



Magnesium deficiency decreases biomass water-use efficiency and increases leaf water-use efficiency and oxidative stress in barley plants

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Abstract

Aims In water-scarce agro-environments a clear understanding of how plant nutrients like magnesium (Mg) affect plant traits related to water-use efficiency (WUE) is of great importance. Magnesium plays a crucial role in photosynthesis and is thus a major determinant of

biomass formation. This study investigated the effect of Mg deficiency on leaf and whole plant water-use efficiency, $\delta^{13}\text{C}$ composition, hydrogen peroxide (H_2O_2) production and the activity of key enzymes involved in ROS scavenging in barley.

Methods Barley (*Hordeum vulgare*) was grown in hydroponic culture under three different levels of Mg supply (0.01, 0.1, 0.4 mM Mg). WUE was determined on the leaf-level (leaf-WUE), the biomass-level (biomass-WUE) and via carbon isotope discrimination ($\delta^{13}\text{C}$). Additionally, concentrations of Mg, chlorophyll and H_2O_2 , and the activities of three antioxidative enzymes (ascorbate peroxidase, glutathione reductase and superoxide dismutase) in youngest fully expanded leaves were analyzed.

Results Dry matter production was significantly decreased (by 34 % compared to control) in Mg deficient barley plants. Mg deficiency also markedly reduced leaf Mg concentrations and chlorophyll concentrations, but increased H_2O_2 concentrations (up to 55 % compared to control) and the activity of antioxidative enzymes. Severe Mg deficiency decreased biomass-WUE by 20 %, which was not reflected regarding leaf-WUE. In line with leaf-WUE data, discrimination against ^{13}C (indicating time-integrated WUE) was significantly reduced under Mg deficiency.

Conclusions Mg deficiency increased oxidative stress indicating impairment in carbon gain and decreased biomass-WUE. Our study suggests that biomass-WUE was not primarily affected by photosynthesis-related processes, but might be dependent on effects of Mg on night-time transpiration, respiration or root exudation.

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Introduction

The increasing world population and the concomitant decrease in water availability due to global climate change and land use change (Pachauri and Meyer 2014) raise an increasing challenge for higher agricultural crop productivity. A key factor limiting plant productivity under drought stress is water-use efficiency (WUE) of crop plants. In general, WUE describes the ratio of assimilated carbon to water used by the plant and is thus, a measure for the efficiency in optimizing carbon assimilation while minimizing water use (Bramley et al. 2013). Usually, plants transpire much higher amounts of water compared to relatively small amounts of carbon that are fixed; between 200 and 1000 g of water are transpired per g of assimilated carbon (Bramley et al. 2013). Manipulating the ratio towards higher efficiency might contribute to the stabilization of yields under present and future conditions of diminishing fresh water supply and increasing food demand.

Water-use efficiency is a rather flexible term depending on the scale being considered, e.g. at the leaf or whole plant level. Biomass-WUE takes the whole plant into account and is defined as plant dry matter production per unit of water loss via transpiration during the vegetation period (Tallec et al. 2013). Biomass-WUE is affected by numerous factors related to biomass formation (e.g. photosynthesis, respiratory carbon loss) and whole plant transpiration (transpiration and unproductive water-loss by e.g. nocturnal transpiration) (Claussen 2002; Wang et al., 2013). Under field conditions, only aboveground biomass is considered for calculation of WUE (shoot-WUE), whereas in greenhouse experiments, where roots can be harvested, total biomass can be used for calculation of WUE (Bramley et al. 2013). Thus, biomass-WUE can be calculated as: Biomass-WUE = Total biomass (g) / Water use (L). Substantial variation in biomass-WUE under variable environmental conditions (e.g. nutrient supply, water scarcity, elevated CO₂ concentrations) have been reported (Cernusak et al. 2009; Lewis et al. 2011).

At the leaf level, intrinsic water-use efficiency (leaf-WUE) is defined as the instantaneous ratio between net CO₂ assimilation rate (A) and stomatal conductance (g_s): Leaf-WUE = A/g_s, whereas g_s is the ratio of transpiration to air-to-leaf vapor pressure deficit. The carbon

stable isotope composition of the plant dry matter ($\delta^{13}\text{C}$) is used as time-integrated indicator for WUE as it represents a measure of plant C assimilation over the period during which dry matter is generated (Wang et al. 2013). Low carbon isotope discrimination ($\Delta^{13}\text{C}$) is seen as indicator of high leaf-WUE (Farquhar et al. 1982) and it has been commonly used as an indicator of leaf-WUE in wheat (Farquhar and Richards 1984), poplar (Rasheed et al. 2013) and tobacco (Brueck and Senbayram 2009). However, the relationship between $\Delta^{13}\text{C}$ and leaf-WUE can be unbalanced by differences in the respective time of integration (Ripullone et al. 2004) or by variable mesophyll diffusion conductances (g_m) (Warren and Adams 2006; Soolanayakanahally et al. 2009).

The different scales at which both types of WUE (at a leaf or whole plant level) are measured, might lead to discrepancies in upscaling leaf-WUE to biomass-WUE (Medrano et al. 2015) because components like canopy effects, night-time transpiration and respiration affect WUE differently. Night-time transpiration increases water use without concomitant carbon assimilation, thus lowering biomass-WUE. Medrano et al. (2015) reported that night-time transpiration accounted for 10 % of the daily transpiration in grapevine. In addition, respiratory processes lead to carbon loss, thus reducing net carbon gain and also lowering biomass-WUE. Plant respiration plays a crucial role in carbon balance and might be a main unknown factor when comparing leaf-WUE and biomass-WUE (Medrano et al. 2015).

Water-use efficiency can be improved by plant breeding or different agronomical practices regarding soil and plant nutrient management (Blum 2009). Positive effects of adequate nitrogen (Shangguan et al. 2000; Brueck and Senbayram 2009) and potassium supply (Fournier et al. 2005; Arquero et al. 2006) on biomass-WUE and leaf-WUE were reported, but knowledge on how Mg deficiency may affect WUE in crop plants is scarce.

Magnesium as one of the essential plant nutrients, is the most abundant divalent cation in cellular systems (Li et al. 2001). Free Mg²⁺ ions stabilize membranes and are involved in activation of numerous enzymes, among them ATPases and ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Li et al. 2001; Shaul 2002). Magnesium is essential for chlorophyll synthesis and up to 10 % of total Mg can be associated with chlorophyll (Wilkinson et al. 1990). Furthermore, Mg is important for grana stacking in chloroplasts (Hall et al. 1972; Ceppi et al. 2012) that may adversely affect photosynthetic performance of plants suffering from

low Mg supply. It was reported that the degree of stacking increases with increasing Mg concentrations (Stys 1995) and Mg deficiency leads to disruption of grana stacks (Hall et al. 1972). Such involvements of Mg demonstrate its irreplaceable functions in photosynthesis. A reduction of dry matter production under Mg deficiency was observed in various plants, such as bean (Fischer and Bremer 1993; Cakmak et al. 1994a), sugar beet (Hermans et al. 2004), rice (Ding et al. 2006), *Arabidopsis* (Hermans and Verbruggen 2005). One of the earliest responses of plants to Mg deficiency is impaired phloem loading (Cakmak et al. 1994a). Photo-assimilates are not sufficiently transported from source leaves into sink tissues such as shoot tips, seeds and roots, leading to an accumulation of sucrose and starch in source leaves. This accumulation of carbohydrates commonly occurs before visible Mg deficiency symptoms develop, e.g. interveinal chlorosis and necrotic lesions on leaves and reduction in shoot biomass and also before any distinct change in photosynthetic performance of plants is detectable (Cakmak and Kirkby 2008; Verbruggen and Hermans 2013). The sucrose accumulation may trigger a downregulation of genes involved in photosynthesis (Jang and Sheen 1994) or a carbon-metabolite feedback inhibition of photosynthesis, whereas both processes inevitably decrease assimilation. A decrease in assimilation is also caused by reduced activities of enzymes involved in CO₂ fixation (Cakmak and Kirkby 2008). Decreased capacity to fix CO₂ leads to an overreduction of the photosynthetic electron transport chain, initiating the photoreduction of dioxygen (O₂) to superoxide (O₂⁻) and subsequently the catalysis of superoxide to H₂O₂ by superoxide dismutase (Asada 1999). H₂O₂ is suggested to act as a signal messenger (Møller et al. 2007), being involved in abiotic and biotic stress response (Cheeseman 2007). However, increased levels of H₂O₂ cause oxidative damage to cell components (Foyer and Noctor 2011). Additionally, the presence of H₂O₂ inhibits the activity of enzymes involved in photosynthesis, in particular RubisCO (Asada 1999), further limiting the photon-utilization capacity and ultimately enhancing the production of reactive oxygen species. Enhanced H₂O₂ generation is also typical under low water supply, contributing to ABA-induced stomatal closure (Hu et al. 2006; Wang and Song 2008), lipid peroxidation and chlorophyll degradation (Farooq et al. 2009; Foyer and Shigeoka 2011). Under such conditions, detoxification of H₂O₂ is extremely important and it is achieved by

ascorbate peroxidase (APX) in the chloroplast (Foyer and Noctor 2011). APX reduces H₂O₂ to H₂O at the expense of ascorbate which is subsequently regenerated either by oxidation of monohydroascorbate or by reduction of dehydroascorbate. For the latter, glutathione serves as the reductant which is generated from reducing glutathione sulfide by glutathione reductase (GR) (Polle 2001). Enhanced activities of the antioxidant enzymes indicate photooxidative stress as commonly observed under Mg deficiency (Cakmak and Kirkby 2008). An up-regulation of antioxidant enzyme activities and antioxidant metabolites was observed in common bean (Cakmak and Marschner 1992), citrus (Tang et al. 2012), wheat (Mengutay et al. 2013) and maize (Tewari et al. 2006).

In the future, incidences and duration of drought are predicted to increase, posing a risk to agricultural production and yield stability. Plants suffering Mg deficiency are assumed to be more sensitive to drought and adequate Mg supply is needed for optimal yield formation under drought situations (Senbayram et al. 2015a). Hence, a clear understanding of how Mg affects plant traits related to water-use efficiency (WUE) is of great importance. To our knowledge, there are no published reports on effects of Mg-deficiency on leaf- and biomass-WUE. For several reasons, WUE of plants can be adversely affected by the Mg nutritional status of plants. For example, it is well known that an adequate Mg nutrition is required for stomatal conductance of plants (Laing et al. 2000; Cakmak and Kirkby 2008). In addition, well-documented reductions in root growth due to impairments in carbon partitioning into roots (Cakmak et al. 1994a; Hermans et al. 2004) may greatly affect root water uptake from growth medium as suggested by Cakmak and Kirkby, (2008) and Senbayram et al. (2015a, b). This study has been conducted to increase knowledge regarding direct effect of varying Mg supply on biomass-WUE and related parameters, including carbon assimilation, carbon isotope discrimination and ascorbate peroxidase activity in combination with the capacity to form H₂O₂ under non-limiting water supply.

Materials and methods

Plant culture

Seeds of *Hordeum vulgare* L cv. Şahin-91 were germinated in wetted paper rolls in the greenhouse and

seedlings were transferred to hydroponic plant culture using 5 l pots (2 plants per pot). In order to avoid osmotic shock, seedlings were grown in half-strength nutrient solution for the first 5 days, then transferred to 75 % of full-strength nutrient solution for another 6 days before supplying full-strength nutrient solution. Full-strength nutrient solution contained 2 mM K_2SO_4 , 3 mM NH_4NO_3 , 1 mM $MgSO_4 \cdot 7 H_2O$, 1 mM $CaCl_2 \cdot 2 H_2O$, 0.25 mM $Ca(H_2PO_4)_2 \cdot H_2O$, 0.1 mM Fe-EDTA, 25 μM H_3BO_3 , 2 μM $ZnSO_4 \cdot 7 H_2O$, 2 μM $MnSO_4 \cdot H_2O$, 0.5 μM $CuSO_4 \cdot 5 H_2O$, 0.075 μM $H_{24}Mo_7N_6O_{24} \cdot 4 H_2O$. 24 days after germination, three magnesium levels were introduced: 0.01 mM (Mg_{low}), 0.1 mM (Mg_{med}) and 0.4 mM $MgSO_4 \cdot 7 H_2O$ (Mg_{high}). In order to avoid nutrient depletion, nutrient solutions were renewed every 5 days at the beginning of the experiment and every 2 days at later growth stages. Nutrient solutions were permanently aerated.

Determination of Mg concentrations, SPAD and $\delta^{13}C$

Plants were harvested 34 days after onset of treatments (DAO), separated into roots and shoots and dried at 60 °C for dry matter (DM) determination. In order to assess chlorophyll concentrations, SPAD readings (Konica Minolta, Japan) were taken on youngest fully expanded leaves. For determination of magnesium concentration, 100 mg of dried plant material was digested in 4 ml concentrated HNO_3 and 2 ml 30 % H_2O_2 at 200 °C and 15 bar for 75 min. Magnesium concentrations were measured by ICP-OES (Vista RL, CCD simultaneous ICP-OES, Varian Inc., USA) and atomic absorption spectrometry (220 FS, Varian Inc., USA).

The carbon isotope discrimination ($\Delta^{13}C$) was analyzed as a measure of time-integrated leaf-WUE. The ratio of ^{13}C to ^{12}C in shoot dry matter and in young leaves was determined after Dumas combustion on a ThermoFinnigan Delta Plus IRMS (ThermoFinnigan, Bremen, Germany). $\delta^{13}C$ was calculated by relating the measured isotopic ratio to Vienna PeeDee Belemnite lime stone formation (VPDB) (according to Smith and Epstein 1971). For analyzing the relationship between leaf-WUE and leaf $\delta^{13}C$ values, mean leaf-WUE was calculated via temporal integration. For analyzing the relationship between total biomass-WUE and leaf $\delta^{13}C$ values, two additional replications of Mg_{low} and Mg_{high} were included in the dataset. These two additional replications were harvested on 20 DAO and total biomass-WUE was determined as described below.

Determination of hydrogen peroxide concentrations and ascorbate peroxidase activity

Determination of H_2O_2 concentration was conducted using ferrous ammonium sulfate xylenol orange (FOX) solution described by Wolff (1994) and modified by Cheeseman (2009). Briefly, 3 leaf discs were taken from young leaves using a cork borer (0.46 cm²) and were transferred to 1 ml acetone acidified by adding 25 mM H_2SO_4 . Samples were frozen in liquid nitrogen. For measurements, FOX solution containing 250 μM ferrous ammonium sulfate, 100 mM sorbitol, 100 μM xylenol orange and 25 mM H_2SO_4 was prepared prior to thawing the samples at room temperature for 45 min. 1 ml of FOX solution was added to 50 μl of each sample which were previously dissolved in acidified acetone. Samples were incubated at room temperature for 30–45 min. H_2O_2 was quantified spectrometrically (EPOCH, BioTec, USA/8453 UV-VIS Spectroscopy System, Agilent, USA) at 550 nm and subtracting the background at 850 nm using a standard curve ranging from 0 to 100 μM .

For measurement of ROS scavenging enzyme activities, leaf samples were harvested and immediately frozen in liquid nitrogen. 0.5 g of samples were homogenized in 5 ml phosphate buffer (pH 7.6) including 1 % polyvinylpyrrolidone (PVP) and 0.1 mM EDTA and centrifuged for 20 min at 16,000 g at 4 °C. The supernatant was collected and used as crude extract in the reaction mixtures of the enzyme activity assays.

For ascorbate peroxidase (APX) assay, the 0.3 ml reaction mixture contained 0.5 mM ascorbic acid, 50 mM phosphate buffer, 1 mM EDTA, 0.5 mM H_2O and 10–15 μl of the supernatant. The reaction was started by adding 10 μl of 15 mM of H_2O_2 and APX was assayed spectrometrically (EPOCH, BioTec, USA/8453 UV-VIS Spectroscopy System, Agilent, USA) following the decrease of absorbance at 290 nm (Nakano and Asada 1981).

Glutathione reductase (GR) was assayed according to Halliwell and Foyer (1978) with slight modifications. The 0.3 ml reaction mixture contained 0.2 mM nicotinamide adenine dinucleotide phosphate (NADPH), 1 mM GSSG (glutathione disulfide; oxidized form of glutathione), 50 mM K-P buffer (pH 7.6) with 0.1 mM EDTA and 10–15 μl of crude extract. GR was determined following the decrease in absorbance at 340 nm as NADPH was oxidized.

The background was corrected by observing the non-enzymatic oxidation of NADPH in the absence of GSSG. Superoxide dismutase (SOD) activity was determined according to Giannopolitis and Ries (1977) with small modifications. The 0.3 ml reaction mixture contained 50 mM phosphate buffer, 0.1 mM EDTA, 50 mM Na₂CO₃, 12 mM L-methionine, 75 μM nitroblue tetrazolium (NBT), 2 μM riboflavin and 10–20 μl of the enzyme extract. Riboflavin was added at last and the samples were placed under fluorescent light (4000 lx) for 10 min. Following that, the inhibition of photoreduction of NBT by SOD was measured at 560 nm. Blank samples with no crude extract were considered having the highest reaction rate of super oxide with NBT. One unit of SOD activity is defined as the amount of enzyme required to cause 50 % inhibition of the rate of NBT reduction at 560 nm.

Gas exchange measurements and calculation of leaf water-use efficiency

Net assimilation and stomatal conductance were determined on youngest fully expanded leaves (GFS-3000, Heinz Walz GmbH, Germany). Cuvette conditions were set as follows: 22 °C, 55 % rel. humidity, 380 ppm CO₂, photosynthetic photon flux density of 1000 μmol m⁻² s⁻¹ generated by blue and red LEDs. After reaching stable values due to leaf adjustment to cuvette conditions (after 30 to 45 min.), fluxes were averaged over 5 min. Leaf water-use efficiency (leaf-WUE) was determined by relating net assimilation to stomatal conductance.

Calculation of transpiration and biomass water-use efficiency

Daily whole plant transpiration was assessed by measuring daily weight differences of the pots. Each pot was placed on a balance (TQ30, ATP Messtechnik, Germany), automatically recording the weight in an interval of 30 min. in a one-gram-resolution. As pots were sealed, weight reduction of pots was solely caused by transpiration of plants. Cumulative transpiration was calculated by summing up daily weight differences. Shoot biomass water-use efficiency (shoot biomass-WUE) was determined by relating the shoot dry mass to the cumulative transpiration and total biomass water-use efficiency

(total biomass-WUE) was determined by relating the total plant dry mass to the cumulative transpiration.

Modelling plant growth and daily shoot water-use efficiency

To allow comparison of daily shoot-WUE an empirical model procedure was developed to estimate daily biomass production from leaf area (LA) development. For this purpose, 30 pots of barley plants were grown in a preliminary trial under conditions similar to those of the main experiment. Plant images were taken from a fixed position (defined distance and angle) at least twice a week in front of a black background using a digital single-lens reflex camera (Canon EOS 600D, Canon Inc., Japan). The area of green pixels in each picture was calculated using ImageJ software (Rasband 1997). After the imaging procedure, plants of two pots were harvested and total leaf area per pot was determined using a desktop scanner together with ImageJ. Measured LA of the harvested pots was plotted against the respective area of green pixels and a second order polynomial was fitted (see Online Resource 1). The parameters of the polynomial were used for calculating LA per pot from plant images only. The linear regression between observed and predicted LA together with the mean absolute predictive discrepancy (MD) were used to indicate the goodness of fit ($r^2 = 0.98$, $p < 0.001$, MD = 87.1 cm²). During the main experiment, LA was imaged at least once a week. To obtain daily values of LA development, three-parametric logistic growth curves of the shape $f(x) = \frac{a}{1+b e^{(-kx)}}$ (where a, b, k are the estimated parameters of the function and t is temporal component) were calculated from iterative non-linear least-square regression using the *nls*-function implemented in R (R Core Team 2014). Daily shoot dry matter was estimated assuming that growth curves of shoot dry matter production follow the same curve progression as leaf area production.

The logistic curves obtained from the imaging of leaf area development were fitted to three discrete data points along the experimental period where DM per pot was known (start of experiment, second harvest) or could be estimated (first harvest). Parameters a and b were re-fitted, k was considered constant. For estimating dry matter at the first harvest date, the ratio of LA to shoot DM was calculated from plants harvested on that day. Mean ratio per treatment was then used to calculate

shoot DM for each pot not harvested on that day with respect to treatments. The resulting curves of total daily dry matter per pot were differentiated to obtain values of dry matter production per day and pot (daily DM) (see Online Resource 2).

Daily shoot-WUE was calculated by relating daily DM increase to daily whole plant transpiration.

Statistical analyses

Statistical analyses were performed using R version 3.0.3 (R Core Team 2014). Analysis of variance (ANOVA) was performed to determine whether effects of treatments on the respective factor were significant, followed by Duncan's post-hoc test ($\alpha = 0.05$) where ANOVA indicated significance. Data were tested for normal distribution with Shapiro-Wilk-Test and, where necessary, transformed logarithmically. Data are displayed untransformed.

Results

Plant dry matter formation, Mg leaf concentrations and chlorophyll content

At the final harvest, 34 days after onset of treatments (DAO), plant total dry matter (DM), shoot DM and root DM decreased significantly with decreasing rate of Mg supply (Table 1). Total DM was about 73 % and 34 % lower in plants supplied with 0.01 mM Mg (Mg_{low}) and 0.1 mM Mg (Mg_{med}) as compared to plants treated with 0.4 mM Mg (Mg_{high}). The shoot/root ratio was unaffected by the rate of Mg supply (Table 1). Over all treatments, total DM production correlated well with

shoot Mg uptake ($r^2 = 0.923$) (Fig. 1) and leaf Mg concentrations were significantly lower in Mg_{low} and Mg_{med} when compared to Mg_{high} treated plants. This effect was more severe in older leaves than in younger leaves (Table 1). Highest Mg concentrations were measured in youngest fully expanded leaves in Mg_{high} treatment ($1.09 \pm 0.07 \text{ mg g}^{-1} \text{ DM}$). Mg concentrations in leaves of medium and low Mg supplied plants were 37 % and 62 % lower than in plants treated with Mg_{high} 34 DAO, respectively. However, in older leaves, Mg concentrations in Mg_{low} ($0.25 \pm 0.01 \text{ mg g}^{-1} \text{ DM}$) and Mg_{med} ($0.45 \pm 0.08 \text{ mg g}^{-1} \text{ DM}$) treated plants were 74 % and 53 % lower compared to plants supplied with Mg_{high} . Leaf SPAD values that were recorded to assess leaf chlorophyll content were similar in all treatments until 9 DAO. Then, until 33 DAO, SPAD readings decreased steadily to 64 % and 57 % in Mg_{low} and Mg_{med} , but remained almost constant in Mg_{high} (Fig. 2).

Hydrogen peroxide concentration and ROS scavenging enzyme activity

Hydrogen peroxide concentrations in youngest fully expanded leaves of Mg_{low} plants were 40 % and 55 % higher than in Mg_{high} on 13 DAO and 33 DAO (Fig. 3a). Overall, the activities of ROS scavenging enzymes were higher in Mg deficient plants. 13 DAO, the activities of APX and GR were 4- and 3-fold higher in Mg_{low} than in Mg_{high} , and 33 DAO, they were 5- and 3-fold higher (Fig. 3b, c). Superoxide dismutase, the enzyme being responsible for the reduction of superoxide to hydrogen peroxide, showed highest activity in Mg deficient plants. In contrast to APX and GR, the SOD activity of Mg_{low} and Mg_{med} treatments did not differ from each other (Fig. 3d).

Table 1 Effect of Mg supply on total DM (g per pot), shoot DM (g per pot), root DM (g per pot), shoot-to-root ratio, Mg concentration (young and old leaves; $\text{mg g}^{-1} \text{ DM}$), $\delta^{13}\text{C}$, total biomass water-use efficiency (biomass-WUE, g L^{-1}) and shoot-WUE

(g L^{-1}) in low Mg (0.01 mM Mg), medium Mg (0.1 mM Mg), and high Mg (0.4 mM Mg) treated barley plants. Values are means \pm SE ($n = 3$). Means followed by the same small letter are not significantly different ($\alpha = 0.05$)

Mg supply (mM)	Dry matter			Shoot/root ratio	Mg concentration		Biomass-WUE	
	total (g)	shoot (g)	root (g)		young leaf ($\text{mg g}^{-1} \text{ DM}$)	old leaf ($\text{mg g}^{-1} \text{ DM}$)	total (g DM L^{-1})	shoot (g DM L^{-1})
0.01	$17.9 \pm 1.38 \text{ c}$	$15.56 \pm 1.22 \text{ c}$	$2.38 \pm 0.15 \text{ c}$	$6.53 \pm 0.11 \text{ a}$	$0.41 \pm 0.00 \text{ c}$	$0.25 \pm 0.01 \text{ b}$	$3.77 \pm 0.12 \text{ b}$	$3.27 \pm 0.10 \text{ b}$
0.1	$38.8 \pm 1.63 \text{ b}$	$33.36 \pm 1.21 \text{ b}$	$5.42 \pm 0.43 \text{ b}$	$6.20 \pm 0.29 \text{ a}$	$0.69 \pm 0.06 \text{ b}$	$0.45 \pm 0.08 \text{ b}$	$4.49 \pm 0.28 \text{ a}$	$3.86 \pm 0.22 \text{ a}$
0.4	$53.2 \pm 0.50 \text{ a}$	$46.06 \pm 0.59 \text{ a}$	7.08 ± 0.50	$6.58 \pm 0.54 \text{ a}$	$1.09 \pm 0.07 \text{ a}$	$0.96 \pm 0.14 \text{ a}$	$4.55 \pm 0.13 \text{ a}$	$3.95 \pm 0.14 \text{ a}$

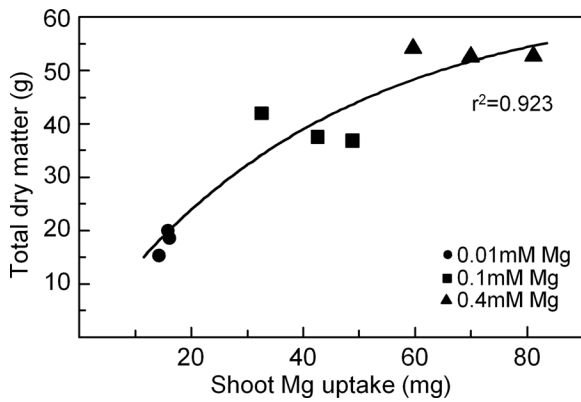


Fig. 1 Relationship between total DM (g per pot) and shoot Mg uptake in low Mg (0.01 mM Mg), medium Mg (0.1 mM Mg), and high Mg (0.4 mM Mg) treatments of barley plants

Leaf water-use efficiency

Temporal courses of net assimilation rate (A_N) and stomatal conductance (g_s) measured on youngest fully expanded leaves are presented in Fig. 4. In treatments Mg_{high} and Mg_{med} , mean A_N were 26.8 ± 0.64 and $21.2 \pm 0.27 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, and remained almost constant throughout the experimental period. However in Mg_{low} , A_N decreased rapidly and already starting 8 DAO, it was significantly lower than in the other Mg treatments. Here, A_N was $11.7 \pm 1.14 \mu\text{mol m}^{-2} \text{ s}^{-1}$ in Mg_{low} , being 59 % lower than in Mg_{high} . Stomatal conductance (g_s) decreased in all three Mg-supply treatments during the experimental period. However, the

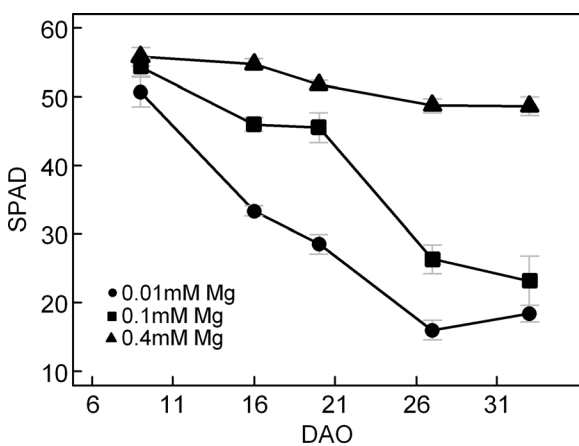


Fig. 2 SPAD values measured on old leaves in low Mg (0.01 mM Mg), medium Mg (0.1 mM Mg), and high Mg (0.4 mM Mg) treatments of barley plants. Means \pm SE ($n = 3$). DAO = days after onset of treatment

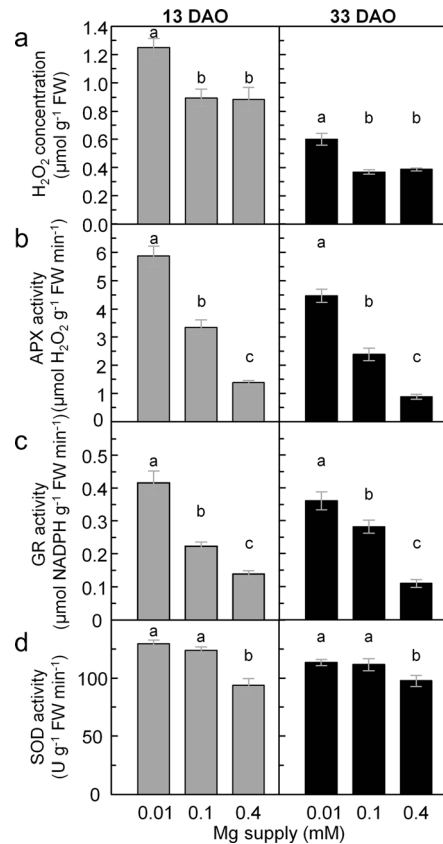


Fig. 3 Hydrogen peroxide (H_2O_2) concentrations (a), ascorbate peroxidase (APX) activity (b), glutathione reductase (GR) activity (c), and superoxide dismutase (SOD) activity (d) in low Mg (0.01 mM Mg), medium Mg (0.1 mM Mg), and high Mg (0.4 mM Mg) treatments of barley plants at 13 and 33 days after onset of treatment (DAO). Error bars represent standard errors ($n = 6$, $n = 12$ for hydrogen peroxide). Means labelled with the same small letter are not significantly different ($\alpha = 0.05$)

decrease in g_s was more pronounced in Mg_{low} . From 6 to 13 DAO in Mg_{low} , the g_s values decreased from $267.5 \pm 19.6 \text{ mmol m}^{-2} \text{ s}^{-1}$ to $125.8 \pm 24.9 \text{ mmol m}^{-2} \text{ s}^{-1}$ and remained almost constant thereafter. Interestingly, the decrease in g_s was more pronounced than the decrease in A_N and thus, affected the leaf-WUE (A_N/g_s) significantly. In Mg_{low} , leaf-WUE was $79.29 \pm 3.87 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ at 6 DAO, being already slightly higher than in Mg_{high} , and over time, it increased to $92.51 \pm 3.94 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ on 33 DAO. In Mg_{high} , leaf-WUE was $56.38 \pm 2.67 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ on 8 DAO and increased gradually to $88.8 \pm 10.44 \mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$ until 33 DAO (Fig. 4c). In Mg_{med} , leaf-WUE remained almost constant during the experiment, being similar to leaf-WUE in Mg_{high} , except for the measurement on 13 DAO.

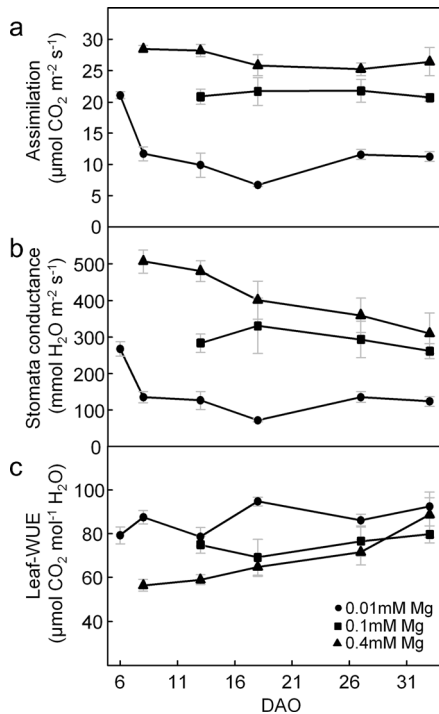
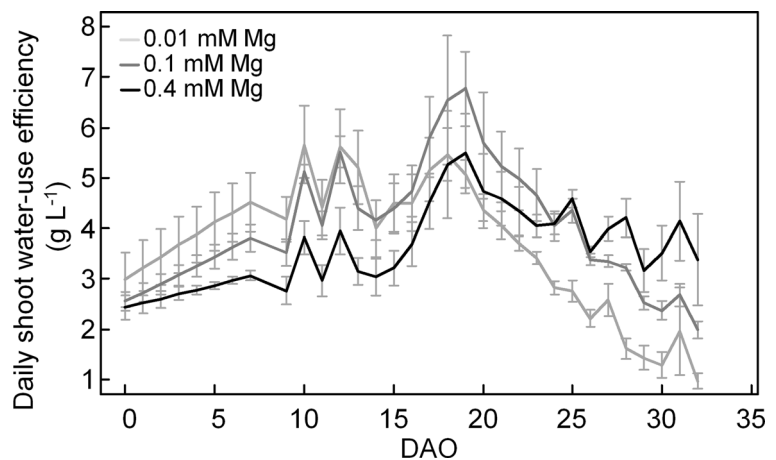


Fig. 4 Net assimilation (a), stomatal conductance (b) and leaf water-use efficiency (leaf-WUE) (c) in low Mg (0.01 mM Mg), medium Mg (0.1 mM Mg), and high Mg (0.4 mM Mg) treatments of barley plants. Symbols represent means \pm SE ($n = 3$). DAO = days after onset of treatment

Biomass water-use efficiency

Overall, total biomass-WUE of barley plants ranged from 3.77 to 4.55 g DM L⁻¹ H₂O on 34 DAO (Table 1). Here was no significant difference when comparing Mg_{high} and Mg_{med}, however, total biomass-WUE in Mg_{low} was significantly lower (17 % lower

Fig. 5 Daily shoot water-use efficiency in low Mg (0.01 mM Mg), medium Mg (0.1 mM Mg), and high Mg (0.4 mM Mg) treatments of barley plants. Represented are means \pm SE ($n = 3$). Error bars represent standard errors. DAO = days after onset of treatment



total biomass-WUE) than in both Mg_{high} and Mg_{med} (Table 1). The effect of Mg supply on shoot biomass-WUE followed the same trend as with total biomass-WUE (Table 1).

Daily shoot water-use efficiency

Daily shoot-WUE (DM production day⁻¹/transpiration day⁻¹ pot⁻¹) was calculated by estimating the biomass production and relating it to the measured daily transpiration. The daily shoot-WUE of all three treatments increased from treatment start and showed quite simultaneous maxima at day 18 and 19 (Fig. 5). These maximum daily shoot-WUE of Mg_{high}, Mg_{med} and Mg_{low} were 4.9, 5.8 and 5.6 g DM L⁻¹ H₂O. Subsequently, the daily shoot-WUE decreased until 32 DAO in all three treatments, however, the decline was much more pronounced in Mg_{low}. Here, daily shoot-WUE declined by 75 % to 1.4 g DM L⁻¹ H₂O at 32 DAO, thus having a daily shoot-WUE of only 44 % compared to the control.

Carbon isotope composition

The $\delta^{13}\text{C}$ values of the shoot biomass decreased with increasing Mg supply (Fig. 6a). Highest discrimination was observed in control plants and plants with medium Mg supply ($-30.47 \pm 0.37 \text{‰}$ and $-29.26 \pm 0.37 \text{‰}$). In Mg deficient plants, $\delta^{13}\text{C}$ values were significantly higher ($-27.66 \pm 0.14 \text{‰}$) compared to the control. $\delta^{13}\text{C}$ values of young leaves showed significant positive correlation with integrated leaf-WUE (Fig. 6b). However, there was no common relationship between shoot $\delta^{13}\text{C}$ values and total biomass-WUE (Fig. 6c), but

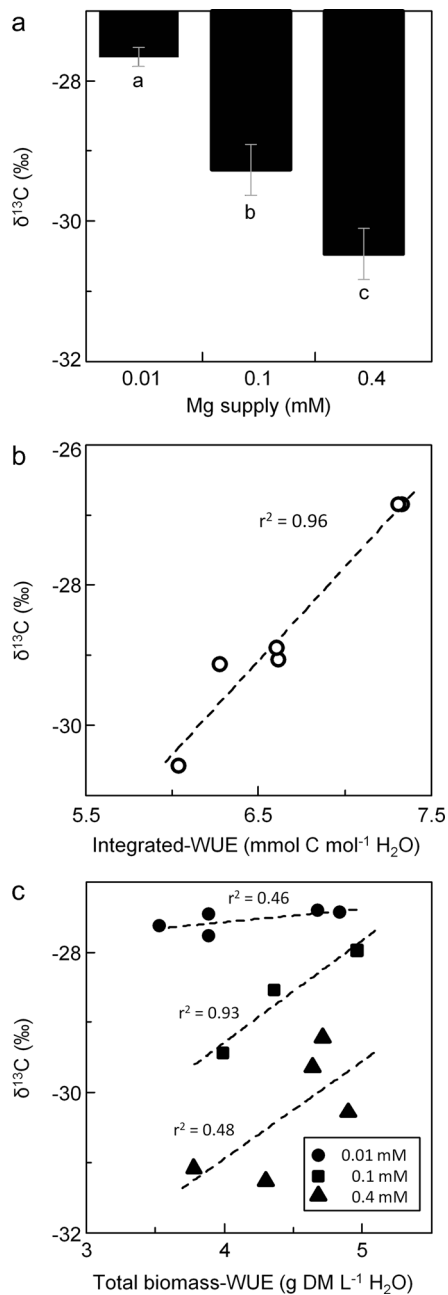


Fig. 6 $\delta^{13}\text{C}$ values of shoot (a), relationship between $\delta^{13}\text{C}$ of young leaves and integrated leaf water-use efficiency (leaf-WUE) (b), relationship between $\delta^{13}\text{C}$ of shoot and total biomass water-use efficiency (total biomass-WUE) (c) in low Mg (0.01 mM Mg), medium Mg (0.1 mM Mg), and high Mg (0.4 mM Mg) treatments of barley plants. DAO = days after onset of treatment. Error bars represent standard errors ($n = 3$). Means labelled with the same small letter are not significantly different ($\alpha = 0.05$)

individual linear relationships were found between shoot $\delta^{13}\text{C}$ and total biomass-WUE within plants of the same Mg treatment.

Discussion

Effect of Mg supply on chlorophyll content and dry matter formation

Being involved in many physiological and biochemical processes, Mg is an essential element for plant growth and development (Cakmak and Kirkby 2008; Cakmak and Yazici 2010; Cakmak 2013). As expected, plant total dry matter (DM) formation responded significantly to the various Mg supply regimes (Table 1). In a similar study on wheat, authors reported a decrease of 21 % in total DM within 23 DAO when concentration of supplied Mg decreased from 0.45 to 0.015 mM (Mengutay et al. 2013). Magnesium leaf concentrations were lower in older leaves than in younger leaves, an effect that is well known to be attributable to the phloem-mobile nature of Mg within the plant: Mg is thought to be relocated from the older to the younger leaves enabling the plant to start the generative phase in order to finish its life cycle. One of the major functions of Mg in plants is its role as the central atom of the chlorophyll molecule in the light absorbing complex of chloroplasts and its contribution to the photosynthetic carbon dioxide fixation (Cakmak and Kirkby 2008). In the present study, chlorophyll content of Mg-deficient plants was remarkably lower than in adequate Mg-supplied plants. Visual leaf symptoms of Mg deficiency appeared as interveinal leaf chlorosis being apparent first on older leaves. The latter progressed to the young leaves as the deficiency became more severe over time, which might be attributable to the phloem-mobile behavior of Mg (Hermans et al. 2013). Chlorosis and following necrosis might be, among other reasons, a consequence of increased ROS generation, such as hydrogen peroxide (H_2O_2). Increased levels of H_2O_2 under Mg deficiency might be attributed to a lower demand for reductants in the Calvin cycle and concomitant fully reduced photosynthetic electron carriers which results in an increased electron flow to O_2 . The reduced oxygen is then dismutated to H_2O_2 . In line with our data, higher H_2O_2 concentrations in Mg deficient maize leaves (Tewari et al. 2004) and mulberry plants (Tewari et al. 2006) were reported. An increased activity of key ROS scavenging enzymes (APX, SOD and GR) indicates an activation of the antioxidant machinery and an increased effort to reduce, viz. detoxify H_2O_2 in the chloroplasts. In line with the present study, enhanced activities of ROS scavenging enzymes under Mg deficiency were observed in maize

(Tewari et al. 2004), wheat (Mengutay et al. 2013), citrus (Tang et al. 2012; Yang et al. 2012) and bean (Cakmak 1994). The fact that H_2O_2 concentrations in Mg_{low} were higher than in Mg_{med} and Mg_{high} despite highest APX and GR activities indicates that the capacity of H_2O_2 scavenging was insufficient and thus, the generation of H_2O_2 might be one of the stress factors that contribute to growth reductions under severe Mg deficiency.

In the present experiment, the shoot/root ratio was similar in all treatments indicating no significant effect of Mg supply on DM allocation (Table 1). A number of reports showed an increased shoot/root ratio under Mg deficiency, for example in wheat and maize (Mengutay et al. 2013), bean (Cakmak et al. 1994b) and coffee (Meireles da Silva et al. 2014). Such increase in shoot/root ratio is commonly attributed to the negative effect of Mg deficiency on phloem loading and assimilate translocation. In contrast, in studies on *Arabidopsis* (Hermans and Verbruggen 2005) and sugar beet (Hermans et al. 2005) the shoot/root ratio did not change. So far, we do not have a clear explanation for this discrepancy; however, it may be related to the duration of the Mg deficiency and the vegetation period in our experiment. Verbruggen and Hermans (2013) suggested a more severe impact on root growth than on shoot growth, hence an increase in shoot/root ratio can only be observed when young plants are exposed to Mg deficiency. We may speculate that when plants are grown with sufficient Mg concentrations before exposing them to Mg deficiency, like in our study, no or less pronounced changes may occur. Currently, knowledge on the effect of Mg-deficiency on root development is still scarce, thus further research in this area is needed.

Leaf gas exchange and leaf water-use efficiency affected by Mg supply

In the present study, low Mg supply decreased both mean A_N and g_s significantly. Similarly, Terry and Ulrich (1974) reported a rapid decline in A_N due to Mg deficiency in sugar beet already 7 DAO. Similar results were obtained in studies on *Pinus* (Laing et al. 2000; Sun et al. 2001), citrus (Tang et al. 2012; Yang et al. 2012), sugar beet (Terry and Ulrich 1974) and maize (Jezek et al. 2015). Tang et al. (2012) proposed non-stomatal reasons for a decrease in A_N in citrus, as the internal CO_2 concentration did not differ from that of

plants supplied with adequate amounts of Mg. Significant decreases of A_N in Mg_{low} indicate that Mg deprivation did not only decrease the rate of leaf area expansion but also photosynthetic carbon gain per unit leaf area. This decrease in photosynthetic efficiency, which was most prominent in the Mg_{low} -treated plants, might also explain why excessive H_2O_2 formation was highest in the Mg_{low} treatment: it is very likely that an incomplete or blocked photosynthesis causally triggered the formation of oxygen radicals due to higher light intensities than sufficient to saturate photosynthetic processes under the experimental conditions. Mg deficiency induced decreases in A_N are commonly attributed to i) a decrease in chlorophyll content (Peaslee and Moss 1966), and ii) an accumulation of carbohydrates in leaves suffering Mg deficiency causing feedback inhibition of RubisCo activity, and higher mesophyll resistance towards CO_2 diffusion (Terry and Ulrich 1974). Furthermore, Mg deficiency leads to changes in the ultrastructure of chloroplasts as thylakoid stacks are disrupted (Hall et al. 1972). Ceppi et al. (2012) observed a decrease in maximum fluorescence intensity (F_M) during measurement of fast chlorophyll fluorescence induction kinetics on sugar beet. The authors attributed the decrease of F_M to the disruption of grana stacks and an increasing spillover of energy from photosystem (PS) II to PS I (Ceppi et al. 2012). Grana disruption diminishes the segregation of PSI and PSII (Stys 1995) and leads to quenching of PSII fluorescence (Murata 1969). This and the above mentioned reasons might explain the detrimental effects of Mg deficiency on photosynthesis.

Tang et al. (2012) and Yang et al. (2012) reported lower g_s in Mg deficient *Citrus* plants. A decrease of g_s was also reported by Kobayashi et al. (2013). In their study on rice plants, Mg deficiency had a more pronounced effect on transpiration rates, as the latter declined earlier than assimilation rates. Similarly in our study, the stomatal conductance was distinctly affected, as the decrease in g_s in Mg_{low} compared to Mg_{high} was more pronounced than the decrease in A_N , causing higher leaf-WUE. To our knowledge, there are no studies that analyze effects of varying Mg supply on leaf-WUE. For the first time, the presented data clearly indicate that severe Mg deficiency enhances leaf-WUE. Certainly, more details on the genetic nature of this relationship are needed to understand whether this is a common relationship or whether it is specific for barley.

Effect of Mg supply on biomass water-use efficiency

At the whole-plant level, WUE is defined as plant dry matter production per unit of water loss by transpiration (biomass-WUE). As in the calculation of shoot biomass-WUE root biomass production is not considered, shoot biomass-WUE is lower than total biomass-WUE in all three treatments, but significant differences remain comparing Mg_{low} and Mg_{high} . This trend is as expected since the shoot/root ratio did not change significantly with differing Mg levels. In the present study, there were no significant differences in either shoot- or total biomass-WUE measured on 34 DAO when comparing Mg_{med} and Mg_{high} , although biomass was about 34 % lower (34 DAO), but severe Mg deficiency decreased shoot- and total biomass-WUE by about 20 % on 34 DAO. It is commonly accepted that biomass-WUE increases under elevated nitrogen (Ripullone et al. 2004; Brueck and Senbayram 2009) and potassium supply (Römheld and Kirkby 2010; Grzebisz et al. 2013), but to our knowledge until today, there are no studies reporting on effects of Mg nutrition on biomass-WUE. Yield depression of barley in Mg_{med} was significant (34 %) when compared to Mg_{high} , however, biomass-WUE was similar in both treatments. This clearly suggests that only severe Mg deficiency affects biomass-WUE.

Surprisingly, shoot- and total biomass-WUE and leaf-WUE were showing opposite trends: shoot- and total biomass-WUE decreased under Mg deficiency whereas leaf-WUE showed an increase under Mg deficiency. Discrepancies between whole plant-WUE and leaf-WUE were also reported by Tomás et al. (2012) and Senbayram et al. (2015b). In their studies on grapevine cultivars and tobacco, the large variability in whole plant-WUE was not reflected in leaf-WUE as determined by gas exchange and $\delta^{13}\text{C}$. This discrepancy might be due to the complexity of factors which are involved in regulating biomass-WUE, but are not directly addressed when measuring instantaneous leaf gas exchange. Compared to leaf-WUE, biomass-WUE takes into account carbon loss (e.g., respiration, root exudates) and unproductive water loss (from non-photosynthesizing parts, and/or night-time transpiration from the leaves), which are two additional parameters that might substantially contribute to the variation in biomass-WUE. One may speculate that higher night-time respiration specifically from the leaves (due to high soluble sugar content) or excessive rates of root

exudation in severely Mg deficient plants may contribute to the decline in biomass-WUE. Root exudation may release remarkable amounts of fixed carbon into the rhizosphere; meaning a pronounced loss of reduced carbon assuming that most of the exudates are not re-absorbed by retrieval mechanisms (Kuzyakov and Xu 2013). Usually, retrieval of exuded carbonic compounds such as sugars is partly driven by an H^+ electrochemical gradient which is established by H^+ -ATPase activity. The generated proton gradient drives the uptake of sugars by means of H^+ /sugar-cotransporter (Jones et al. 2009). Enhanced net release of sugars was found when plasma membrane ATPase was inhibited and consequently, the proton gradient degraded (Mühling et al. 1993). As the activity of H^+ -ATPase is strongly dependent on the presence of Mg^{2+} ions (Palmgren 2001), the retrieval mechanism might be disturbed under Mg deficiency. Enhanced root exudation due to mineral deficiency such as potassium deficiency (Krafczyk et al. 1984), iron-, phosphorous- or nitrogen deficiency was observed in maize (Carvalhais et al. 2011).

Increased dark respiration under progressing Mg deficiency was reported in sugar beet (Terry and Ulrich 1974), and in *Phaseolus vulgaris* (Fischer and Bremer 1993). In the latter study, dark respiration rates of Mg deficient plants were 50 % higher than in control plants 6 days after onset of Mg deficiency. In conclusion, the decrease in biomass-WUE under severe Mg deficiency may be attributed to possible excessive carbon loss from the root (as exudates) and/or from leaves (night-time respiration).

Shoot- and total biomass-WUE can only give insight into the time-integrated responses at one specific time-point, but not into any dynamics. Thus, in order to understand the dynamics of progressing Mg deficiency and biomass-WUE, we studied variation in daily shoot-WUE throughout the vegetation period via empirical modeling. The strong decrease of daily shoot-WUE in Mg deficient plants after 18 DAO might be partly attributable to the decline in biomass production as the latter occurred as well after 18 DAO (see Online Resource 2). Furthermore, unproductive water loss at night was significantly higher in Mg deficient plants (data not shown), which is another factor that may cause lower biomass-WUE under Mg deficiency. In our study, night-time water loss in Mg deficient plants reached 35 % of daytime transpiration. Night-time transpiration rates may vary

between 5 and 15 % of daytime transpiration and, in some cases, rates of up to 30 % were reported (Benyon 1999; Snyder et al. 2003). Night-time transpiration without simultaneous carbon gain imposes carbon costs to the plant; however, it might be beneficial by increasing transpiration-driven mass flow to the root rhizosphere, thus enhancing nutrient availability during night, which positively affects plant productivity and growth (Caird et al. 2007). Here, higher night-time transpiration without concomitant biomass production might partly contribute to reduced biomass-WUE.

Effect of Mg supply on carbon isotope composition

Carbon isotope discrimination reflects the time-integrated CO_2 partial pressure at the carboxylation site (c_c) and therefore can be used to calculate time integrated c_c/c_a ratios which are sensitive to A , g_s and mesophyll conductance (Seibt et al. 2008; Buckley and Warren 2014). Overall, shoot $\delta^{13}\text{C}$ values decreased with increasing Mg supply indicating a change in the c_c/c_a ratio possibly due to the restriction in CO_2 diffusion by either stomatal (g_s) or mesophyll (g_m) conductance. The significant positive relationship between $\delta^{13}\text{C}$ values of young leaves and leaf-WUE (Fig. 6b) is in accordance with the linear model introduced by Farquhar et al. (1982), where $\delta^{13}\text{C}$ represents a temporal integration of leaf-WUE and where variability in CO_2 -diffusion is determined by c_i/c_a , the ratio of leaf internal to atmospheric concentration, alone. As seen in Fig. 6b, about 96 % of the variation in $\delta^{13}\text{C}$ values is explained by the change in leaf-WUE. Thus, we may speculate that in our study g_m was not significantly affected by varying Mg supply, although changes of leaf density by e.g. increased concentrations of non-structural carbohydrates, which are commonly associated with Mg deficiency (Cakmak et al. 1994a), can cause a reduction of g_m (Flexas et al. 2012). Surprisingly, a common relationship could not be established between average shoot $\delta^{13}\text{C}$ and total biomass-WUE. However, significant individual linear relationships were found between shoot $\delta^{13}\text{C}$ and total biomass-WUE when plotting each Mg treatment separately. The linear response of average shoot $\delta^{13}\text{C}$ to total biomass-WUE differed in slope and intercept with respect to Mg supply.

Both $\delta^{13}\text{C}$ and leaf-WUE data clearly showed that the decrease in biomass-WUE in Mg_{low} treatment was not solely due to the variation in photosynthesis or

photosynthesis-related g_s . Therefore, we conclude that lower biomass-WUE in Mg_{low} treatment might be attributable to the increase in nocturnal stomatal conductance, respiration or excessive root exudation as discussed above. Thus, this study is the first to establish a direct positive effect of Mg supply on biomass-WUE.

Conclusion

The aim of the current study was to improve our understanding on the direct effect of Mg supply on biomass-WUE and related parameters, e.g. leaf-WUE, and carbon isotope discrimination under non-limiting water supply. In this context, we draw the following conclusions:

- H_2O_2 concentrations were increased in Mg_{low} plants, although the activities of the ROS scavenging enzymes APX, GR and SOD were highest in in this experimental treatment. Plants that suffered from moderate Mg-deficiency maintained H_2O_2 on the level of plants treated with adequate amounts of Mg. Thus we conclude that the capacity of the antioxidative machinery to detoxify ROS is exhausted only under conditions of severe Mg-deficiency.
- Leaf-WUE and $\delta^{13}\text{C}$ values (showing time integrated leaf-WUE) were higher under Mg deficiency.
- In contrast to leaf-WUE, our experiment clearly showed that shoot- and total biomass-WUE decreased under severe Mg deficiency. Mild Mg deficiency caused a significant decrease in DM production (34 %), but did not affect biomass-WUE. We speculate that a possible variation in nocturnal stomatal conductance, night respiration and/or excessive root exudates may be responsible for the decrease in biomass-WUE under severe Mg deficiency.

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