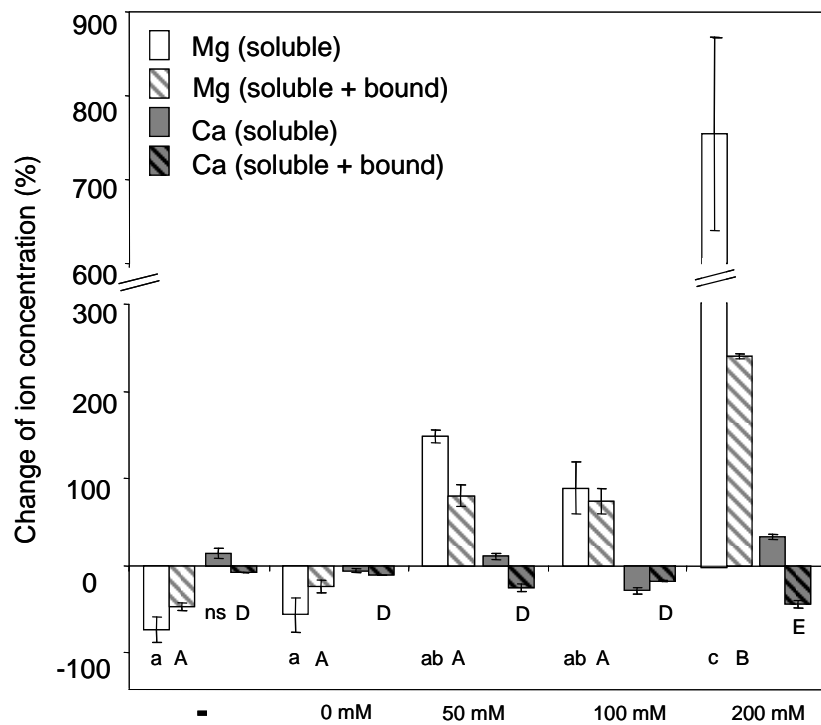


Figure 5

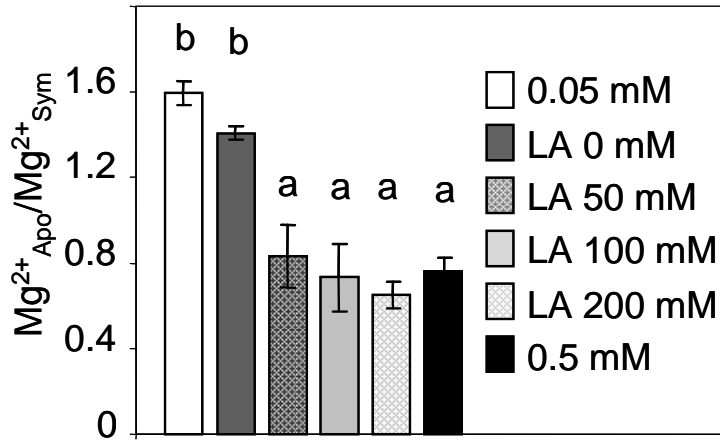
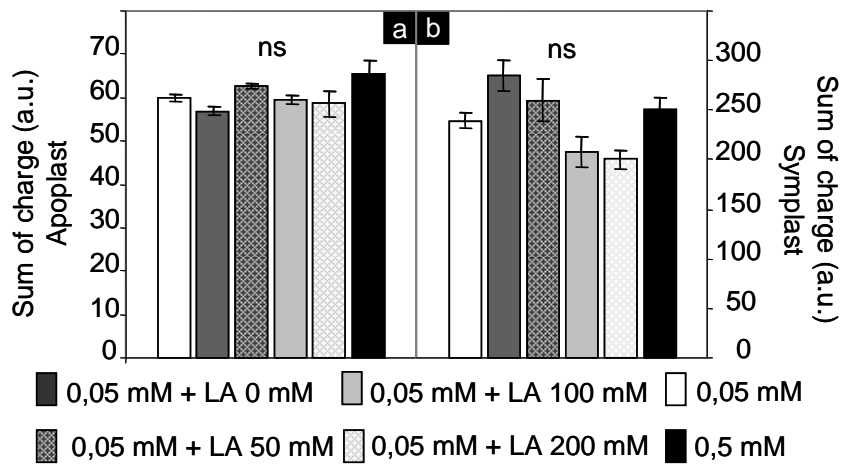
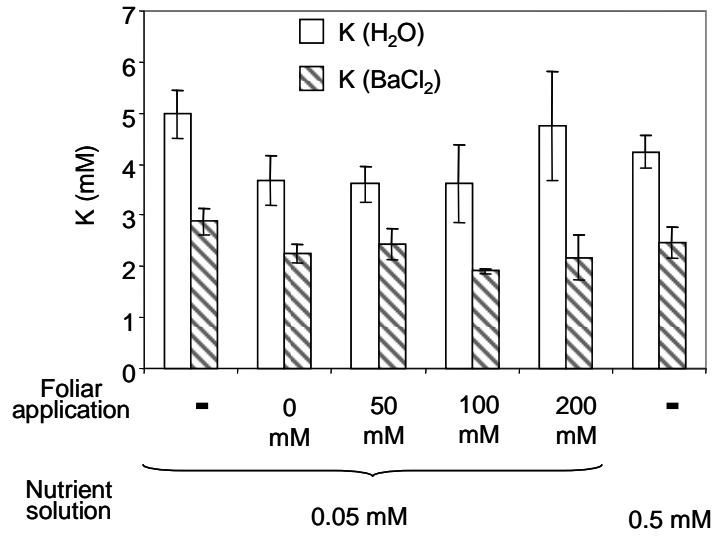


Figure 6



Supplemental Figure



Chapter 3

Transcript expression of Mg-chelatase and H⁺-ATPase isoforms in *Vicia faba* leaves as influenced by root and foliar magnesium supply

Christina Neuhaus, Christoph-Martin Geilfus, Christian Zörb and Karl-Hermann Mühling

Plant and Soil (2013) 368, 41-50

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Chapter 4

Increasing vegetative growth and yield formation in magnesium-deficient faba beans (*Vicia faba* L.) by MgSO₄ foliar fertilization

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Journal of Plant Nutrition and Soil Science (submitted)

4. Increasing vegetative growth and yield in Mg-deficient faba beans (*Vicia faba* L.) by MgSO₄ foliar fertilization

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4.1 Abstract

Magnesium deficiency reduces yield and quality of crops as Mg is involved in carbohydrate and protein anabolism. Foliar application of MgSO₄ represents a means of ameliorating the nutritional status of crops growing under conditions of deficiency. Foliar Mg application has been demonstrated to increase the chlorophyll concentration and vegetative yield of plants. However, in crop plants used for seed production, the limited number of studies provides only conflicting results in terms of the efficiency of Mg leaf application on yield and quality. We have examined the effect of increasing Mg foliar application rates on differentially developed leaves and leaf stages with regards to Mg concentration, chlorophyll concentration and biomass formation. Yield and seed quality have also been investigated. Our data reveal that foliar Mg application increases yield in Mg-deficient faba beans. Moreover, seed quality is improved because of a rise in protein concentration.

Keywords: chlorophyll concentration, foliar fertilization, magnesium nutrition, protein concentration, yield,

4.2 Introduction

As a result of its high content of protein and energy, the faba bean is a valuable component for human and animal nutrition (Crepon et al. 2009). Its protein composition is of good nutritional quality because of its relatively high content of lysine, which is an essential amino acid in human and animal diets. Additionally, the seeds are rich in vitamins and minerals (Crepon et al. 2009). Moreover, faba beans have a positive effect on the cropping system by enriching the soil with N via symbiotic N₂ fixation and by diversifying crop rotation (Jensen et al. 2010).

In order to obtain good quality and a high yield of faba beans, an adequate nutrient supply is needed. Magnesium deficiency reduces faba bean yield (Hariadi and Shabala 2004) and is a widespread problem in agriculture and forestry (Hermans et al. 2004). Not only low soil concentrations, but also environmental conditions affecting the availability of Mg, e.g. low water supply or high concentration of other cations in the soil solution (Marschner 1995), can inhibit sufficient uptake of Mg by crops.

Under deficiency conditions, the supply of magnesium via foliar fertilization can be a solution, since foliar fertilization is known to improve the efficiency and rapidity of nutrient utilization in times of need (Oosterhuis and Weir 2010). Foliar fertilization with MgSO₄ has been shown to increase the chlorophyll content in leaves of maize (Szulc et al. 2001), soybean (Teklic et al. 2009) and oregano (Dordas 2009). In accordance, Neuhaus et al. (2013, *in revision*) have recently found that chlorophyll concentrations and photosynthesis rise following Mg leaf application in fully developed faba bean leaves. Nevertheless, effects on biomass production remain relatively small. We hypothesize that the effect of foliar MgSO₄ application becomes more pronounced during seed development, as the Mg supply is suggested to be especially important at the reproductive stage of plant development (Hariadi and Shabala 2004). Foliar application is known to improve yield in some species in which vegetative material has been harvested (Obatolu 1999; Barlog and Grzebisz 2001; Dordas 2009; Rao and Rajput 2011). In addition, Teklic et al. (2009) have demonstrated an improvement of soy bean seed yield and Vrataric et al. (2006) have reported an effect of foliar application depending on the soybean cultivars. On the contrary, Reinbott and Blevins (1995) and Yang-Yuen et al. (1999) have found no such effect.

Other than its effect on yield quantity, Mg can also have an effect on yield quality (Merhaut 2007). Magnesium is essential for the synthesis of proteins, as it is needed for the aggregation of the ribosomal subunits (Maathuis 2009). Furthermore, the stabilization of RNA is dependent upon Mg, with a further major function of Mg being the activation of glutamine

synthase (Marschner 1995). The latter explains the importance of a Mg supply for the efficiency of nitrogen use, an interrelation that has been reviewed by Grzebisz et al. (2010). In this study, we have therefore investigated the impact of increasing MgSO₄ foliar fertilization rates on Mg-deficient faba beans with special regard to the quantity and quality of the yield as represented by protein concentration.

4.3 Material and Methods

Plant cultivation

Vicia faba L. plants of the cultivar Fuego (NPZ, Hohenlieth, Germany) were grown hydroponically in the greenhouse. After being soaked in 1 mM aerated CaSO₄ solution for one day, seedlings were imbedded into sterile quartz sand. After 7 days, the seedlings were transferred to 4.5 L plastic pots (4 plants per pot) containing one-quarter-strength nutrient solution, which was raised to full concentration in a stepwise fashion. The full nutrient solution had the following composition: 2.0 mM Ca(NO₃)₂, 1.0 mM K₂SO₄, 0.1 mM KH₂PO₄, 0.01 mM NaCl, 10 μM H₃BO₄, 2.0 μM MnSO₄, 0.5 μM ZnSO₄, 0.2 μM CuSO₄, 0.05 μM (NH₄)₆Mo₇O₂₄, 60 μM Fe-EDTA, and was changed every second day to avoid nutrient depletion. Plants were subjected to five different treatments, with each consisting of three independent biological replications. Control plants were supplied with 0.5 mM MgSO₄ over the roots. The remaining four treatment groups were grown in nutrient solution containing only 0.015 mM MgSO₄, of which three treatment groups were subjected to MgSO₄ leaf applications. The leaf application solutions contained 0 mM, 50 mM or 200 mM MgSO₄ and additionally the wetting agent ARMA (0.1% (v/v); Agroplanta, Langenpreising-Zusdorf, Germany). Foliar applications were started after a growing period of four weeks at the beginning of flowering and were replicated three times at one-week intervals. To each pot, 3.5 g of the solution was applied at each time point. After sixty days of growth, leaf samples were taken and, after 100 days, the plants were harvested. Of each plant, the number of pods and the number of seeds per pod was counted. Subsequently, the seeds, pods and vegetative plant material were dried at 30°C and weighed.

Leaf measurements

For each treatment, the 2nd, 12th, 22nd and the youngest leaflet or leaf were sampled and rinsed with deionized water to remove nutrients that might be attached to the leaf surface, e.g. ion contamination that had originated from the leaf spraying procedure and that did not enter the leaf. The leaves were weighed and the relative chlorophyll concentration was measured by

using a portable chlorophyll meter (SPAD-502, Minolta, Japan). Subsequently the leaf area was measured with a leaf area meter (Li-3100; Li-Cor, Inc., Lincoln, Nebraska) and the material was stored frozen for further ion or protein analysis.

Analysis of nutrients and proteins in leaves and seeds

For the measurement of magnesium in leaves and seeds, the material was ground and ashed for 4 h at 550°C. Magnesium, calcium and potassium were then extracted with 4 M nitric acid for 4 h, the nitric acid then being diluted to 0.8 M for measurements with an atomic absorption spectrometer (AAS 5EA Thermo Electron S, Carl Zeiss, Jena, Germany). N and S concentrations were analysed in whole bean flour with a CNS analyser (vario MAX CNS, Hanau, Germany).

The protein concentration in the seeds was determined by using a NanoDrop 1000 spectrophotometer (Thermo Fisher, Delaware, USA) which measured light absorbance at 280 nm. For the extraction of proteins, 0.1 g of whole bean flour was first defatted by using 100% acetone. Proteins were then extracted twice with 1 ml 0.01 M NaOH for 20 min at 4°C. After precipitation with chloroform and methanol, proteins were resuspended in 0.1 M NaOH for measurements.

Statistics

Statistical analysis was carried out by using R (R version 2.11.1, The R foundation for statistical computing, 2010). 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.

4.4 Results

4.4.1 Effect of MgSO₄ application on concentrations of Mg in leaves

Magnesium deficiency treatment resulted in a decline of Mg concentrations in all four sampled leaf stages (Fig. 1a). The Mg fresh weight concentration in the adequately supplied plants (0.5 mM Mg²⁺ over the roots; black bar in Fig. 1a) ranged from a maximum of 0.68 mg Mg g⁻¹ in the oldest leaves (2nd leaf) to a minimum of 0.34 mg Mg g⁻¹ in the youngest leaves, whereas Mg concentrations in the deficiency treatment (0.015 mM Mg²⁺ over the root, no leaf application; white bar in Fig. 1a) were 0.11 mg Mg g⁻¹ and 0.16 mg Mg g⁻¹, respectively. Leaf application of 50 mM MgSO₄ resulted in a slight increment of Mg concentrations in the older leaves but did not lead to statistically significant mean differences. In contrast, leaf

application of 200 mM MgSO₄ resulted in an increase of the Mg concentration at leaf stages 2 and 12, with the same concentration level being attained as in adequately (0.5 mM Mg over root) supplied plants. However, this effect did not occur in the younger leaves.

4.4.2 Influence of MgSO₄ foliar application on chlorophyll concentration

In response to Mg deficiency, the chlorophyll concentration decreased significantly in the 2nd and 12th leaf stage, whereas in the younger leaves, no effect could be observed (Fig. 1b). In the 2nd leaf stage, the Mg foliar application did not affect the chlorophyll concentration but a significant increase in the leaves of the 12th stage could be achieved by application of 50 mM and 200 mM MgSO₄ over the leaves. This pronounced effect of foliar application was also readily visible (Fig. 2).

4.4.3 Influence of MgSO₄ folia application on leaf fresh weight

In the oldest leaves, fresh weight was not affected by any treatment. The younger the leaves, the more pronounced became the differences in fresh weight between the adequately supplied plants and the plants growing under Mg deficiency conditions. Significant differences developed in the 22nd and the youngest leaves. Foliar application of MgSO₄ did not significantly affect the fresh weight of the leaves (Fig. 1c).

4.4.4 Influence of MgSO₄ foliar applications on seed ions and proteins

The magnesium concentration in the seeds was significantly reduced in response to the magnesium deficiency treatment. The application of MgSO₄ to the leaves did not affect the seed Mg concentration (Fig. 3a) or the Ca and K concentrations in the seeds (Supplement figure 1). The sulfur concentration in the seeds was significantly reduced by the MgSO₄ deficiency treatment. In the variants grown in low-Mg nutrient solution, 0.18-0.2% S was found compared with 0.24% S in seeds with adequate MgSO₄ in the nutrient solution. A supply of 200 mM MgSO₄ to the leaves led to a slight increment of S in the seeds (Fig. 3b). The N concentration in the seeds decreased from 3.2% under a high-Mg supply to 2.7% under a low-Mg supply. In response to the 200 mM MgSO₄ leaf application treatment, we found a slightly higher N concentration in the seeds compared with those under deficiency conditions (Fig. 3c). Concomitantly, the protein concentration decreased in the seeds under Mg deficiency and the foliar application of 200 mM MgSO₄ led to a significant increase in protein concentration (Fig. 3d).

4.4.5 Influence of MgSO₄ foliar applications on yield parameters

Foliar MgSO₄ application increased the yield per Mitscherlich pot, being significantly raised by the application of 200 mM MgSO₄. In low-Mg treatments, only 7-8 g was harvested, compared with 15 g in treatments receiving 200 mM over the leaves and 31 g in well-supplied plants (Fig. 4). Neither the deficiency treatment nor the foliar application of MgSO₄ affected the number of seeds per pod (Fig. 4b) but the single seed weight responded to the application of MgSO₄, being 0.34-0.43 g in the deficiency treatments and 0.52-0.54 g in the treatments receiving foliar 50 or 200 mM MgSO₄ (Fig. 4c). The application of 50 or 200 mM MgSO₄ also significantly raised the number of pods per plant (Fig. 4d).

4.4.6 Formation of shoot and root dry weight in response to magnesium treatment

Both shoot and root dry matter were significantly reduced in plants growing in low-Mg nutrient solution compared with plants adequately supplied with Mg (Fig. 5). The root dry matter was about 68% lower, whereas the shoot dry matter was reduced about 40%. In both root and shoot, foliar application of 200 mM MgSO₄ led to a significant increase of dry matter of up to 5.2 g and 27.5 g, respectively, compared with 2.8 g and 19.3 g in the low-Mg treatment.

4.5 Discussion

4.5.1 Impact of increasing foliar Mg supply on Mg and chlorophyll concentrations in vegetative tissue

In this study, we investigated the effect of increasing Mg foliar application rates on Mg concentration, chlorophyll concentration and biomass formation. Mg deficiency became most pronounced in the oldest leaves in which the Mg concentration and chlorophyll concentration were lowest (Figs 1 and 2). The decrease in Mg concentration in the Mg-deficient plants compared with the well supplied plants was significant in all leaf stages but declined with leaf age, being 84%, 64%, 48% and 31% in the 2nd, 12th, 22nd and the youngest leaf stage, respectively (Fig. 1a). This effect is attributable, on the one hand, to a remobilization of Mg from the older leaves into the growing tissues, since Mg is a phloem mobile plant nutrient (Marschner 1995) and, on the other hand, to a decline in the fresh weight in the plants under magnesium deficiency treatment, and becomes more pronounced in the younger the leaves (Fig.1c). Moreover, the magnesium concentration declines from older to younger leaves in plants growing in a high-Mg nutrient solution. This is in accordance with Fischer (1998) and

Elamin and Wilcox (1985) who have also observed this changed relationship of magnesium concentration in older versus younger leaves.

MgSO₄ foliar application (200 mM) increases the Mg leaf concentration in the 2nd and 12th leaf stages significantly but has no effect on the Mg concentration in younger leaves. The uptake of foliar-applied Mg becomes most pronounced in the 12th leaf stage in which the chlorophyll concentration increases simultaneously (Fig. 1b). These measurements agree well with the visually observed amelioration of the Mg nutritional status (Fig. 2). In general, the breakdown of chlorophyll under magnesium deficiency is believed to be connected to the accumulation of sugars and starch in the cells of deficient leaves (Hermans and Verbruggen 2005; Cakmak and Kirkby 2008). This causes an over-reduction of the photosynthetic electron transport chain, which leads to the formation of reactive oxygen species and therewith to the destruction of the chlorophyll (Cakmak and Kirkby 2008). In this context, the increased destruction of the D1 protein in photosystem II has also been proposed not to be compensated in Mg-deficient plants, because of the lack of Mg needed for protein synthesis (Fischer 1998). The higher chlorophyll concentration in leaves supplied with 50 or 200 mM Mg via the leaves (Fig. 1) therefore possibly leads to an enhanced translocation of sugars and concomitantly to a reduced destruction of chlorophyll. These results show that the foliar-applied Mg is taken up by the leaves and becomes physiologically available, as recently confirmed by measuring Mg concentrations in the symplast of plants that have been supplied with Mg over the leaf (Neuhaus *et al.*, in revision). Nevertheless, the absence of an effect of the foliar application on the younger leaves with regards to the magnesium concentration and the fresh weight indicates that the Mg taken up is not transported to the younger leaves. Curiously, the chlorophyll concentration is stable in young leaves that suffer under Mg deficiency (Fig. 1b). However, this can be explained by a dilution effect. The reduction in fresh weight leads to an increased concentration of chlorophyll.

4.5.2 Seed nutrient and protein concentration in response to MgSO₄ foliar application

The magnesium concentration in the seeds is significantly reduced under magnesium deficiency treatment (Fig. 3a). However, the concentrations still lie in the range reported as normal in the literature (Hill-Cottingham 1983; Jensen *et al.* 2010). Although Mg is known to be a phloem mobile mineral nutrient, the foliar-applied Mg is not translocated from the older leaves into the younger leaves and does not elevate the Mg concentration in the seeds. However, because of the increased seed yield, the total seed Mg is increased. This might be attributable to an enhanced translocation of Mg toward the reproductive organs. The relatively

low yield nevertheless shows that the foliar-applied Mg doses were too small, to balance the low supply by the roots.

With regard to N and S in the seeds, not only the total content, but also the N and S concentration rises under the influence of a 200 mM application (Figs 3 b and c). This is reflected by the protein concentrations in the seeds, which also significantly increases (Fig. 3d). Furthermore, Kiss and Poszar (1975) and Vrataric et al. (2006) have found a higher protein content in the leaves and seeds when plants are supplied with MgSO₄ via leaf application. Kiss and Poszar (1975) have explained this effect on the basis that magnesium maintains the ribosomal structure and thus amino acid synthesis. In agreement with this, Ruan et al. (2012) have assumed protein biosynthesis to be inhibited, as they have measured an accumulation of free amino acids in Mg-deficient tea leaves. This has not been confirmed here, as the N and protein concentrations in the seeds increase in a linear relationship in response to an increasing supply of Mg (Supplement figure 2). The reduction of protein content in the seeds might therefore be attributable to a reduced translocation of amino acids to the seeds (see below).

As we have applied not only Mg²⁺, but also SO₄²⁻, we should consider that S fertilization might also have an effect on yield and protein concentration. However, the S concentration in the shoot is not significantly reduced in the plants under deficiency treatment compared with the adequately supplied plants (Supplement figure 3). Further, leaf S concentrations are higher than the critical levels for deficiency of 0.08 to 0.16% reported for various plant species (Hitsuda et al. 2005). Moreover, an additional S supply to plants that are adequately supplied with S is known to have no quantitative effects on protein concentration (Zörb et al. 2009; 2010).

4.5.3 Foliar application ameliorates yield and increases number of pods in faba bean grown under Mg-deficient conditions

The yield depression caused by magnesium deficiency is significantly ameliorated by the foliar application of 200 mM MgSO₄ (Fig. 4a). This amelioration is mainly attributable to the increase in the number of pods, which correlates the strongest to the yield (Fig. 4d). These findings agree well with published data in which the number of pods is often the most important determinant of seed yield under various conditions (Karamanos and Gimenez 1991; Stoddard 1993; Nachi and Le Guen 1996; Lopez-Bellido et al. 2005). In addition, a significant rise is also observed in the mean seed weight, whereas the number of seeds does not change significantly (Figs 4 b and c). The number of seeds per pod and the mean seed

weight are largely determined by the genotype (Lopez-Bellido et al. 2005); hence, these values are more stable and are not as greatly reduced by environmental stresses as the number of pods. Nevertheless, the mean seed weight is the last yield component to be determined and can partly compensate for pod loss and thus increase yield significantly (Lopez-Bellido et al. 2005).

As the number of pods is highly variable, Stoddard (1993) has highlighted the importance of the supply of assimilates at the time of pod setting for the development of yield. In this context, Mg plays an important role. The translocation of amino acids and sugars from sink to source might be inhibited under magnesium deficiency because of the effect of Mg on the H⁺-ATPase (Cakmak and Kirkby 2008). Mg-ATP is the direct substrate for the H⁺-ATPase and is thus indispensable for establishing the electrochemical H⁺ gradient across the plasma membrane, the driving force for sugar and amino acid H⁺-co-transport and thus translocation via the phloem (Cakmak and Kirkby 2008; Patrick and Stoddard 2010). Additionally the enhanced chlorophyll concentration under magnesium deficiency might lead to an increased photosynthesis rate and provision of developing pods with assimilates.

4.6 Conclusions

Foliar application of 200 mM MgSO₄ significantly raises the yield of faba beans grown under suboptimal Mg supply, an effect possibly contributable to rises in the Mg and chlorophyll concentration of leaves directly subjected to the foliar treatment. The yield trait that responds most is the number of pods per plant, assumedly because of a higher concentration of chlorophyll that allows for a higher photosynthesis rate and therewith a higher supply of assimilates. The magnesium content of the seeds is not influenced by foliar MgSO₄ application but the seeds of plants treated with 200 mM MgSO₄ are significantly higher in protein concentration than seeds from untreated plants. This might be explained by the better translocation of amino acids via the phloem in plants with a higher Mg concentration in their vegetative tissue.

Acknowledgements

C. Neuhaus is grateful for the receipt of a Kiel University federal state grant.

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Figures

Fig. 1

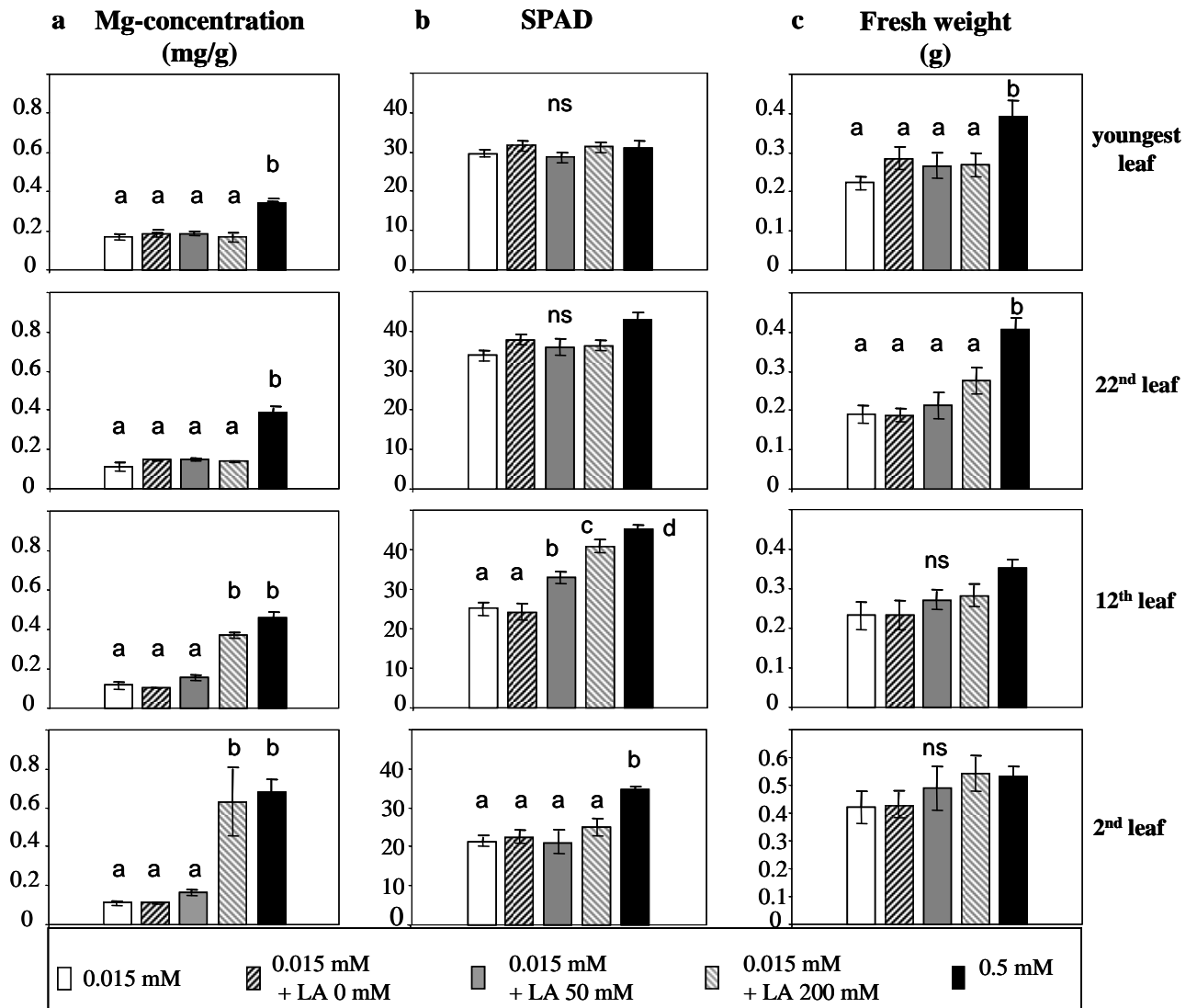


Fig. 2

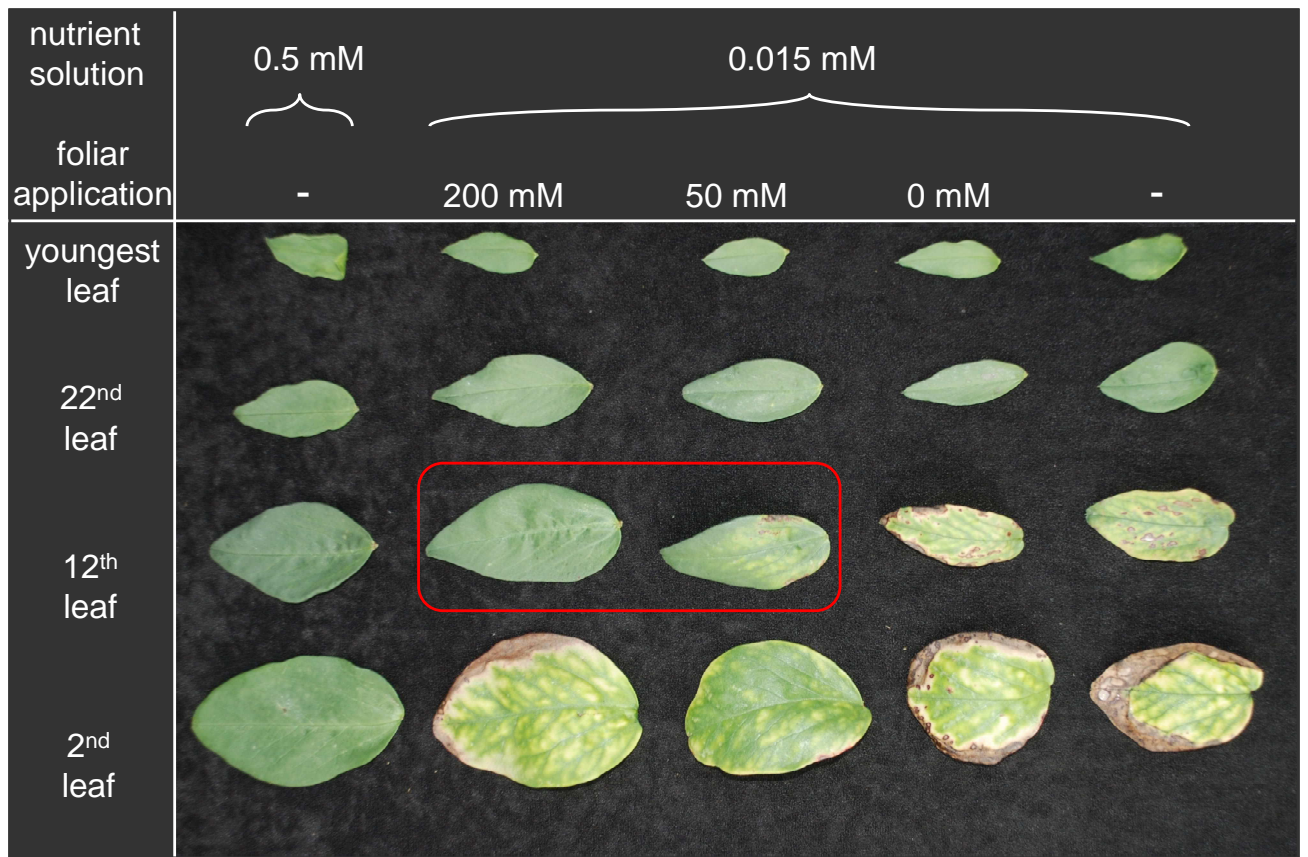


Fig. 3

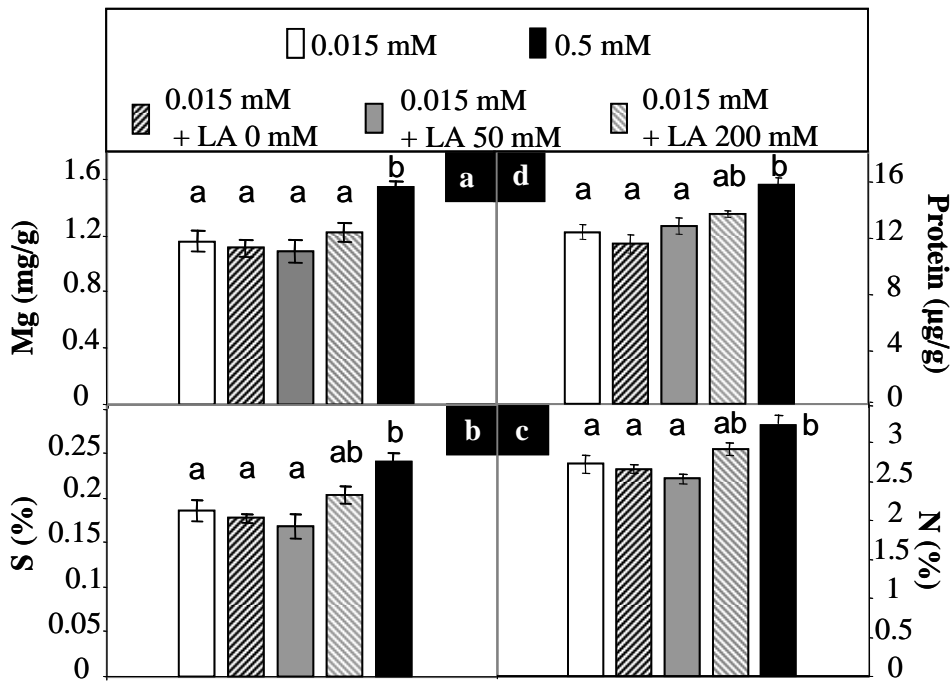


Fig. 4

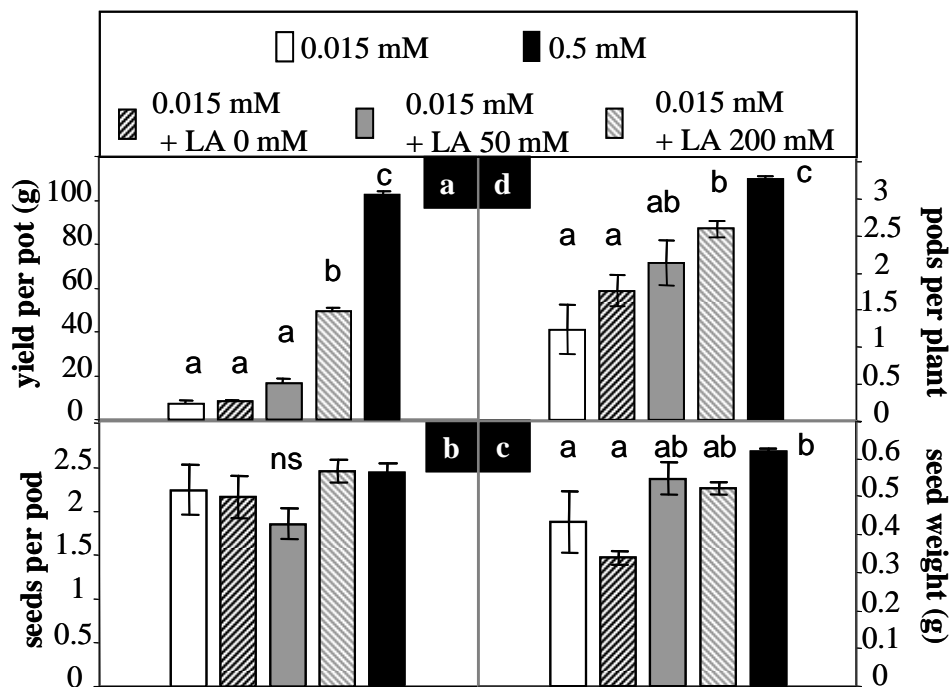


Fig. 5

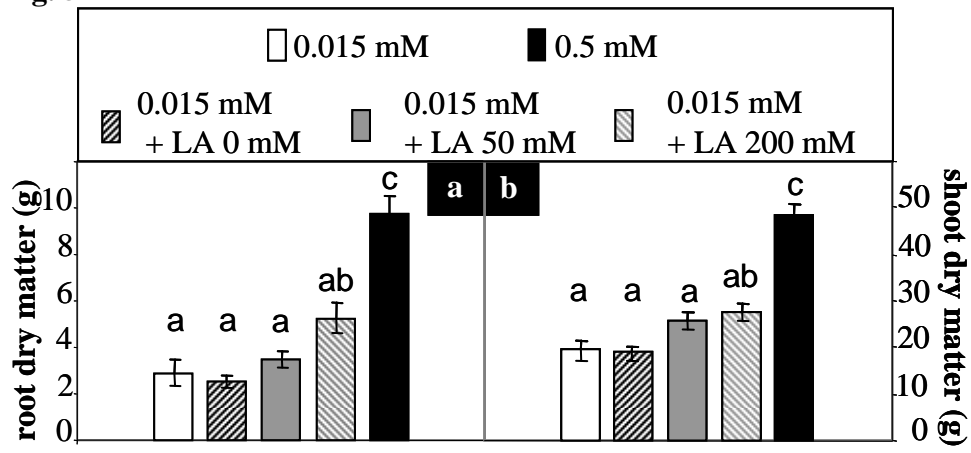


Figure legends

Fig. 1: Impact of MgSO₄ foliar application on the magnesium concentration (mg/g) in the fresh weight (a), the chlorophyll concentration expressed as SPAD-values (b) and the fresh weight (c). Samples were taken in the 2nd, 12th, 22nd and the youngest leaves of plants. a, b, c: significant differences ($p \leq 0.05$) between treatments, LA: leaf application (with the MgSO₄ concentration of the solution being 0 mM, 50 mM or 200 mM), error bars represent mean \pm SE of four independent replications

Fig. 2: Representative leaves of different ages showing magnesium deficiency symptoms in response to the concentrations in the nutrient solution (0.5 mM or 0.015 mM MgSO₄) and foliar application (0 mM, 50 mM or 200 mM MgSO₄). The *red box* accentuates 12th leaves of plants supplied with 50 or 200 mM MgSO₄ in which the chlorophyll concentrations were raised. LA: leaf application (with the MgSO₄ concentration of the solution being 0 mM, 50 mM or 200 mM)

Fig. 3: Seed concentration of (a) magnesium (mg/g), (b) sulfur (%), (c) nitrogen (%) and (d) proteins in response to magnesium deficiency and foliar MgSO₄ application. a, b, c: significant differences ($p \leq 0.05$) between treatments, LA: leaf application (with the MgSO₄ concentration of the solution being 0 mM, 50 mM or 200 mM), error bars represent mean \pm SE of four independent replications

Fig. 4: Impact of magnesium deficiency and foliar MgSO₄ application on (a) the seed yield per pot (g), (b) the number of seeds per pod, (c) the seed weight (g) and (d) the number of pods per plant. a, b, c: significant differences ($p \leq 0.05$) between treatments, LA: leaf application (with the MgSO₄ concentration of the solution being 0 mM, 50 mM or 200 mM), error bars represent \pm SE of four independent replications

Fig. 5: Root (a) and shoot (b) dry matter formation influenced by MgSO₄ supplied to the roots and the leaves. a, b, c: significant differences ($p \leq 0.05$) between treatments, LA: leaf application (with the MgSO₄ concentration of the solution being 0 mM, 50 mM or 200 mM), error bars represent mean \pm SE of four independent replications

Supplemental material

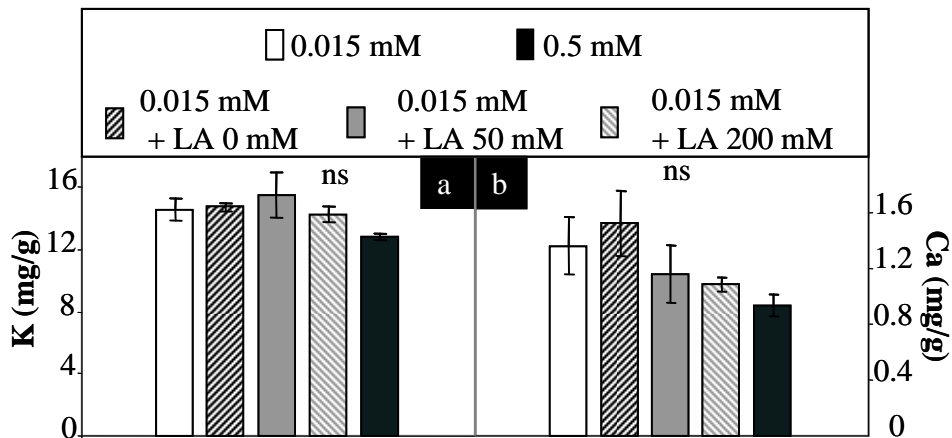


Figure 1

Seed concentrations of (a) potassium (mg/g) and (b) calcium (mg/g). ns: no significant differences ($p \leq 0.05$) between treatments, LA: leaf application (with the MgSO₄ concentration of the solution being 0 mM, 50 mM or 200 mM), error bars represent mean \pm SE of four independent replications

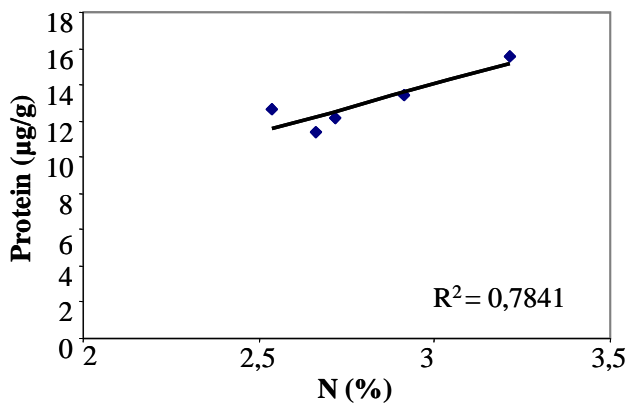


Figure 2

Protein concentration (μ g/g) in relation to the N concentration (%) in the seeds

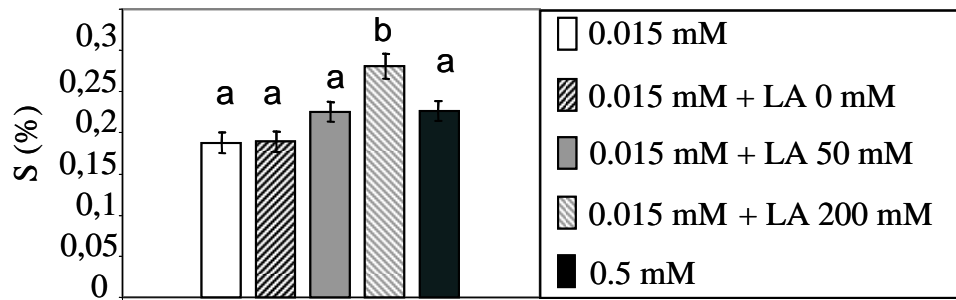


Figure 3

Impact of magnesium deficiency and foliar MgSO₄ application on the sulphur concentration (%) of the vegetative tissue. a, b, c: significant differences ($p \leq 0.05$) between treatments, LA: leaf application (with the MgSO₄ concentration of the solution being 0 mM, 50 mM or 200 mM), error bars represent \pm SE of four independent replications

Chapter 5

General Discussion

5. General discussion

Magnesium deficiency leads to growth reduction and reduced yield in crops. One possibility to supply magnesium is the application via the leaves. As the leaves are not the organs specialized on the uptake of high amounts of nutrients, the supply of foliar fertilizers might cause physiological perturbations in relation to the ionic composition of different plant compartments. This in turn, might affect on processes such as the gene transcription, the development of deficiency symptoms and the biomass accumulation.

In this thesis, some aspects about the influence of foliar application of MgSO_4 on ion relations, gene transcription of enzymes that depend on the supply of magnesium in the leaves and yield formation, were studied. Therefore, plants were grown in nutrient solution containing suboptimal levels of magnesium and subjected to foliar MgSO_4 applications of different concentrations (0 mM, 50 mM, 200 mM). These were compared to control plants growing in either sufficient or suboptimal magnesium levels in the nutrient solution, which received no foliar fertilization.

5.1. Response of *Vicia faba* to magnesium deficiency with regards to ion relations

As expected, the deficiency treatment reduced the magnesium concentrations in the old leaves of plants significantly, down to $0.6 \text{ mg g}^{-1} \text{ Mg}$ in the dry matter. Concomitantly, severe deficiency symptoms developed in these leaves. In those plants with sufficient magnesium supply, $5.2 \text{ mg g}^{-1} \text{ Mg}$ were found (chapter 3, fig. 1d).

The magnesium concentration sufficient for optimal growth varies with species, e.g. Beringer and Forster (1981) reported $1.5\text{-}2.0 \text{ mg g}^{-1}$ in the dry matter to be sufficient for barley and Mehne-Jakobs and Gülpen (1997) found $0.9\text{-}1.7 \text{ mg g}^{-1}$ sufficient for Norway spruce. However, the results obtained in this study agree well with the general threshold line for the occurrence of magnesium deficiency determined by Kirkby and Mengel (1976) of about $2 \text{ mg g}^{-1} \text{ Mg}$ in the dry matter, as magnesium in the leaves of sufficiently supplied plants always ranged above this value (chapter 5, fig. 1a).

It is a known phenomenon, that the amount of a cation supplied to the plant does not only influence the uptake and concentration of the cation itself, but also the concentration of other cations. This effect has often been shown for high concentrations of K^+ in the nutrient solution, influencing the uptake of Ca^{2+} or Mg^{2+} (e.g., Ologunde and Sorensen 1982; Spear et al. 1987; Miller 1999; Troyanos et al. 2000). High Mg^{2+} supply has also been shown to

change Ca^{2+} and K^+ concentrations (Ologunde and Sorensen 1982; Grant et al. 1988; Troyanos et al. 2000).

With regards to low supply of magnesium, results reported in literature are contrasting. magnesium deficiency was shown to cause a concomitant rise of Ca^{2+} and K^+ (Fischer and Bussler 1988; Hermans et al. 2004; Hermans et al. 2010). This differs from results obtained by Cai et al. (2012) and Fischer and Bremer (1993) as well as from the results obtained in this study, in which alterations in leaf Ca^{2+} and K^+ have not been observed (chapter 2, fig 3).

These differing results may be attributable to differences in the experimental setup. For instance, Hariadi and Shabala (2004) showed that effects on Ca^{2+} and K^+ were dependent on the severity of the magnesium deficiency and to sampling time. In their study, the effects on Ca^{2+} and K^+ concentrations in leaves of broad bean were induced in magnesium-deficient plants at early sampling times, but at later sampling times this effect prevailed only in severely deficient plants. Another reason might be the use of different ion ratios, Shewmaker et al. (2008) and Narwal et al. (1985) observed that Mg^{2+} could have synergistic, but also antagonistic effects on the uptake of K^+ , depending on the Mg^{2+} concentration, and vice versa. Additionally, considerable variation in ion content can also occur due to differences in species (Broadley et al. 2008).

5.2 Impact of magnesium foliar application on leaf and seed ion concentrations

In this study, an extraction procedure was used, that allowed for the discrimination of the ion concentrations in 3 fractions (Mühling and Sattelmacher 1995). These were: ions bound to the fixed anions in the apoplast, free ions in the apoplast and ions in the symplast. This discrimination was introduced, because ion concentrations in these compartments differ significantly, which is due to the specific functions of the cations. For instance, the cytosolic Ca^{2+} concentration is low (100-200 nM), because it acts as a second messenger in signal transduction (Marschner 1995). In contrast, high concentrations of Ca^{2+} are found in the apoplast, as it functions in bridging the pectins of the cell wall, thereby ensuring cell wall stability (Grignon and Sentenac 1991). Also Mg^{2+} binds to these apoplastic negative charges. Therefore the foliar MgSO_4 application might also lead to a fixation of Mg^{2+} in the apoplast, rather than to an uptake into the symplast. The differentiation between the apoplastic and the symplastic fraction, realized in this study, showed that in both the apoplast and the symplast, the Mg^{2+} concentration was significantly raised (chapter 2, fig. 3). Concomitantly, the concentration of Ca^{2+} , bound to the negative apoplastic charges, was significantly reduced and in the symplast, the concentration of K^+ significantly decreased. The removal of Ca^{2+} might

lead to a solubilization of the pectins (Grignon and Sentenac 1991) and the decrease of K^+ might affect physiological processes such as enzyme activation, protein synthesis or on water homeostasis which depend upon K^+ (Maathuis 2009), this displacement of the cation relations might have detrimental effects. However, the increases in seed yield and plant biomass gained by foliar application (chapter 4, figs 4 and 5), showed that these effects on the cation relations are not adverse. Furthermore, the Ca^{2+} and K^+ concentrations in the seeds were not significantly affected (chapter 4, supplemental material 1). The exchange of one cation by another when the supply varied has also been observed in other investigations (Bedi and Sekhon 1977; Ologunde and Sorensen 1982; Ding et al. 2006) and led to the assumption, that in part, they can replace each other in their functions. However, limitations of this conclusion result from the fact that experiments on cation concentrations in the leaves and on yield were conducted independently, and different concentrations in the nutrient solutions were used (0.05 and 0.015 mM $MgSO_4$).

Instead of a discrimination in leaf fractions, in the second chapter, a discrimination between leaf age was introduced. This revealed that the foliar application did not lead to a rise in magnesium in the younger leaves. This is astonishing, as magnesium is phloem mobile and therefore, translocated from the older leaves to the young developing organs. However, foliar application could raise the seed yield and therewith the total seed magnesium. This might indicate an enhanced translocation of magnesium, from the old leaves during the development of the reproductive organs and possibly also underlines the observation made by Hariadi and Shabala (2004) that magnesium becomes crucial at the reproductive stage of plant development.

5.3 Influence of deficiency treatment on biomass, chlorophyll and photosynthesis

Due to the magnesium deficiency treatment (0.015 mM $MgSO_4$ in the nutrient solution), a reduction in dry matter could be observed. Both the root and the shoot dry matter were reduced to 68% and 40% respectively (chapter 4, fig. 5). Nutrient deficiencies often result in changed biomass allocation to roots and shoots (Hermans et al. 2006). Inhibition of assimilate transport reduces root growth, as under magnesium deficiency, but the perpetuation of root growth is beneficial, as it ameliorates the nutrient acquisition. Under magnesium deficiency, plants showed differential reactions with regards to the root and shoot growth. Cakmak et al. (1994) showed on *Phaseolus vulgaris*, that root growth declined before any reaction of shoot growth, whereas Ding et al. (2006) observed only a reduction in shoot growth of *Oryza sativa*. Also, Hermans et al. showed a more pronounced decrease of shoot fresh weight compared

with root fresh weight in *Beta vulgaris* (2004) and *Arabidopsis thaliana* (2005), whereas Ruan et al. (2011) showed that the root to shoot ratio remained unaffected in *Camellia sinensis*. Differences in the plants reaction to magnesium deficiency might be attributable to the species, but also to the varying growth conditions with respect to the onset and severance of magnesium deficiency.

The decreased formation of dry matter under magnesium deficiency can be explained by the reduced chlorophyll concentration (chapter 3, fig. 1b) and the reduced photosynthesis rate of magnesium deficient plants (chapter 3, fig. 1c). Loss of chlorophyll and photosynthesis rate under magnesium deficiency have also been reported in other plant species (e.g. Fischer and Bremer 1993; Hermans et al. 2004; Laing et al. 2000). The causes for these reductions in turn have been under debate. It is known that the reduction of chlorophyll is not due to a deficit of magnesium at the chloroplasts (at least at the beginning deficiency), as the relative amount of magnesium associated to chlorophyll rises with decreasing total magnesium concentration (Marschner 1995). The reduction in photosynthesis rate seems to be associated with several factors. As sugars and starch accumulate under magnesium deficiency, photosynthesis is suggested to be reduced due to negative feedback inhibition (Cakmak and Kirkby 2008). Due to the accumulation of photoassimilates, the photosynthetic electron transport chain becomes over-reduced, which leads to the formation of reactive oxygen species. These are highly reactive and lead to the degradation of proteins and pigments. Fischer (1998) suggests that the turnover of the D1 protein in the photosystem II might play an important role. The D1 protein is degraded, due to the reduction of the plastochinones. It cannot however, be resynthesized in an adequate amount under magnesium deficiency, as the synthesis of proteins is dependent upon the adequate supply of magnesium. A reduced stomatal conductance has also been associated with the reduced photosynthesis (Hariadi and Shabala 2004), though this was not always the case (Lasa et al. 2000; Yang et al. 2012). An additional factor might be the activity of RuBP carboxylase, which is highly influenced by magnesium (Portis et al. 1992).

Additionally, it has been shown that under magnesium deficiency the transcription of chlorophyll associated proteins is reduced. This was shown for several chlorophyll A/B binding proteins and light harvesting chlorophyll-protein complexes I & II (Hermans et al. 2010). In this study, it was demonstrated that under magnesium deficiency, the transcription of Mg-chelatase subunit H is also reduced (chapter 3, fig. 2). This enzyme is essential for the synthesis of chlorophyll, as it incorporates the Mg^{2+} into the chlorophyll precursor molecule and is supposed to have a regulatory function in the chlorophyll metabolism (Masuda 2008).

Furthermore, the transcriptional control of Mg-chelatase subunit H is a key regulatory point of Mg-chelatase activity (Masuda 2008). Therefore the reduced transcript abundance of Mg chelatase H could contribute to the lower chlorophyll concentration under magnesium deficiency.

The expression of chlorophyll A/B binding protein 2 is already reduced before the development of visual deficiency symptoms (Hermans and Verbruggen 2005). This was also the case for Mg chelatase H, which was already downregulated in the young leaves that were still green and contained a higher amount of magnesium relative to the old leaves (chapter 3, fig. 2).

As stated above, the appearance of chlorosis under magnesium deficiency is related to the accumulation of sugars and starch in the leaves. The reason for this is assumed to be an impaired activity of the PM H⁺-ATPase (Hermans et al. 2006; Cakmak and Kirkby 2008). The PM H⁺-ATPase pumps H⁺ into the apoplast, creating an electrochemical gradient that provides the driving force for solutes to enter the cells (Palmgren 2001). This is also needed for the uptake of sugars in the phloem of *Vicia faba*, which is conducted via H⁺ symport (Peat 1983). The activity of PM H⁺-ATPase depends on the supply of magnesium as its substrate is Mg-ATP (Palmgren 2001).

An analysis of the transcription of different PM H⁺-ATPase isoforms revealed that under magnesium deficiency the isogenes *vha1* and *vha5*, which belong to the same family of PM H⁺-ATPases, are downregulated in their abundance (chapter 3, fig. 4). Though these data have to be interpreted with care, as they do not reflect the actual activity of the PM H⁺-ATPase, this reduced transcript abundance might contribute to a reduced export capacity of carbohydrates in plants under magnesium deficiency. Contrarily, this is not true for the third PM H⁺-ATPase isoform, which was measured in this study. Isoform *vha4* belongs to a different subgroup and its transcription was upregulated in response to magnesium deficiency (chapter 3, fig. 4). It was not yet possible to assign a specific function to one of the subgroups, likewise their expression could not be connected to a specific tissue. But the higher transcription of isoform *vha4* could be an attempt of the plant to cope with the low supply of magnesium, as it has been suggested that H⁺-ATPases can display differences in H⁺/ATP ratios which might capacitate them to adapt to differential environmental conditions (Arango et al. 2003).

5.4 Chlorophyll, photosynthesis and biomass as influenced by foliar MgSO₄ application

Foliar application of magnesium led to increased chlorophyll concentrations in leaves (chapter 4, fig. 1). This effect is similar the observations of other authors in oregano (Dordas 2009),

soybean (Teklic et al. 2009), maize (Szulc et al. 2001) and apples (Reay et al. 1998). In this study it was shown that this effect depends on the leaf age. In 2nd leaves no influence of magnesium foliar application was measurable, although the magnesium concentration was significantly higher under application of 200 mM Mg (chapter 4, figs 1a and b). This may be due to beginning senescence processes, since these leaves already began to show deficiency symptoms at the time the foliar application was started. The chlorophyll concentration in the youngest leaves, in contrast, was not significantly affected by any treatment, as the lower total amount of chlorophyll is balanced by the reduced biomass (chapter 4, fig. 1b and c). In the 5th leaves (chapter 3, fig. 1b) and 12th leaves (chapter 4, fig. 1b) foliar application could significantly raise the chlorophyll concentration. Concomitantly, the photosynthesis rate was increased (chapter 3, fig. 1c).

A reason for higher chlorophyll concentrations under magnesium foliar application could be an enhanced production of chlorophyll and chlorophyll associated proteins. Our measurements of the transcript abundance of *Mg chelatase H* do not support this idea, as no influence of magnesium foliar application on the transcription of this gene could be observed (chapter 3, fig. 2). It may therefore be possible that although the synthesis of chlorophyll is still reduced, the degradation of the chlorophyll due to the loss of magnesium for protein synthesis or assimilate export, is eased. Of course, this relation will need further elucidation, as we did not investigate on the transcription and activity of other genes such as chlorophyll binding proteins A/B.

Despite the enhanced chlorophyll concentration and the rise in photosynthesis rate the biomass accumulation was only slightly increased. Interestingly, foliar application also led to an enhanced root growth (chapter 4, fig. 5). This might indicate a risen translocation of sugars and might lead to an ameliorated nutrient acquisition.

5.5 Impact of foliar MgSO₄ application on yield of *Vicia faba*

The yield depression due to magnesium deficiency was significantly ameliorated by foliar application of 200 mM MgSO₄. This rise in yield was mainly due to the enhanced number of pods, which responded the most to the treatment (chapter 4, fig. 4). This conforms well with published data, in which the number of pods often is the most important determinant of seed yield under various conditions (Karamanos and Gimenez 1991; Stoddard 1993; Nachi and Le Guen 1996; Lopez-Bellido et al. 2005). Additionally, the single seed weight was significantly raised under the influence of foliar application, whereas the number of seeds per pod remained stable (chapter 4, fig. 4). The number of seeds per pod and the mean seed weight are

largely determined by the genotype (Lopez-Bellido et al. 2005) for that cause they are more stable and not reduced as much by environmental stresses as the number of pods. Nevertheless, the mean seed weight is the last yield component to be determined and it can partly compensate for pod loss and increase yield significantly (Lopez-Bellido et al. 2005).

As the number of pods is varies greatly, Stoddard (1993) highlights the importance of assimilate supply at the time of pod setting for the development of yield. The application of MgSO_4 might have led to an enhanced production of assimilates, as the chlorophyll concentration in some leaves was raised, and concomitantly the photosynthesis rate was enhanced. Additionally, as aforementioned the translocation of assimilates, as well as amino acids, was possibly ameliorated by an enhanced supply of magnesium (Cakmak and Kirkby 2008; Patrick and Stoddard 2010).

Besides the positive effect on seed yield, the quality of the seeds was also affected by foliar MgSO_4 application. Faba bean is a valuable component of human and animal nutrition, as its seeds provide high contents of protein and energy. Due to the relatively high contents of lysine, an essential amino acid in human and animal diet, its protein is of good nutritional quality. In response to the 200 mM MgSO_4 foliar application the protein concentration of the seeds could be significantly raised (chapter 4, fig. 3d), as compared to the deficiency treatment. Kiss and Poszar (1975) also found a higher protein content in leaves and seeds in plants subjected to magnesium foliar application. They explained this effect with the role of magnesium in maintaining the ribosomal structure and amino acid synthesis. It has also been suggested that sugars delivered from the shoots to the roots might act as signals and regulate the expression of nitrate transporters (Peuke 2010). An enhanced assimilation and transport of sugars, due to the foliar supply of magnesium, could therefore induce a higher uptake of nitrate.

5.6 Evaluation of magnesium foliar application

Not only the suboptimal availability of magnesium, but also high magnesium concentration in plant tissues has been reported to cause a reduction in plant biomass (Rao et al. 1987) or chlorophyll content (Hariadi and Shabala 2004; Ding et al. 2006). Nevertheless, the application of high salt concentrations is needed to obtain sufficient uptake of the applied nutrient. This is also indicated by our experiments in which the application of 50 mM MgSO_4 did not raise the magnesium concentrations significantly in deficient plants and only led to a slight increase in chlorophyll concentration and photosynthesis rate (chapter 3, fig. 1). The application of 200 mM MgSO_4 yielded better results with regards to the parameters mentioned

and it was possible to increase the seed yield significantly. Additionally, negative effects of the 200 mM MgSO₄ application could not be observed. Nevertheless, the foliar application of MgSO₄ did not compensate entirely for the low magnesium concentration in the nutrient solution, as indicated by the seed yield (chapter 4, fig. 4). As mentioned above, by application of foliar MgSO₄, it may also be possible to raise the quality of vegetable food. magnesium is involved in enzymatic reactions involved in the energy, protein and fatty acid metabolism (Broadley et al. 2008), and foliar application has proved to raise the oil concentration in soybean (Vrataric et al. 2006), the sugar concentration in sugar beet (Kristek 2000) and the protein concentration in soybean (Vrataric et al. 2006) and faba bean (chapter 4, fig. 3). Besides this, foliar application of MgSO₄ is a possibility to raise the concentration of magnesium in vegetable foods. Biofortification with magnesium could become an important issue with regards to the decreasing human daily magnesium intake, which is already below the recommended amount (Grzebisz 2011). These experiments indicated that this could be especially valuable for crops whose vegetative parts are harvested, as the magnesium concentration could only be raised in the plant parts directly subjected to the application and did not raise the magnesium concentrations in the seeds (chapter 4, figs 1 and 3).

As it has been suggested earlier (e. g. Oosterhuis and Weir 2010), foliar fertilization could be beneficial if water supply or nutrient uptake is limited. Here, foliar magnesium fertilization led to enhanced root growth (chapter 4, fig. 5) and therefore might not only ameliorate the provision with magnesium, but also might capacitate plants to ameliorate water and nutrient acquisition.

This study demonstrates that magnesium deficiency in plants can be relieved by foliar MgSO₄ application with regards to the vegetative and generative growth and leads to higher protein concentrations in the seeds.

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Chapter 6

Summary

6.1 Summary

Being a macronutrient with essential functions in plant metabolism, a sufficient supply of magnesium to crops is important to gain high yields and quality. In agriculture and forestry magnesium deficiency has become a problem in some areas. Besides low soil concentrations especially in light and sandy soils, also other parameters such as high concentrations of Ca^{2+} , K^+ , H^+ , Al^{3+} or low soil humidity can lead to suboptimal provision of magnesium to crops. This will lead among other things, to the formation of chlorosis in the older leaves, reduced growth and the accumulation of sugars in source leaves. Instead of soil amendment, magnesium can also be applied via foliar fertilization. This can be valuable if the magnesium soil availability of magnesium is reduced or if a rapid improvement of the plants nutrient status is needed.

Magnesium foliar application has been demonstrated to improve chlorophyll concentration and biomass accumulation in oregano, maize, soybean and apples (Reay et al. 1998; Szulc et al. 2001; Dordas 2009; Teklic et al. 2009). With regards to responses of yield and quality, however, studies present differential results. The impact of foliar MgSO_4 application on plant physiology has also been studied scarcely.

In this study, the influence of foliar MgSO_4 fertilization on different aspects, such as the distribution of the major cations Mg^{2+} , Ca^{2+} and K^+ in different plant compartments, the transcript abundance of enzymes related to the appearance of deficiency symptoms and the formation of yield and seed quality in *Vicia faba* was examined. For this purpose, faba beans were grown in nutrient solution containing either sufficient (0.5 mM) or low (0.05/0.015 mM) MgSO_4 . Plants growing in nutrient solution containing only low concentrations of MgSO_4 were subjected to foliar applications of MgSO_4 with either 0 mM, 50 mM or 200 mM.

Foliar application proved to change the cation relations in the apoplast and symplast in diverse manners. The rise in apoplastically bound Mg^{2+} led to a decrease of Ca^{2+} while in the symplast the risen Mg^{2+} concentration affected on the K^+ concentration, if high concentrations of MgSO_4 (200 mM) were applied. Although both cations hold important functions in plant structure and metabolism, negative effects could not be observed with regards to the other parameters measured. It was therefore proposed that these cations can replace each other partially.

The concentration of chlorophyll and the photosynthesis rate proved to be enhanced by application of 200 mM MgSO_4 . However, the reduced transcript abundance of Mg chelatase H under magnesium deficiency was not changed by foliar magnesium application. This might indicate that under magnesium deficiency chlorophyll synthesis is reduced which is not influenced by foliar application. Instead, a reduced chlorophyll breakdown due to ameliorated magnesium supply is possible. This study revealed that the transcription of the plasmalemma H^+ -

ATPase isoforms is differentially regulated under magnesium deficiency. However, specific functions of different isoforms have not been revealed yet.

Magnesium foliar application resulted in a significant increase of seed yield of plants growing in magnesium deficient nutrient solution. This increase was mainly attributable to the increase in the number of pods per plant. Additionally, the quality of the seeds was raised due to magnesium foliar application as significantly higher protein concentrations could be found. This might be associated to the role of magnesium in nitrogen assimilation and protein synthesis.

6.2 Zusammenfassung

Als Makronährstoff wird Magnesium in relativ hohen Konzentrationen benötigt und erfüllt vielfältige Funktionen im pflanzlichen Stoffwechsel. Eine ausreichende Versorgung von Kulturpflanzen mit Magnesium ist wichtig um hohe Erträge und eine hohe Qualität der Ernteprodukte sicherzustellen. In Land- und Forstwirtschaft wurde in einigen Regionen, eine suboptimale Versorgung mit Magnesium festgestellt. Neben geringen Mg-Konzentrationen im Boden, können auch andere Faktoren, wie eine hohe Konzentration an Ca^{2+} , K^+ , H^+ , Al^{3+} oder eine geringe Bodenfeuchte zu einem Auftreten von Magnesiummangel beitragen. In Pflanzen führt dies unter Anderem zu der Entwicklung von Chlorosen in den älteren Blättern, zu einer Wachstumsverringerng und zu einer Anstauung von Zuckern in Blättern. Neben einer Mg-Düngerzufuhr über den Boden, kann Magnesium auch durch Blattdüngung bereitgestellt werden. Besonders in Situationen in denen die Verfügbarkeit des Magnesiums im Boden eingeschränkt ist, oder eine schnelle Verbesserung der Ernährungssituation herbeigeführt werden soll, kann Blattdüngung hilfreich sein.

In einigen Studien konnte bereits gezeigt werden, dass Mg-Blattdüngung die Chlorophyllkonzentration und die Biomasseakkumulation in Oregano, Mais, Soja und Apfel erhöht (Reay et al. 1998; Szulc et al. 2001; Dordas 2009; Teklic et al. 2009). In Bezug auf die Wirkung der Mg-Blattdüngung auf den Ertrag und die Qualität gibt es jedoch unterschiedliche Ergebnisse. Auch zu der Wirkung der Mg-Blattdüngung auf die Physiologie der Pflanzen gibt es nur wenige Studien.

In dieser Untersuchung wurde der Einfluss einer MgSO_4 -Blattdüngung auf verschiedene Parameter wie die Verteilung der wichtigsten Nährkationen in verschiedenen Blattkompartimenten, die Transkriptabundanz von Enzymen die mit dem Auftreten von Magnesiummangelenzymen in Verbindung stehen, sowie den Ertrag und die Samenqualität von *Vicia faba* untersucht. Ackerbohnen wurden zu diesem Zweck unter kontrollierten Bedingungen in Nährlösungen angezogen, in denen entweder ausreichende (0,5 mM) oder geringe (0,05/0,015 mM) Konzentrationen an MgSO_4 enthalten waren. Zusätzlich wurden die Pflanzen, die gering mit Magnesium versorgt wurden, mit einer Blattapplikation von entweder 0 mM, 50 mM oder 200 mM MgSO_4 gedüngt.

Die Blattdüngung wirkte sich unterschiedlich auf die Ionenverhältnisse im Apoplasten und Symplasten aus. Durch ein Ansteigen des apoplastisch gebundenen Magnesiums, kam es zu einer Verringerung der Ca-Konzentration im Apoplast. Dagegen führte der Anstieg der Mg-Konzentration im Symplast zu einer Verringerung der K-Konzentration. Obwohl beide Kationen wichtige Funktionen im pflanzlichen Stoffwechsel ausführen, konnten negative Effekte auf die

anderen gemessenen Parameter nicht beobachtet werden. Es ist daher möglich dass diese Kationen sich teilweise in ihrer Funktion ersetzen können.

Die Chlorophyllkonzentration und die Photosyntheserate wurden durch die Blattapplikation von 200 mM MgSO₄ signifikant gesteigert. Die unter Magnesiummangel gesunkene Transkriptabundanz der Mg chelatase H wurde jedoch nicht von der Blattdüngung beeinflusst. Dies könnte ein Hinweis darauf sein, dass unter Mg-Mangel die Synthese von Chlorophyll reduziert wird. Dies wird nicht von der Blattdüngung beeinflusst, aber möglicherweise wird durch die Magnesium Zufuhr ein weiterer Abbau des Chlorophylls verringert. In dieser Studie konnte gezeigt werden, dass die Transkription von verschiedenen Isoformen der Plasmalemma H⁺-ATPase unterschiedlich reguliert wird. Allerdings konnte den Isoformen noch keine spezifische Funktion zugewiesen werden.

Mg-Blattdüngung führte zu einer signifikanten Steigerung des Samenertrags von Ackerbohnen die in Mg-Mangelnährlösung wuchsen. Diese Ertragssteigerung wurde vor allem durch eine Steigerung der Anzahl der Schoten hervorgerufen. Auch die Samenqualität in Bezug auf die Proteinkonzentration konnte gesteigert werden. Dies kann auf die Funktion von Magnesium bei der Assimilation von Stickstoff und der Proteinsynthese zurückgeführt werden.

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Danksagung

An erster Stelle danke ich meinem Doktorvater Herrn Prof. Dr. Karl Herrmann Mühling für die Ermöglichung dieser Arbeit und für die Unterstützung während der Bearbeitung.

Mein Dank gilt ebenso Herrn Prof. Dr. Christian Zörb für die Einweisung in verschiedene Labormethoden und für die vielfachen Hinweise und Anregungen.

Mein besonderer Dank gilt Dr. Christoph-Martin Geilfus für die Einweisung in Labormethoden, die Unterstützung und die vielfachen hilfreichen Anregungen die zu der Entstehung dieser Arbeit beigetragen haben. Danke auch für die Hilfsbereitschaft und die Gesellschaft im Büro.

Frau Prof. Dr. Sylvia Lindberg danke ich für die freundliche Aufnahme in Stockholm und für die fachliche Betreuung während meines Aufenthaltes. Ebenso danke ich ihr für die sehr schönen gemeinsam unternommenen Ausflüge in Stockholm.

Mein herzlicher Dank gilt meinen Doktorandengeschwistern Christoph-Martin, Sajid, Shahzad, Sherif, Jan-Reent, Marcus, Caro, Mareike, Li und Cheng, sowie Reiner Krähmer für das angenehme Arbeitsumfeld und die schöne gemeinsam verbrachte Zeit.

Auch den Mitarbeitern im Labor, insbesondere Martina Bach, Stefanie thor Straten, Bärbel Biegler und Thoja Heimbeck, sowie Anne Putbrese möchte ich danken für die angenehme Zusammenarbeit und die Hilfsbereitschaft die mir entgegengebracht wurde.

Dem DAAD möchte ich danken für die Ermöglichung meines Forschungsaufenthaltes am Institut für Botanik der Universität Stockholm. Herzlich bedanke ich mich außerdem bei der Universität Kiel für die finanzielle Unterstützung in Form eines Graduiertenstipendiums, welches diese Arbeit ermöglicht hat.

Meiner Familie und meinen Freunden, danke ich für die Unterstützung während dieser Zeit und für den Ausgleich nach getaner Arbeit.

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