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Institute of Plant Nutrition and Soil Science, Kiel University, Kiel, Germany

Foliar Magnesium supply increases the abundance of RuBisCO of Mg-deficient maize plants

Julian Wolf, Stefanie thor Straten, Britta Pitann, Karl-Hermann Mühling*

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Summary

Magnesium is a vital macronutrient for plants and is involved in a series of essential physiological processes, e.g., carbohydrate partitioning and photosynthesis. The latter is strictly Mg-dependent, as Mg is the central atom of chlorophyll, and is also required for the *de novo* synthesis of sugars; this pathway revolves around the activity of the enzyme RuBisCO. When plants are subject to Mg-limiting conditions, development as well as yield is reduced. The foliar resupply of Mg, contrary to traditional resupply to the roots, has the advantage of delivering the element directly to the site of highest physiological demand. Thus, the aim of this research is to compare the effects of both resupply methods on the physiology and nutritional status of Mg-deficient plants to see whether foliar application compared to root fertilization can properly improve plant growth after Mg deficiency conditions.

Maize plants were cultivated in hydroponics in order to set up a Mg-deficient root environment. $MgSO_4$ was then resupplied alternatively to the leaves or to the root.

Under Mg-limiting conditions, RuBisCO abundance, as well as the total content of Mn, Zn, Fe, and Cu were severely reduced. This state was significantly ameliorated by the foliar resupply of $MgSO_4$, although the highest increase in biomass production was observed in response to root resupply. The foliar resupply of $MgSO_4$ upregulated the process of photosynthesis in Mg-deprived plants. In this context, the foliar $MgSO_4$ application was able to return RuBisCO abundance to control levels in Mg-deficient plants.

Key words: Magnesium deficiency, maize, foliar application, photosynthesis, RuBisCO, micronutrients

Introduction

Magnesium (Mg) is an essential macronutrient for the optimal growth and development of plants. Although Mg is mostly known for its role as the central atom of chlorophyll, this element is utilized for numerous other physiological and biochemical processes. The loading of photosynthates into the phloem and their redistribution throughout the plant, for example, are Mg-dependent processes (Cakmak and Kirby, 2008; Cakmak and Yazici, 2010). The driving force of these mechanisms is the activity of the plasma membrane H^+-ATPases, which require Mg-ATP as a substrate (Hanstein et al., 2011). Moreover, Mg is known to be involved in the activity of over 300 enzymes, including photosynthetic enzymes, such as ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO) and phosphoenolpyruvate carboxylase (PEPC) (Chen et al., 2018). Mg is crucial for the process of protein synthesis, as it is required for the association of ribosomal subunits and the maintenance of their activity (Chen et al., 2018).

Due to the multiple roles of Mg in the plant system, Mg deficiency (MGD) is detrimental for crop growth and yield (Farhat et al., 2016). This nutritional stress is very common in acidic soils that have a high content of aluminum (Al) and manganese (Mn), and in saline soils with a high content of sodium (Na). These elements are present in the soil as cations and can replace and cause Mg to be leached, since Mg is only weakly bound to negatively charged soil particles. Sandy soils are also particularly prone to leaching as Mg adheres mainly to clay minerals. Moreover, since Mg reaches the root surface through diffusion and mass flow, Mg nutrition is additionally hampered if the soil's water content is low (Jezek et al., 2015a). A way to overcome these issues is by delivering Mg directly to the site of highest nutritional demand through foliar application. The efficacy of this resupply method has been previously studied in faba bean and soybean (Vrataric et al., 2006; Neuhaus et al., 2013, 2014). Here it was shown that a greater share of Mg is taken up by the plant following foliar application when compared to root application (Neuhaus et al., 2013, 2014; Jezek et al., 2015a, b). This is due to the fact, that Mg can enter the plant more directly *via* the stomata and cuticula, while application to the soil causes a greater portion of the fertilizer to not reach the root due to secondary soil interactions and leaching (Chen et al., 2018).

Moreover, soil fertilization is best performed either directly at the moment of sowing or at the seedling stage, as the field is more easily accessible (Kannan, 2010). Delivering the fertilizer to the plant any later via soil application during the growing season can be more labor intensive and cause mechanical damage to the shoot. Conversely, Mg can be delivered to the leaves of more numerous plants at once via spraying the plants or by taking advantage of the water irrigation sprinklers.

The shoot is where the main processes that affect biomass production take place, namely the light-dependent and -independent reactions of photosynthesis. Both pathways rely on Mg and take place in the chloroplast, where most of the plant's Mg is found (15-35%) (Wanli et al., 2016). The former converts the sun's radiation energy into plant-available chemical energy, namely adenosine triphosphate (ATP). Chlorophyll plays a crucial role as it captures photons and channels their energy along the electron transport chain (ETC). The relationship between Mg-availability and chlorophyll content is substantial, as multiple authors observed a decrease in the content of the pigment under MGD (Cakmak and Yazici, 2004; Mengutay et al., 2013; Broadley et al., 2012). This is most likely caused by the inhibition *de novo* synthesis of chlorophyll, as well as the degradation of existing chlorophyll in response to MGD (Chen et al., 2018). Through the latter, in particular, the plant gains access to the organically-complexed Mg pool, which can then be reallocated from mature tissues to the young shoot (Senbayram et al., 2015; Wanli et al., 2016). The light-independent reactions of photosynthesis revolve around the fixation of carbon dioxide (CO_2), using the newly-generated ATP. The first step of this pathway is catalyzed by the enzyme RuBisCO. This complex globular protein has both a carboxylase activity, which is prevalent under physiological conditions and accepts CO_2 as a substrate, as well as an oxygenase activity, requiring molecular oxygen (O2). A prerequisite to enable the carboxylation reaction to occur is that a Mg atom anneals to the active site of the enzyme; only then can a CO_2 molecule bind to the enzyme. Moreover, Mg is required also indirectly, during the day, to

* Corresponding author

balance the charge difference across the thylakoid membrane and ensure the stroma reaches the correct pH for optimal catalytic activity (SPREITZER and SALVUCCI, 2002). However, little is known regarding the response of RuBisCO to MGD. A study by YUGUAN et al. (2009a) observed how MGD in spinach caused the expression level of the genes coding for RuBisCO's big and small subunit to decrease, as well as an overall reduction of its catalytic activity. These results are in accordance with the decreased net CO_2 assimilation rate observed by JEZEK et al. (2015a) in response to MGD in maize.

This study was performed using maize, as it is one of the most extensively cultivated cereal crops in the world (PECHANOVA et al., 2013). The grain of this plant, as well as the whole portion growing above-ground, are used as silage material. In addition to this, its high starch content, on average 72%, justify its use as raw material for the production of biofuel and biogas (KSIEZAK et al., 2008; KSIEZAK et al., 2012; RANUM et al., 2014).

The aim of this research was firstly to verify that severe MGD had a negative effect on the photosynthetic machinery, specifically on RuBisCO abundance. Secondly, in this research the effects of foliar and root resupply of Mg on the physiology and nutritional status of Mg-deficient plants were investigated. Additionally, the question should be answered whether the foliar resupply of magnesium sulphate ($MgSO_4$) is comparably or even better suited than the re-supply to the root to amend the detrimental effects of MGD.

Material and methods

Experimental setup

Maize (*Zea mays* L.) plants from the cultivar Susann (Nordsaat Saatzucht, Langstein, Germany) were cultivated hydroponically in a greenhouse from November to December 2017. Plants were grown under an artificial light system (Lamps: Professional Lighting, SON-K 400, Philips Deutschland GmbH, Hamburg, Germany; bulbs: Philips SON-T Agro 400 Watt, Philips Deutschland GmbH; light intensity: 350 µmol m^{-2} s^{-1}) with a 16/8 h day/ night regime, at 20-25 °C and 65% relative humidity.

Seeds were soaked in an aerated 2 mM $CaSO_4$ solution overnight and germinated between two sheets of filter paper moistened with 2 mM $CaSO_4$ solution.

Six-day-old seedlings were transferred to 10 L containers (four plants per container) containing 25% strength nutrient solution (NS); this day is hereafter considered as day 0. Within the next 3 d, the NS was increased step-wise up to 100%. The full-strength NS was exchanged weekly and had the following composition: 1.3 mM $Ca(NO_3)_2$, 0.7 mM NH_4NO_3, 2.0 mM $CaCl_2$, 1.0 mM K_2SO_4, 0.2 mM KH_2PO_4, 200 µM Fe-EDTA, 5 µM H_3BO_3, 2 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.3 µM $CuSO_4$, and 0.01 µM $(NH_4)_2Mo_7O_{24}$.

Plants were divided into four discrete experimental groups with four independent biological replicates per treatment ["Positive control" (CONTR+), "Negative control" (CONTR–), "Root application" (ROOT) and "Foliar application" (LEAF)]. Containers were set up in a completely randomized design. CONTR+ plants were given sufficient Mg, namely the full NS with the addition of 0.5 mM $MgSO_4$, throughout the experiment. The other three variants were supplied with 0.02 mM $MgSO_4$ for the first week, in order to ensure proper seedling development, and only 0.01 mM $MgSO_4$ from thereafter. This concentration was chosen in order to induce clear MGD symptoms. Two weeks after the transfer to 10-L containers (day 14), the first MGD symptoms became clearly visible on the leaves of plants growth in Mg-deficient NS, e.g., intervenial chlorosis. The same day, and until the end of the experiment, the Mg concentration of ROOT was raised to the level of CONTR+. Concurrently and over the course of two weeks, LEAF was sprayed with a 200 mM $MgSO_4$ solution, splitted into four single applications; each one of these had a total volume of 80 mL. By this, the total Mg amount applied to the leaves was stepwise increased to avoid salt effects (e.g., burning) possibly caused by a single higher dose. Silwet (Spiess-Urania Chemicals GmbH) in the dose of 0.1% was added as a wetting agent to the foliar application solution.

Plants were harvested on day 29 and shoots and roots were sampled separately. Moreover, the shoots were additionally subdivided into two discrete samples: the two youngest leaves (without the stalk) were sampled as "Young"; the following three leaves were sampled as "Old". In order to calculate the total shoot fresh weight of one plant, the weight of the discrete shoot fractions ("Young" and "Old") were summed up. Thus, for each treatment, 12 samples were taken (4 "Old", 4 "Young", and 4 "Root"), for a total of 48 samples.

Samples were homogenized with a mortar and pestle in the presence of liquid N_2 before being stored at -80 °C for further analysis.

Determination of growth parameters

During harvest, the average height and the weight of both shoots and roots were measured for each container. The height was defined as the distance from the top of the hydroponic culture container to the tip of the highest leaf. The same portion was subsequently weighted to obtain the weight of the shoot. The leaves of the LEAF variant were thoroughly rinsed with deionised water before storage to remove adhering spraying solution.

Protein analysis

Total protein extraction

Total protein was extracted using the TCA/acetone protocol adapted from ZÖRB et al. (2004). For this, aliquots of 300 mg ground material were extracted with 1.6 mL of ice-cold 10% (w/v) trichloroacetic acid in acetone with the addition of 50 mM dithiothreitol (DTT). After incubating at -20 °C overnight, the protein fraction was separated from the liquid phase by centrifugation (19,125 g, 4 °C, 15 min). Pellets were suspended in 1 mL of ice-cold acetone plus 50 mM DTT and incubated again overnight at -20 °C. Vacuum-dried protein pellets were suspended in 1 mL of lysis buffer (urea 8 M, thiourea 2 M, DTT 30 mM, Tris base 20 mM, CHAPS 4%) to which 0.625 µL of protease inhibitor (Sigma) was added. After incubating for 2 h at 3 °C, the protein fraction was separated by centrifugation (19,125 g, 4 °C, 30 min) and stored at -80 °C.

The protein concentration was determined using 2D Quant Kit according to the manufacturer's manual (GE Healthcare Life Sciences). The calibration curve was created using bovine serum albumin (BSA) as a standard.

Western Blot

A Western Blot (WB) analysis was performed on the samples associated with old and young leaves; for this, the method suggested by ZÖRB et al. (2005) was used. Briefly, the RuBisCO large subunit (55 kDa) was employed as the primary antibody (RuBisCO, antibodies-online.com). The four polyacrylamide gels used for this analysis were a combination of a 6.3% stacking gel and a 15% separation gel. The equivalent of 8 µg of protein was loaded into each well. Page Ruler Pre-stained Marker (Thermo Fisher Scientific) was used as a protein ladder. The gels were run at 20 mA per gel in an electrophoresis chamber. Once the gels had been run (20 mA per gel for 1 h), they were incubated in Towbin buffer (for 1 L: 3.03 g Tris base, 14.4 g glycine and 100 mL of methanol, pH 8.3) for 15 min. Simultaneously, four gel-sized pieces of polyvinylidene difluoride (PVDF) membrane were soaked for 3 s in methanol and subsequently incubated in Towbin buffer for 10 min. The "blot-sandwich" was run at 110 mA for 1 h using the Hoefer Semiphor chamber (Pharmacia Biotech). After the transfer, the PVDF membranes were incubated in a 5% BSA/TBS solution (Bovine Serum Albumin, Merck; for 1 L: 1.21 g Tris-HCl

and 8.77 g NaCl) for 2 h. After 3 washing steps with TBS-T (TBS and 0.1% Polysorbate 20 (Tween 20, Sigma)), the solution containing the antibody for RUBISCO, diluted 1:5.000 with PBS (for 1 L: 8 g NaCl, 0.2 g KCl, 0.76 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4.), was added to the PVDF membranes. The membranes were incubated 1 h at RT and at 4 °C overnight. The following morning, the membranes were washed twice for 10 min with TBS-T and once for 10 min with a 1.5% milk powder in TBS-T solution. Next, the membranes were incubated for 2 h in a solution of the second antibody (Anti-Rabbit IgG, Alkaline Phosphatase Conjugate, Sigma), diluted 1:30.000 in PBS.
To remove the second antibody, the membranes were washed for 10 min a total of 5 times, 4 of which with TBS-T and one with TBS. After the last washing step, the membranes were incubated for 5 min in the AP-buffer (for 1 L: 12.11 g Tris base, 5.84 g NaCl and 1.02 g $MgCl_2$, pH, 9.5). Finally, the AP-buffer was removed and the membranes were covered with the developing solution (10 mL AP-buffer, 32 µL BCIP solution (100 mg BCIP in 1.9 mL H_2O), 66 µL NBT solution (100 mg of NBT in 1.9 mL 70% DMF/H_2O solution)). Membranes were washed multiple times with purified H_2O and incubated overnight in the dark. The developed PVDF membranes were scanned with an Epson Perfection V700 PHOTO scanner and the Silverfast Epson SE program. Subsequently, the pictures were analyzed using the GelAnalyzer software (Lazer software, version 2010a).

Determination of nutritional status

Between 280 and 330 mg of frozen sample were dissolved in 10 mL of 69% HNO_3 solution. Digestion of the sample occurred using a microwave (CEM, One Touch Technology, Mars 6) with the following regime: 2 min at 100 °C, 1 min at 120 °C, 20 min at 180 °C, and allowed to cool for 20 min. The digested samples were diluted with purified water to a total volume of 50 mL.
The Mg, Ca, and K contents of each sample were measured using the Atomic Absorption Spectrometer (AAS, Thermo Electron Corporation, S Series). Wavelength was set to 285.5, 422.9, and 766.5 nm, respectively.
Mn, Zn, Cu, Fe and Mo content, on the other hand, was measured using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS, Agilent Technologies 7700 Series, Böblingen, Germany).
Two different analytical methods were used to measure the macro- and micro-nutrient contents of the samples because of the different abundance at which these elements are present in plants; micronutrients make up only 0.02% of the total dry weight and are, thus, 1-4 orders of magnitude less abundant than macronutrients (Sabeeha, 2010). Accordingly, the AAS and ICP-MS were chosen to account for these differences while preserving the necessary precision of the measurements.

Statistical treatment

Significant differences (p-value ≤ 0.05) between means were identified through a one-way analysis of variance (ANOVA) using SPSS (version 17.0).
Post-Hoc analysis for multiple comparisons (Tukey's range test) was additionally carried out to discriminate among the significant differences of the variant's means.
Shown values are given as means of at least three replicates with standard deviations (SD).

Results

Effect of MGD and resupply on overall plant growth

Plant height was significantly reduced after prolonged exposure to Mg-limiting conditions; on average, maize plants grew up to 75.5 cm which accounted for 63.3% of CONTR+ plants (Fig. 1A). Of the two resupply variants, only the ROOT variant did not differ significantly from the positive control; the plants that were treated with foliar $MgSO_4$ grew less than the ROOT variant and were statistically indistinguishable from the negative control. Similar results were observed for the shoot's fresh weight (FW) (Fig. 1B): MGD led to a drastic reduction in plant's FW, namely 90.1% of the positive control. The ROOT and LEAF variant, on the other hand, reached only 45% and 29%, respectively, of the FW of CONTR+. With this, the resupply variants exhibited an intermediate value between the two controls. In the end, only ROOT was significantly different from the controls. Finally, when measuring the root biomass formation, both resupply variants showed a higher mass than the negative control, although not statistically different (Fig. 1B).

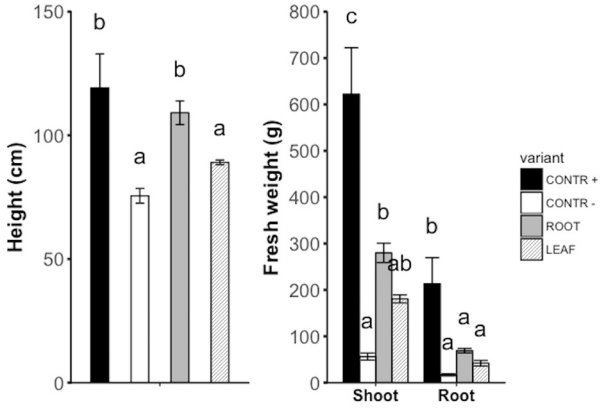

Fig. 1: Heights and weights (FWs) of shoot and root. **(A)** Height (cm) of shoots and **(B)** FWs of shoot and root at harvest (35 days after germination). CONTR+, 0.5 mM $MgSO_4$ in NS; CONTR–, 0.01 mM $MgSO_4$ in NS; ROOT, 0.01 mM $MgSO_4$ in NS for 2 weeks and 0.5 mM $MgSO_4$ in NS for 2 weeks; LEAF, 0.01 mM $MgSO_4$ in NS and foliar application of 200 mM $MgSO_4$ over 2 weeks; mean (n = 4 biological replicates) ± SD; small letters indicate significant difference between treatments (ANOVA, with Tukey adjustment, p ≤ 0.05).

Effect of MGD on macro- and micronutrient composition

The concentrations of Mg, Ca, and K were measured for aliquots of each sample. In order to eliminate dilution effects, the total content of each element was displayed.
Positive control plants were supplied with 0.5 mM of $MgSO_4$ throughout the experiment and always displayed the highest Mg contents. These values were the highest in the old leaves with 67.8 mg $plant^{-1}$, and decreased in the roots (40.0 mg $plant^{-1}$) and young leaves (11.1 mg $plant^{-1}$) (Fig. 2A). Conversely, the growth of maize plants deficient in Mg severely limited total Mg content across all organs.
In the old leaves, ROOT was the only variant to show a significantly increased Mg content (13.1 mg $plant^{-1}$ against 1.7 mg $plant^{-1}$ of the negative control); in contrast, in the young leaves it was the LEAF variant, with more than double the content of the negative control, which diverged most significantly from CONTR–.
The fluctuation of the total K content: MGD led to a very pronounced decrease in total nutrient accumulation (10.8%, 4.4% and 5.4% of the CONTR+, respectively), while the resupply variants did not differ significantly from the negative control (Fig. 2B). The only exception was the total content of K accumulated in the old leaves after resupply of $MgSO_4$ to the NS; this value was significantly higher than the negative control, yet lower than the positive control.
The Ca content in the old leaves, as well as in the young leaves and the roots, did not exhibit any particular differences (Fig. 2C). Overall, the positive control represented the highest value (176.9, 159.1 and 140.7 mg $plant^{-1}$, respectively), while the contents of all other variants

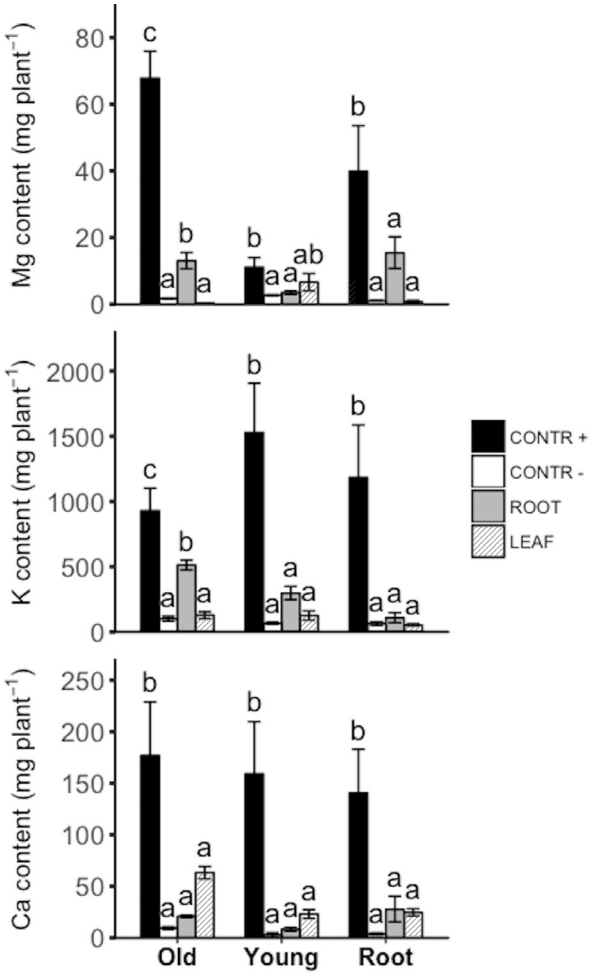

Fig. 2: Macronutrient content in various leaves and roots. **(A)** Total content of Mg, **(B)** K and **(C)** Ca per plant at harvest (35 days after germination). CONTR+, 0.5 mM $MgSO_4$ in NS; CONTR–, 0.01 mM $MgSO_4$ in NS; ROOT, 0.01 mM $MgSO_4$ in NS for 2 weeks and 0.5 mM $MgSO_4$ in NS for 2 weeks; LEAF, 0.01 mM $MgSO_4$ in NS and foliar application of 200 mM $MgSO_4$ over 2 weeks; mean (n = 4 biological replicates) ± SD; small letters indicate significant difference between treatments (ANOVA, with Tukey adjustment, $p \leq 0.05$).

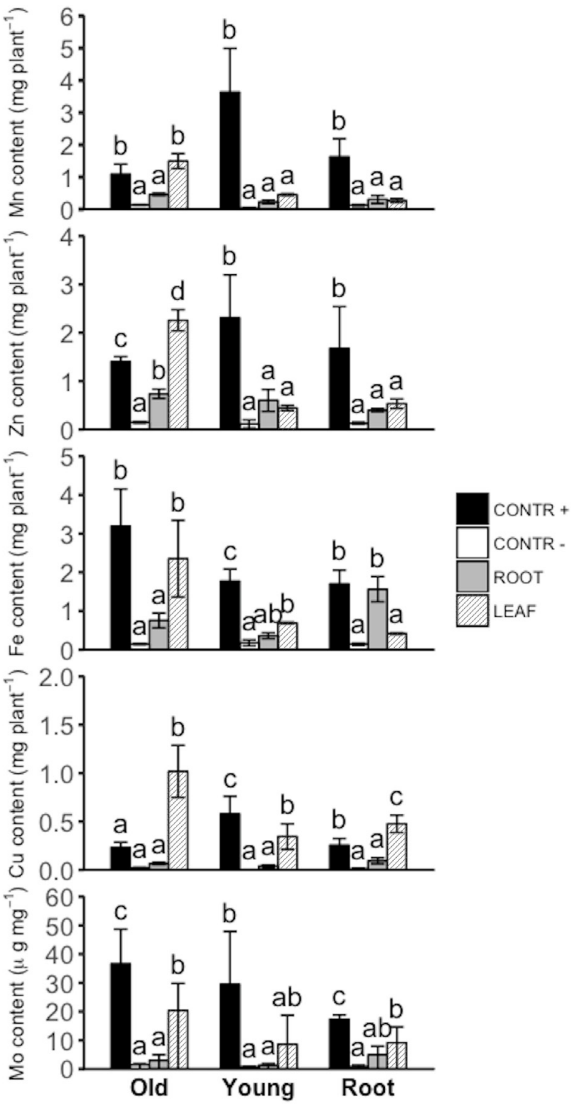

Fig. 3: Micronutrient content in various leaves and roots. **(A)** Total content of Mn, **(B)** Zn**, (C)** Fe, **(D)** Cu and **(E)** Mo per plant at harvest (35 days after germination). CONTR +, 0.5 mM $MgSO_4$ in NS; CONTR –, 0.01 mM $MgSO_4$ in NS; ROOT, 0.01 mM $MgSO_4$ in NS for 2 weeks and 0.5 mM $MgSO_4$ in NS for 2 weeks; LEAF, 0.01 mM $MgSO_4$ in NS and foliar application of 200 mM $MgSO_4$ over 2 weeks; mean (n = 4 biological replicates) ± SD; small letters indicate significant difference between treatments (ANOVA, with Tukey adjustment, $p \leq 0.05$).

were drastically lower (on average, 5%, 2%! and 3% of the CONTR+, respectively) and did not differ significantly from each other.

The total amount of Mn accumulated in the plants showed significant differences only in the old leaves: here, the LEAF variant yielded the highest Mn content (1.49 mg plant^{-1}), yet no significant difference to the positive control could be found (1.08 mg plant^{-1}) (Fig. 3A). Additionally, the value of the ROOT variant (0.46 mg plant^{-1}) was increased compared to the negative control (0.13 mg plant^{-1}) but not enough so to be considered different.

The total content of Zn was measured as well (Fig. 3B). The old leaves were the only organ to show a particular response, specifically in the LEAF variant: the Zn content was markedly increased (2.26 mg plant^{-1}) compared to the positive control (1.41 mg plant^{-1}). Moreover, the ROOT variant also accumulated a significantly higher amount of Zn compared to the negative control (0.74 and 0.15 mg plant^{-1}, respectively), yet less than the positive control.

The pattern of Fe accumulation differs among the three organs (Fig. 3C). In the old leaves, the value of LEAF (2.35 mg plant^{-1}) was increased to the point of showing no significant difference to the positive control (3.2 mg plant^{-1}). Also in the young leaves, the LEAF variant showed an increased value (0.69 mg plant^{-1}), not nearly as large as the positive control (1.77 mg plant^{-1}), yet sufficiently high to be considered different from both controls. Lastly, in the radical apparatus, the ROOT variant (1.56 mg plant^{-1}) reached the iron content of the positive control (1.7 mg plant^{-1}). No statistical difference could be found between these two measurements.

The Cu content of the old leaves is the highest in the LEAF variant (1.02 mg plant^{-1} against 0.23 mg plant^{-1} of CONTR+) (Fig. 3D); the other variants do not differ significantly from one another. In the young leaves, the highest Cu content is one of the positive controls (0.58 mg plant^{-1}). In this organ, the LEAF variant represents an intermediate (0.34 mg plant^{-1}) between the positive control and the negative control (0.001 mg plant^{-1}) and ROOT (0.04 mg plant^{-1}). Finally, the roots exhibit a similar patter as the old leaves with the LEAF variant showing the highest Cu content (0.48 mg plant^{-1}). The positive control (0.25 mg plant^{-1}) is statistically independent from the

LEAF, as well as from the CONTR– (0.01 mg plant^{-1}) and ROOT (0.09 mg plant^{-1}).
Finally, Mo content showed a similar trend across all organs; the amount stored by the LEAF variant was always higher than the negative control but lower than the positive control (Fig. 3E). Additionally, in the roots, also the ROOT variant showed an increased value compared to the negative control.

RuBisCO abundance as influenced by Mg deficiency and resupply

Due to the tight link between Mg nutrition and the process of photosynthesis, the abundance of RuBisCO was measured through a WB analysis. Since no RuBisCO is present in the roots, the analysis was performed only on old and young leaves.
In the old leaves, MGD caused RuBisCO abundance to decrease by a 0.7 fold change. The resupply variants displayed discrete results: the foliar $MgSO_4$ application was able to return RuBisCO abundance to control levels; this was not the case for the ROOT variant, which scored an intermediate value between the two controls (Fig. 4).
A slightly different situation was visible in the young leaves. Here the two control variants were not significantly different from each other. The behavior of the resupply variants was similar to the old leaves: the ROOT application did not manage to return the enzyme's abundance to control levels, while the LEAF application did much better, scoring the best overall.

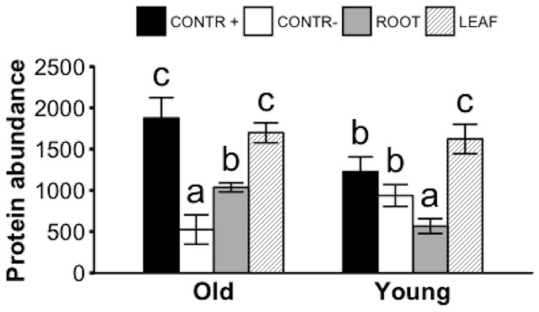

Fig. 4: RuBisCO abundance in old and young leaves. **(A)** RuBisCO abundance, measured in raw pixel volume, in the old and **(B)** young leaves at harvest (35 days after germination). CONTR+, 0.5 mM $MgSO_4$ in NS; CONTR–, 0.01 mM $MgSO_4$ in NS; ROOT, 0.01 mM $MgSO_4$ in NS for 2 weeks and 0.5 mM $MgSO_4$ in NS for 2 weeks; LEAF, 0.01 mM $MgSO_4$ in NS and foliar application of 200 mM $MgSO_4$ over 2 weeks; mean (n = 4 biological replicates) ± SD; small letters indicate significant difference between treatments (ANOVA, with Tukey adjustment, $p \leq 0.05$).

Discussion

Magnesium is involved in a plethora of physiological processes due to its divalent positive charge and ionic radius. Not only does this element serve as a cofactor for over 300 enzymes, it also stabilizes the conformational structure of many different kinds of macromolecules, like proteins, ribosomes, nucleic acids, and the cell membrane and wall (Wanli et al., 2015). Due to its widespread biological function, MGD negatively affects overall plant growth as well as crop yield (Senbayram et al., 2015).
The aim of this research was twofold: on the one hand, it aimed at elucidating how the absence and the resupply of Mg affect the micronutrient content of maize plants. These were expected to fluctuate because of their role as cofactors and as structural elements in the process of photosynthesis. The process of chlorophyll synthesis, in particular, was anticipated to be severely impaired under Mg-limiting conditions; in multiple instances, MGD was associated with a decline of the chlorophyll content (Yuguan et al., 2009a; Jezek et al., 2015a; Wanli et al., 2015).
The effect of the absence and of the resupply of Mg on the RuBisCO abundance was studied because this enzyme plays a pivotal role in the process of photosynthesis and was previously shown to be down-regulated under MGD on a transcriptional level (Yuguan et al., 2009a).

Effect of MGD and Mg resupply on plant growth

The reduction in plant growth is a common consequence of a MGD and has been studied in a wide variety of plants, like *Arabidopsis thaliana, Vicia faba, Beta vulgaris, Spinacia oleracea, Brassica napus* and *Zea mays* (Hermans et al., 2010; Neuhaus et al., 2014; Hermans et al., 2004; Yuguan et al., 2009b; Billard et al., 2016; Jezek et al., 2015a, b).
In accordance to other studies, this research found additional evidence for the deleterious effects of MGD on maize growth, specifically on plant height, as well as on shoot and root weight (Fig. 1). The reduced height and weight of Mg-deficient plants is an indicator of the inability of the plants to redistribute sugars from source organs to the sites of active tissue development, such as young leaves and roots. In this context, it is concluded that the inhibition of the plasma membrane H^+-ATPase primarily limited maize growth under Mg deficiency (Jung et al., 2017) due to the lack of the enzyme's co-substrate Mg. Afterwards, the reduced apoplastic acidification caused a reduced cell-extension growth.
Literature is scarce regarding the effects of resupplying Mg after prolonged MGD. In this experiment, the reapplication of Mg to the root had an overall better effect on plant growth than the foliar application: it returned plant height to the levels of the control and increased shoot and root fresh weight more than the leaf application. This was surprising since the latter delivered Mg straight to the site of the most severe MGD damage, the shoot; the former, on the other hand, was presumed to be less efficient due to the additional time required for nutrient uptake from the NS and subsequent transport of Mg to the shoot through the xylem.
The observed results point towards the ability of root resupply fertilizers to remedy the detrimental effects of MGD more promptly than the foliar resupply. Future research might focus on studying the long-term effect of the two discrete resupply methods by extending the plant's growth period.
These results show that the resupply of Mg after a period of deficiency has a beneficial effect on overall plant development. Additionally, the data indicate that the Mg applied through the leaves was successfully taken up by the leaves and translocated to the site of meristematic tissue growth.

Effect of MGD and Mg resupply on maize plant's total Mg content

In vivo, newly taken-up Mg ions are translocated through the xylem to the shoot (Kobayashi and Tanoi, 2015). This process is driven by the transpiration of water through the leaves and increases in strength relative to the leaf area through which water transpires and the metabolic rate of the tissues (Kramer and Boyer, 1995). With this in mind, it was assumed that newly taken up Mg would reach both the old and young leaves, with a slight preference for the latter due to their greater metabolic activity.
The resupply of Mg to the roots did significantly raise the Mg content in the old leaves (Fig. 2A). The response to root resupply was visible also in the young leaves; here, the Mg content was no significantly different from the negative control due to the inherently higher Mg content in young organs in response to MGD. This physiological

response to MGD is fairly well documented (Senbayram et al., 2015; Wanli et al., 2016); this mechanism implies the reallocation of Mg resources from old tissues, where Mg is being released as a result of catabolic degradation, and their reallocation through the phloem to young developing organs. Keeping this in mind, the fact that the Mg accumulated in the young leaves, as a consequence of root resupply, was not significantly different from the negative control, points to the conclusion that Mg reallocation from the old to the young leaves was not induced. If this process was not initiated, it means that the shoot did not perceive the decrease in Mg content since the xylem transport from the root delivered Mg at a sufficiently high rate.

Another aspect that emerges from the results is that the foliar resupply of Mg leads to an accumulation of Mg solely in the young tissues (Fig. 2A). Previous studies by Jezek et al. (2015a, b) already demonstrated the beneficial effect of reapplying Mg through the leaf on the plant's total Mg content. Contrary to this research, they found that the foliar resupply of Mg enhanced the Mg content only of the leaves that were directly treated with $MgSO_4$ solution. No translocation to the young leaves appeared to occur. In the present study, Mg was applied solely to the old leaves, which were the third, fourth and fifth leaf from the top of the plant; based on this and the available literature, it was expected for the old leaves to show a significant increase in Mg content. However, it was the young leaves that exhibited the highest increase of two and half fold. The measured doubling in Mg content might be seen as proof that the reallocation of Mg to the young leaves is taking place. Moreover, even if the increase in Mg content was not enough to be considered statistically significant, it was sufficient to markedly alter both micronutrient content as well as RuBisCO abundance (Fig. 3 and 4).

MGD and Mg resupply alter the micronutrient content of maize plants

Micronutrients play an important role in the process of photosynthesis. They aid enzymatic activity as prosthetic groups of metalloproteins (Römheld and Marschner, 1991). During this study, the change in leaf content of Mn, Zn, Cu, and Fe in response to varying Mg conditions were investigated.

MGD caused a marked decrease in micronutrient content across all organs; conversely, the foliar resupply of $MgSO_4$ caused these contents to significantly increase (Fig. 3).

Manganese, together with Fe, Cu, and Zn, is involved in the biosynthetic pathway that leads to the formation of chlorophyll (Shenker et al., 2004; Marsch et al., 1963; Maksymiec, 1997; Hu and Sparks, 1991). This process is also heavily influenced by the availability of Mg, as multiple authors have linked MGD to a decrease in chlorophyll content (Cakmak and Yazici, 2010; Mengutay et al., 2013; Broadley et al., 2012). Cu and Fe additionally play an important role in ensuring a continuous flow of electrons along the electron transport chain (ETC), the former as part of plastocyanin, the latter as a constituent of cytochromes (Raven et al., 1999; Broadley et al., 2012). Finally, Mn is also involved in the process of oxygen evolution, as part of the Oxygen Evolving Complex (OEC) of the Photosystem II (PSII). Fashui's group published multiple articles (Yuguan et al., 2009a; Yin et al., 2009; Zhao et al., 2012) in which they linked MGD to a decrease in the rate of oxygen evolution.

The observed decrease in micronutrient content might therefore reflect the reduced requirement for cofactors of the synthesis reactions and the limited flow of electrons along the ETC. In other words, it is suggested that MGD inhibits not only the biosynthetic pathway of chlorophyll but causes the whole process of photosynthesis to be down-regulated.

The foliar resupply of Mg, on the other hand, caused micronutrient content to increase (Fig. 3). This effect was predominantly visible in the old leaves and, to a lesser extent, in the young ones. There is already proof for the positive effect of Mg resupply on the plant's chlorophyll content (Jezek et al., 2015a; Yuguan et al., 2009a). Keeping this in mind, it is reasonable to assume that the observed increase in Mn, Cu, Zn, and Fe content in the old leaves in response to the foliar Mg application might reflect an up-regulation of the *de novo* synthesis of chlorophyll, and possibly of the whole process of photosynthesis.

The effect of MGD and Mg resupply on RuBisCO abundance

The enzyme RuBisCO is referred to as the most abundant globular protein in the world (Ellis, 1979). The importance of this complex oligomer in plant physiology is due to its fundamental role in the process of CO_2 fixation from the atmosphere. Mg^{2+} ions play both a direct and an indirect role in the activity of this enzyme: first of all, for catalytic activity to initiate, Mg has to physically bind to the active site of a RuBisCO. Secondly, Mg is required during the day to balance the charge difference across the thylakoid membrane (Spreitzer and Salvucci, 2002). During this experiment, MGD led to a reduction of RuBisCO abundance in the old leaves (Fig. 4). These results were partially expected since previous studies already showed the negative effects of MGD on RuBisCO's expression levels and catalytic activity (Yuguan et al., 2009a); the novelty of this study lies in proving that MGD affects RuBisCO also on a translational level.

Potentially promising results were obtained for the resupply variants. It was shown in this study for the first time how the resupply of Mg through the root or leaf supply has very discrete effects on the RuBisCO abundance of Mg-deficient plants (Fig. 4). The former caused a slight response only in the old leaves, while the latter was notably able to return enzyme abundance to control levels. This effect was already evident in the old leaves but became even more pronounced in the young leaves.

The reason for the different efficacy of the two resupply methods might be ascribed to the distance that Mg had to travel before interacting with the chloroplasts. The foliar application delivered Mg directly to the site where MGD had the most detrimental effect, which coincidentally is also the location of RuBisCO transcription and translation.

Conclusions

Mg deficiency leads to a large reduction in RuBisCO abundance, as well as in the total content of Mn, Zn, Fe and Cu. The foliar resupply of $MgSO_4$ was shown to cause a marked increase of these physiological traits, proving that Mg application to the leaves up-regulates the process of photosynthesis. While the resupply of Mg to the root was shown to have a better effect on the overall biomass formation of Mg-deficient plants, compared to the foliar application, both methods appear to be suited as a rescue measure against the detrimental effects of MGD.

References

Billard, V., Maillard, A., Coquet, L., Jouenne, T., Cruz, F., Garcia-Mina, J.M., Yvin, J.C., Ourry, A., Etienne, P., 2016: Mg deficiency affects leaf Mg remobilization and the proteome in *Brassica napus*. Plant Physiol. Bioch. 107, 337-343. DOI: 10.1016/j.plaphy.2016.06.025

Broadley, M., Brown, P., Cakmak, I., Rengel, Z., Zhao, F., 2012: Function of nutrients: micronutrients. In: Marschner, H. (ed.), Mineral nutrition of higher plants, 3rd ed., 191-248. New York, U.S.A.: Elsevier. DOI: 10.1016/c2009-0-63043-9

Cakmak, I., Kirby, E.A., 2008: Role of magnesium in carbon partitioning and alleviating photooxidative damage. Physiol. Plant. 133, 692-704. DOI: 10.1111/j.1399-3054.2007.01042.x

Cakmak, I., Yazici, A.M., 2010: Magnesium: a forgotten element in in crop

production. Better Crops 94, 22-24. DOI: 10.1071/cpv66n12_fo

Chen, Z.C., Peng, W.T., Li, J., Liao, H., 2018: Functional dissection and transport mechanism of magnesium in plants. Semin. Cell Dev. Biol. 74, 142-152. DOI: 10.1016/j.semcdb.2017.08.005

Ellis, R.J., 1979: The most abundance protein in the world. Trends Biochem. Sci. 4, 241-244. DOI: 10.1016/0968-0004(79)90212-3

Farhat, N., Elkhouni, A., Zorrig, W., Smaoui, A., Abdelly, C., Rabhi, M., 2016: Effects of magnesium deficiency on photosynthesis and carbohydrate partitioning. Acta Physiol. Plant. 38, 145. DOI: 10.1007/s11738-016-2165-z

Hanstein, S., Wang, X., Qian, X., Friedhoff, P., Fatima, M., Shan, Y., Feng, K., Schubert, S., 2011: Changes in citosolic Mg^{2+} levels can regulate the activity of the plasma membrane H^+-ATPase in maize. Biochem. J. 435, 93-101. DOI: 10.1042/bj20101414

Hermans, C., Johnson, G.N., Strasser, R.J., Verbruggen, N., 2004: Physiological characterization of magnesium deficiency in sugar beet: acclimation to low magnesium differentially affects photosystems I and II. Planta 220, 344-355. DOI: 10.1007/s00425-004-1340-4

Hermans, C., Vuylsteke, M., Coppens, F., Cristescu, S.M., Harren, F.J., Inzé, D., Verbruggen, N., 2010: Systems analysis of the responses to long-term magnesium deficiency and restoration in *Arabidopsis thaliana*. New Phytol. 187, 132-144. DOI: 10.1111/j.1469-8137.2010.03257.x

Hu, H., Sparks, D., 1991: Zinc deficiency inhibits chlorophyll synthesis and gas exchange in 'Stuart' pecan. Horticult. Sci. 26, 267-268.

Jezek, M., Geilfus, C.M., Bayer, A., Mühling, K.H., 2015a: Photosynthetic capacity, nutrient status, and growth of maize (*Zea mays* L.) upon $MgSO_4$ leaf-application. Front. Plant Sci. 5, 781. DOI: 10.3389/fpls.2014.00781

Jezek, M., Geilfus, C.M., Mühling, K.H., 2015b: Glutamine synthetase activity in leaves of *Zea mays* L. as influenced by magnesium status. Planta 242, 1309-1319. DOI: 10.1007/s00425-015-2371-8

Jung, S., Hütsch, B.W., Schubert, S., 2017: Salt stress reduces kernel number of corn by inhibiting plasma membrane H^+-ATPase activity. Plant Physiol. Biochem. 113, 198-207. DOI: 10.1016/j.plaphy.2017.02.009

Kannan, S., 2010: Foliar fertilization for sustainable crop production. In: Lichtfouse, E. (ed.), Genetic engineering, fertilisation, soil quality and organic farming, 371-402. Heidelberg, Germany: Springer. DOI: 10.1007/978-90-481-8741-6

Kramer, P.J., Boyer, J.S., 1995: Water relations of plants and soils, 1st ed., 291-340. New York, U.S.A.: Elsevier. DOI: 10.1016/b978-012425060-4/50003-6

Kobayashi, N.I., Tanoi, K., 2015: Critical issues in the study of magnesium transport systems and magnesium deficiency symptoms in plants. Int. J. Mol. Sci. 16, 23076-23093. DOI: 10.3390/ijms160923076

Ksiezak J., Bojarszczuk, J., Staniak, M., 2008: The productivity of maize and sorghum yields depending on the level of nitrogen fertilization. Polish. J. Agron., 8-20

Ksiezak J., Matyka, M., Bojarszczuk, J., Kacprzak, A., 2012: Evaluation of productivity of maize and sorghum to be used for energy purposes as influenced by nitrogen fertilization. Zemdribyste 99, 363.

Maksymiec, W., 1997: Effect of copper on cellular processes in higher plants. Photosynthetica 34, 321-342.

Marsch, H.V., Evans, H.J., Matrone, G., 1963: Effect of iron deficiency on chlorophyll synthesis. Plant Physiol. 38, 638-642.

Mengutay, M., Ceylan, Y., Kutman, U.B., Cakmak, I., 2013: Adequate magnesium nutrition mitigates adverse effects of heat stress on maize and wheat. Plant Soil 368, 57-72. DOI: 10.1007/s11104-013-1761-6

Neuhaus, C., Geilfus, C.M., Mühling, K.H., 2014: Increasing root and leaf growth and yield in Mg-deficient faba beans (*Vicia faba* L.) by $MgSO_4$ foliar fertilization. J. Plant Nutr. Soil Sci. 177, 741-747. DOI: 10.1002/jpln.201300127

Neuhaus, C., Geilfus, C.M., Zörb, C., Mühling, K.H., 2013: Transcript expression of Mg-chelatase and H^+-ATPase isogenes in *Vicia faba* leaves as influenced by root and foliar magnesium supply. Plant Soil 368, 41-50. DOI: 10.1007/s11104-013-1711-3

Pechanova, O., Takak, T., Samaj, J., Pechan, T., 2013: Maize proteomics: an insight into the biology of an important cereal crop. Proteomics. 13, 637-662. DOI: 10.1002/pmic.201200275

Ranum, P., Peña-Rosas, J.P., Garcia-Casal, M.N.-C., 2014: Global maize production, utilization, and consumption. Ann. N. Y. Acad. Sci. 1312, 105-12. DOI: 10.1111/nyas.12396

Raven, J.A., Evans, M.C.W., Korb, R.E., 1999: The role of trace metals in photosynthetic electron transport in O_2-evolving organisms. Photosynth. Res. 60, 111-149.

Römheld, V., Marschner, H., 1991: Functions of micronutrients in plants. In: Mortvedt, J.J. et al. (eds.), Micronutrients in agriculture, 297-328. SSSA Book Series 4. 2th ed.. Madison, Wisconsin, USA: SSSA. DOI: 10.2136/sssabookser4.2ed.c9

Sabeeha, S., 2010: The elements of plant micronutrients. Plant Physiol. 154, 512-515. DOI: 10.1104/pp.110.161810

Senbayram, M., Gransee, A., Wahle, V., Thiel, H., 2015: Role of magnesium fertilizer in agriculture: Plant-soil continuum. Crop Pasture Sci. 66, 1219-1229. DOI: 10.1071/cp15104

Shenker, M., Plessner, O.E., Tel-Or, E., 2004. Manganese nutrition effects on tomato growth, chlorophyll concentration, and superoxide dismutase activity. J. Plant Physiol. 161, 197-202. DOI: 10.1078/0176-1617-00931

Spreizer, R.J., Salvucci, M.E., 2002: Rubisco: structure, regulatory interactions, and possibilities for a better enzyme. Ann. Rev. Plant Biol. 53, 449-475. DOI: 10.1146/annurev.arplant.53.100301.135233

Vrataric, M., Sudaric, A., Kovacevic, V., Duvnjak, T., Krizmanic, M., Mijic, A., 2006. Response of soybean to foliar fertilization with magnesium sulfate (epsom salt). Cereal Res. Commun. 34, 709-712. DOI: 10.1556/crc.34.2006.1.177

Wanli, G., Nazim, H., Liang, Z., Yang, D., 2015. Magnesium deficiency in plants: an urgent problem. Crop Journal 4, 83-91. DOI: 10.1016/j.cj.2015.11.003

Yin, S., Ze, Y., Liu, C., Li, N., Zhou, M., Duan, Y., Hong, F., 2009: Cerium relieves the inhibition of nitrogen metabolism of spinach caused by magnesium deficiency. Biol. Trace Elem. Res. 132, 247-258. DOI: 10.1007/s12011-009-8392-z

Yuguan, Z., Min, Z., Luyang, L., Zhe, J., Chao, L., Sitao, Y., Yanmei, D., Na, L., Fashui, H., 2009a: Effects of Cerium on key enzymes of carbon assimilation of spinach under magnesium deficiency. Biol. Trace Elem. Res. 131, 154-164. DOI: 10.1007/s12011-009-8354-5

Yuguan, Z., Yin, S., Ji, Z., Luo, L., Liu, C., Hong, F. 2009b: Influences of magnesium deficiency and cerium on antioxidant system of spinach chloroplasts. Biometals 22, 941-949. DOI: 10.1007/s10534-009-9246-z

Zhao, H., Zhou, Q., Chou, M., Li, C., Gong, X., Liu, C., Qu, C., Si, W., Hong, F., 2012: Magnesium deficiency results in damage of nitrogen and carbon cross-talk of maize and improvement by cerium addition. Biol. Trace Elem. Res. 148, 102-109. DOI: 10.1007/s12011-012-9340-x

Zörb, C., Stracke, B., Tramnitz, B., Denter, D., Sümer, A., Mühling, K.H., Yan, F., Schubert, S., 2005: Does H^+ pumping by plasmalemma ATPase limit leaf growth of maize (*Zea mays*) during the first phase of salt stress? J. Plant Nutr. Soil Sci. 168, 550-557. DOI: 10.1002/jpln.200520503

ORCID:
Britta Pitann https://orcid.org/0000-0002-5476-3244
Karl H. Mühling https://orcid.org/0000-0002-9922-6581

Address of the corresponding author:
Prof. Dr. Karl H. Mühling, Institute of Plant Nutrition and Soil Science, Kiel University, Hermann-Rodewald-Straße 2, 24118 Kiel, Germany
E-mail: khmuehling@plantnutrition.uni-kiel.de