Institute of Applied Plant Nutrition
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Magnesium deficiency induced responses in crop physiology: impacts on photosynthesis, light utilization, and photoprotection

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by

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“This work is dedicated to my beloved father who had always been an amazing parent, my friend, my constant source of motivation, and my role-model”

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List of abbreviations

A_n	Net CO_2 assimilation
Ax	Antheraxanthin
APX	Cytosolic ascorbate peroxidase
ATP	Adenosine triphosphate
CAT	Catalase
CO_2	carbon dioxide
Chl	Chlorophyll
^{3}Chl	Triplet chlorophyll
Cu/Zn-SOD	Superoxide dismutase
DEPS	De-epoxidation state of the VAZ pigments
DM	Dry matter
ETC	Electron transport chain
ETR	Electron transport rate
F	Fluorescence emission from dark-adapted leaves
F’	Fluorescence emission from light-adapted leaves
F_o	Minimum fluorescence of dark-adapted leaves
F_o'	Minimal fluorescence yield of light-adapted leaves
F_m	Maximum fluorescence of dark-adapted leaves
F_m'	Maximum fluorescence yield of light-adapted leaves
Φ_{PSII}	Effective PSII quantum yield
F_q’	Difference between $F_{m'}$ and F’
F_v/F_m	Maximum PSII quantum efficiency
Fd	ferredoxin
Fig.	Figure
FNR	Ferredoxin $NADP^+$ oxidoreductase
FW	Fresh weight
GPx	Glutathione peroxidase
GR	Glutathione reductase
H^+	Hydrogen
H_2O_2	Hydrogen peroxide
HPLC	High-performance liquid chromatography
K	Potassium
LHCII	Light harvesting complex II
Mg	Magnesium
mM	Millimolar
NADP	Nicotinamide adenine dinucleotide phosphate

NPQ	Non-photochemical quenching
O^{-}_{2}	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical
$^{1}O_{2}$	Singlet oxygen
Q_A	Primary quinone electron acceptor
qE	Energy-dependent quenching
qP	Photochemical quenching
qPCR	Quantitative real-time PCR
qZ	Zeaxanthin-dependent quenching
RC	Reaction center
ROS	Reactive oxygen species
RuBP	Ribulose-1,5-bisphosphate
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco)
SOD	Superoxide dismutase
VAZ	Sum of xanthophyll cycle pigments (Vx+ Ax+ Zx)
VDE	Violaxanthin de-epoxidase
Vx	Violaxanthin
ZEP	Zeaxanthin epoxidase
Zx	Zeaxanthin

Chapter 1

1. General introduction

Humans have been affected by hunger and diseases throughout history. There have been times of famine which led to starvation. Therefore, fighting hunger and malnutrition became an everlasting challenge. Discovering the nutritional requirements of plants in agriculture was recognized as one of the key resolutions in the battle against hunger in the mid-nineteenth century (Roy et al., 2007). There have been numerous studies since the nineteenth century dedicated to plant nutrition and plant nutrition optimization (Osvalde, 2011). The start of the Green Revolution (late 1950's) brought an increased application of commercial fertilizers (Pimentel, 1996), which in turn led to increased agricultural crop production. The increased crop production notwithstanding, it is still expected that the world population growth (expected to reach 9.2 billion by 2050) (Evans, 2009) can pose problems in meeting the food demand in the near future. Therefore, the sustainable plant production requires integrated nutrient, water, and soil management to feed the growing population (Gruhn et al., 2000).

There are 14 mineral nutrient elements that are beneficial for plant growth (Marschner, 1995), which are divided into two groups known as macro- and micro-nutrients. Almost all the macro- and micro-nutrients are taken up from the soil (Jones and Jacobsen, 2001). Each element supports a different and/or specific plant function and varies in its mobility within the plant. Among the macro nutrient elements, magnesium (Mg) concentrations in agricultural crops and regular diet have gained global attention in food production, food quality, and human nutrition. Mg deficiency reduces the sustainability in agricultural production and has long-term negative effects on animal and human health (de Baaij et al., 2015). Among agricultural crops, cereal grains are reported to be the major source of Mg for human diet. Moreover, adequate Mg nutrition is important for the growth of animals, especially grazing livestock. Sufficient concentrations of Mg should be available in soils as well as in plants to reach the requirements for both livestock and humans to avoid Mg deficiency. Accordingly, it is beneficial to understand the relative amounts of Mg required (the other 13 elements as well) by each crop in order to supply sufficient macro- and micro-nutrients in crop cultivation and fertilization strategies.

1.1 The nutrient element magnesium

Mg is one of the most important macronutrients in plants and yet known as the forgotten element (Cakmak and Yazici, 2010). Mg is taken up by the roots from the soil solution in its ion form (Mg^{2+}) (Kirkby, 2011) where the pool of exchangeable cations is in equilibrium. The concentrations of Mg^{2+} in soil solution vary from micromolar to millimolar. Through the apoplastic pathway, Mg can be passively diffused by water flow in root cortex apoplast. Moreover, the Mg ions can also pass in the epidermis and cortex and be transferred via the

carriers from cell to cell through diffusion by plasmodesmata within the symplast (symplastic pathway) (Hermans et al., 2013). However, application of other fertilizers such as potassium (K) can induce Mg deficiency in crop plants, where K^+ inhibits the Mg^{2+} uptake by the roots (White, 2011).

It is often suggested that optimal Mg concentration in plant tissue is above 2 mg Mg g^{-1} dry matter (DM) and concentrations below 2 mg Mg g^{-1} DM are considered to be critical (Cakmak and Kirkby, 2008). However, critical Mg concentrations vary among diverse physiological and biochemical processes (Hauer-Jákli and Tränkner, 2019). In this respect, Hauer-Jákli and Tränkner (2019) have reported that the critical Mg concentrations for DM formation are lower than concentrations required for net carbon dioxide (CO_2) assimilation. Furthermore, there is no summary about critical tissue Mg concentrations on growth and photosynthesis in various plant species. Yet chloroplast-related processes are expected to react more sensitively to reductions in Mg concentrations (Cakmak and Kirkby, 2008; Jamali Jaghdani et al., 2020).

The total Mg concentration in a plant cell is reported to be between 15-20 mM. Total metabolic Mg-pool is distributed among different cell compartments. About 0.2-0.5 mM Mg can be found in mitochondria, whereas the cytosol contains 0.2-0.4 mM Mg (Hermans et al., 2013). Mg is the central atom of chlorophyll pigments that are located in chloroplasts. Hence chloroplasts contain the highest share of metabolic Mg (15-20 mM) as Mg is the central atom of chlorophyll pigments (Hermans et al., 2013).

The first step of chlorophyll biosynthesis includes the insertion of Mg^{2+} into the porphyrin structure (Walker and Weinstein, 1991). Mg deficiency influences the structure, size, and functionality of chloroplasts. Under Mg deficiency, chlorophyll degradation starts in mature leaves in order to provide Mg for younger leaves. Therefore, the symptoms of Mg deficiency are initially observed in the mature leaves as interveinal chlorosis (Cakmak and Kirkby, 2008).

Mg plays a bridging role in aggregating ribosome subunits (Cammarano et al., 1972), which is necessary for protein synthesis. Several enzymes and enzymatic reactions require Mg or are strongly promoted by it. Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most abundant enzyme on Earth and a key enzyme in photosynthesis. Rubisco's activation is Mg-dependent (Cakmak and Yazici, 2010). Regulating ribulose-1,5-bisphosphate (RuBP) carboxylase in the stroma of chloroplasts is another key role of Mg (Pierce, 1986). Many of the Mg-dependent reactions include phosphate transfer, such as phosphatases and adenosine triphosphate (ATP)-ases, or the transfer of carboxyl group, such as carboxylase (Hawkesford et al., 2011).

Furthermore, Mg plays an important role in CO_2 fixation. In Mg deficient leaves, CO_2 uptake is declined and correspondingly CO_2 fixation is reduced (Terry and Ulrich, 1974). Studies on

Arabidopsis plants (Hermans and Verbruggen, 2005) and sugar beet (*Beta vulgaris* L.; Hermans et al., 2004) under Mg deficiency induced a decline in photosynthesis, which suggest that sugar accumulation in leaves provokes photosynthetic CO_2 fixation and provides a negative feedback on photosynthesis.

The status of Mg in plants has a noticeable influence on transportation of photosynthates. It affects photosynthates' distribution between the source and sink organs (Cakmak and Kirkby, 2008) which leads to a decreased root growth and subsequently to reductions in nutrient uptake.

Carbohydrate accumulation in leaves is also associated with Mg deficiency (Fischer et al., 1998). A possible explanation for the impaired phloem (sucrose) loading is the decline in photosynthesis in Mg deficient leaves. Furthermore, Mg-ATP is known as the major complex of ATP (Igamberdiev and Kleczkowski, 2003) that is required for effective H^+-ATPase operation. The phloem (sucrose) loading is catalyzed by H^+/sucrose co-transporter and includes a proton gradient across the phloem cells and plasma membranes (Cakmak and Kirkby, 2008). In order to load photosynthates into phloem, sufficient levels of cytosolic Mg^{2+} and Mg-ATP are required. Therefore, plants respond to Mg deficiency by reductions in phloem exportation from source to sink organs (Hermans et al., 2013).

The accumulation of photosynthates and impairments in CO_2 fixation leads to failure in absorbed light utilization in photochemistry and promotes the overreduction of electron transport chain (ETR) and consequently reactive oxygen species (ROS) production. Nutrient deficiency contributes to severe photooxidative damage. Nonetheless, sufficient supply of Mg mitigates the overreduction of ETR and photooxidation (more details are given in section 1.3). Figure 1 summarizes the effects of Mg deficiency in a plant.

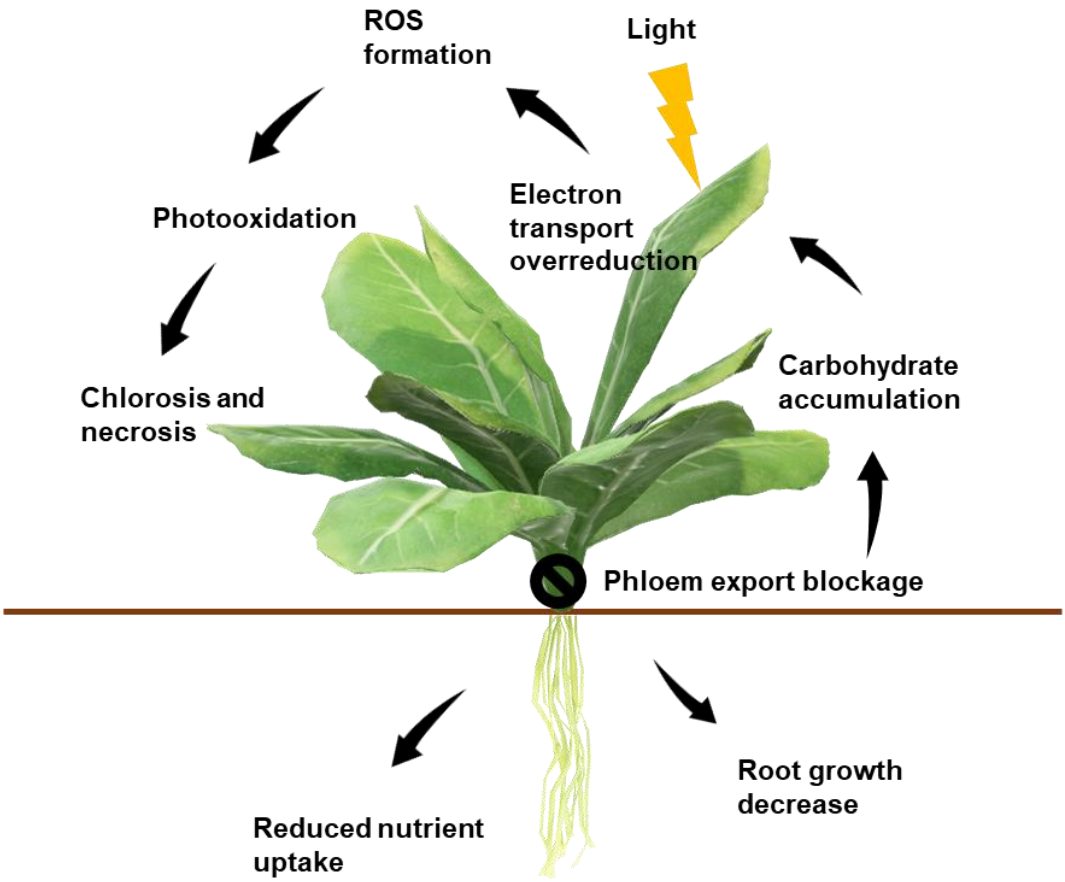

Fig. 1. A summarized illustration of the influences of magnesium deficiency on photosynthesis, photooxidation and declined plant growth following Cakmak and Kirkby (2008).

1.2 Light absorption and chlorophyll fluorescence

Chloroplasts are the chlorophyll containing organelles in the cells of leaves and stems of eukaryotic plants and algae. The chloroplasts contain an inner and outer membrane surrounding the stroma and stacks of coin-shaped thylakoids (plural: grana) (Taiz and Zeiger, 2002). Each granum may contain 2-30 or more thylakoids (Crang et al., 2018). The inner space of thylakoid is called lumen and they are surrounded by stroma from outside. Grana are connected to each other by lamellae (Fig. 2). The membranes of thylakoids contain proteins and pigments (chlorophylls and carotenoids) that function in the photochemical reactions of photosynthesis. Most of the pigments are involved in harvesting light and transferring the energy to the photosystems and reaction centers (RC) and are known as the antenna complex. The core of the RC consists of different proteins including the two membrane proteins D1 and D2 (Zouni et al., 2001). The chlorophyll molecule is a pigment-protein complex that is associated with photosystem II (PSII) and photosystem I (PSI). When light is absorbed by chloroplasts, there are 3 possible routes: 1) it will be utilized in photosynthesis

(photochemistry); 2) it will be re-emitted as chlorophyll fluorescence; or 3) it will be dissipated as heat which is also known as non-photochemical quenching, NPQ (Fig. 2) (Murchie and Lawson, 2013). Photochemistry is in competition with chlorophyll fluorescence and NPQ. When the rate of one mechanism is increased, the other two will be decreased (Baker, 2008). Therefore, the chlorophyll fluorescence rate can provide valuable information about photosynthetic efficiency. Fluorescence is reduced (quenched) when an electron is transferred to PSII to be used in photochemistry (Baker, 2008), which is known as photochemical quenching (qP). Since NPQ serves as a regulated protective process against excessive excitation energy, increased values of NPQ can be a sign of stress in plants (Murchie and Lawson, 2013).

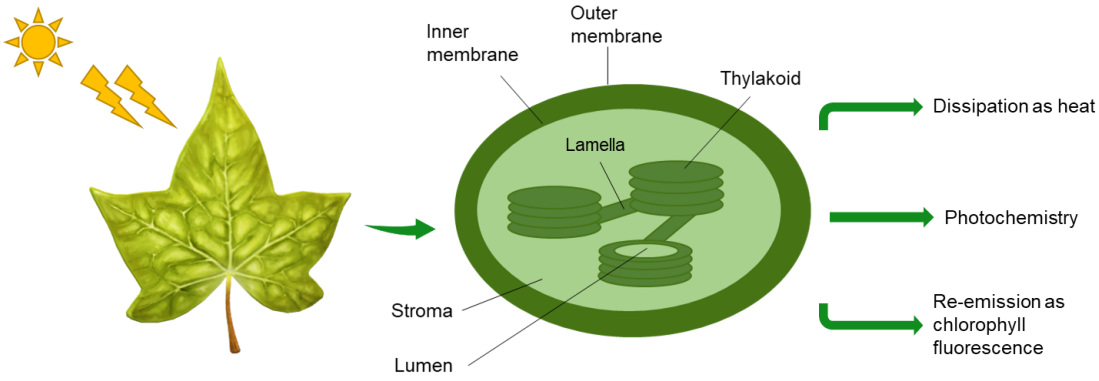

Fig. 2. Illustration of the chloroplast structure and the possible pathways for the absorbed light.

One of the common methods to estimate qP and NPQ is the use of a fluorometer on dark-adapted leaves and/or chlorophyll-containing plant parts. Dark adaptation allows the RCs to be maximally oxidized and therefore open and ready to receive the electrons. Applying a saturating pulse to the dark-adapted material induces the maximum value of fluorescence (F_m) and closes all the RCs within PSII. At this moment no photochemistry is occurring, because the primary quinone electron acceptor (Q_A) of PSII cannot accept any new electrons until it has passed it on to the next electron acceptor. Furthermore, in an unstressed plant, no NPQ is occurring as NPQ is a light-dependent mechanism. The maximum PSII quantum efficiency (F_v/F_m) can be determined with the help of minimum and variable fluorescence of dark-adapted leaves (F_o and F_v, respectively). Measuring light (which is non-actinic) is applied to estimate F_o. F_o intensity is too low to drive ETR but it is high enough to determine minimum fluorescence at the dark-adapted state. F_v/F_m is a fine indicator for maximum quantum yield of PSII. Reductions in F_v/F_m value can be indicators of photooxidation or damaged PSII RC. When the plant is exposed to actinic light, the fluorescence value is reduced (F': fluorescence emission

from light-adapted leaves; Fig. 3). F_m' is an indicator of maximum fluorescence from light-adapted leaves and can be estimated when the saturation pulse is applied. The difference between F_m' and F' is indicated as F_q' (Fig. 3). Minimum fluorescence of light-adapted leaves and variable fluorescence from light-adapted leaves are indicated as F_o' and F_v', respectively (Fig. 3). qP, which is an indicator of the proportion of open RCs, is estimated as F_q'/F_v'. Additionally NPQ, which estimates the rate constant for heat dissipation, is calculated as F_m-F_m'/F_m' (Baker, 2008; Murchie and Lawson, 2013).

NPQ is the reason for the strong quenching in F_m' value during light-adapted state in comparison to F_m. When a saturation pulse is applied in absence of actinic light, the NPQ rate decreases and the fluorescence value increases. Chlorophyll fluorescence measurements are widely used to investigate different types of stress in plants (Kalaji et al., 2014; Krause and Weis, 1984; Moustaka et al., 2015). In chapters 2, 3, and 4, a variety of chlorophyll fluorescence parameters are studied in wheat, sunflower, barley, and spinach under different Mg deficiency levels.

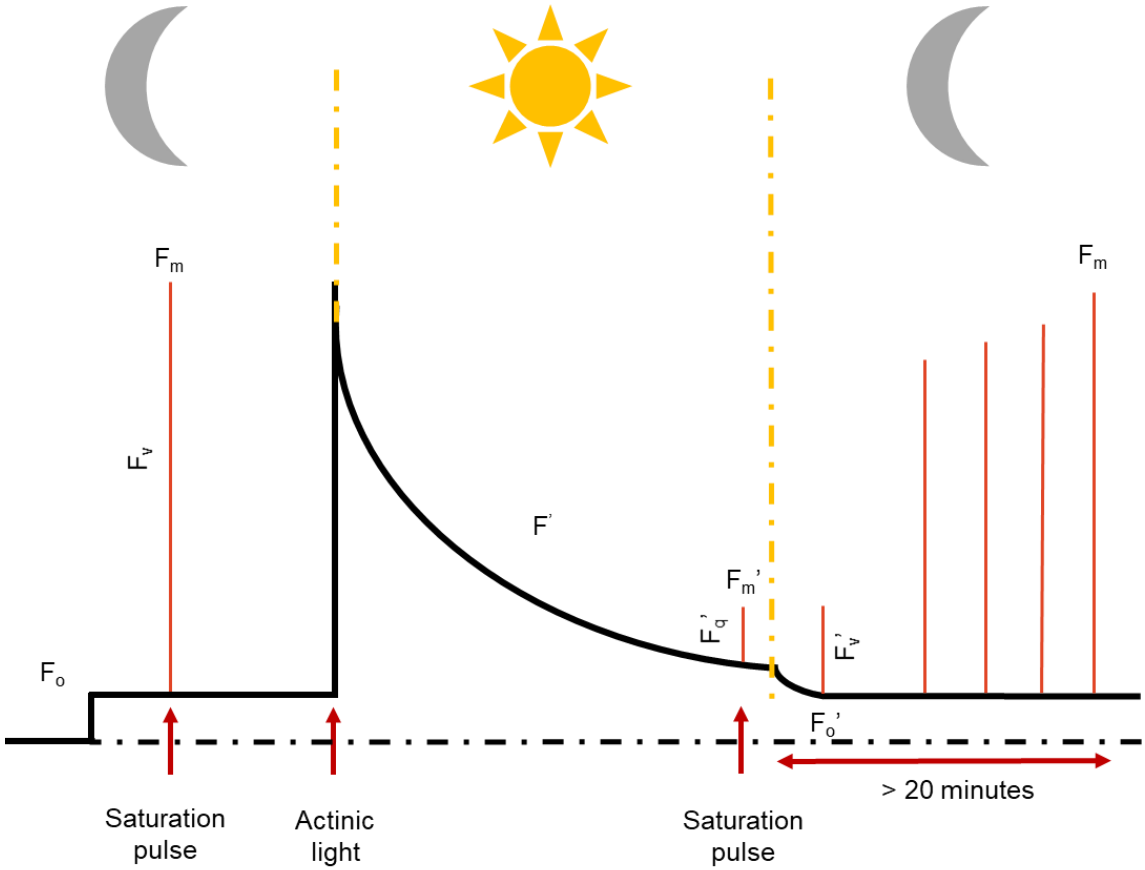

Fig. 3. Fluorescence quenching analysis adapted from Murchie and Lawson, (2013). All parameters **with** (‘) are obtained from a chlorophyll containing plant material exposed to actinic light. The parameters **without** (‘) are obtained from the dark-adapted state. The quenched state is indicated with the sun and the unquenched state is indicated with the moon. Dark-adapted chlorophyll containing plant material is illuminated by a saturation pulse (about 800 milliseconds in our studies) to assess maximum fluorescence (F_m). When actinic light is applied, the F_m is quenched. A saturation pulse is applied to assess the maximum fluorescence (F_m’) after exposure to actinic light. F_o and F_o’ indicate minimum fluorescence from dark and light-adaptation, respectively. F_v and F_v’ represent variable fluorescence from dark and light-adapted chlorophyll containing plant material, respectively. F_q’ indicates the difference between F_m’ and F’. Several minutes (>20 minutes) are required to achieve the steady state of F’ from a dark-adapted state.

1.3 Photoprotection

ROS have always been the uninvited guests of aerobic life (Halliwell, 2006) and their formation is enhanced under unfavorable conditions. They are constantly formed as the byproducts of metabolic pathways. They are produced in various cell compartments such as chloroplasts, peroxisomes and mitochondria (Del Río and López-Huertas, 2016; Smirnoff, 2007). However, their production rapidly increases in chloroplasts in response to nutrient deficiency, drought,

and high-light intensity. Ferredoxin (Fd), which is located in the last step of $NADP^+$ reduction in photosystem I (PSI), becomes highly reduced when chloroplasts are exposed to excessive light energy. Therefore, the regeneration of $NADP^+$ is hindered. Thus, electron acceptance is inhibited, electron transport chain (ETC) is reduced and electron leakage is increased and subsequently ROS formation is enhanced (Uzilday et al., 2012). Also, transferring energy from triplet chlorophyll (^3Chl) to the molecule of oxygen leads to ROS formation (singlet oxygen at this stage) (Krieger-Liszkay, 2005). The most important known ROS molecules formed in plants include singlet oxygen ($^1O_2$), superoxide radical (O^{-}_2), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) (Asada, 2006). ROS are toxic and cause damage to lipids, proteins, and DNA (McCord, 2000) and consequently lead to cell death.

Plants however, have developed photoprotective mechanisms that preserve them from high concentrations of ROS. The antioxidant system against photooxidative stress is one of the known photoprotective mechanisms. The most relevant enzymes involved in the detoxification (scavenging) of ROS include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and catalase (CAT) (Ahmad et al., 2008). The activity of the individual enzymes is independent in each cellular compartment and can occur simultaneously to detoxify a specific ROS molecule. However, the balance between the activities of SOD, APX, and CAT in cells regulates the steady state level of superoxide radicals and hydrogen peroxide (Bowler et al., 1991).

The non-radical molecule H_2O_2 is converted into H_2O and O_2 by the glutaredoxin enzyme system consisting of GR and glutathione peroxidase (GPx); and CAT (Bhatla and Lal, 2018).

Numerous studies have investigated changes in ROS concentration and antioxidant enzyme activity under nutrient deficiency (Mengutay et al., 2013; Tang et al., 2012; Tewari et al., 2006; Tränkner et al., 2016). In chapter 3 and 4 the impacts of Mg deficiency on barley (*Hordeum vulgare* L.) and spinach (*Spinacia oleracea*) have been studied to evaluate the efficiency of antioxidant mechanisms.

Non-photochemical quenching (NPQ) is a photoprotective mechanism. The three carotenoids violaxanthin (Vx), antheraxanthin (Ax), and zeaxanthin (Zx) are involved in the Vx cycle (Jahns et al., 2009) and contribute to the prevention of ROS formation by NPQ. NPQ is associated with a peripheral antenna complex of PSII, the PsbS protein. Under high-light intensities, due to the increase in ETR and the transportation of the H^+ cations to the thylakoid lumen, the lumen pH decreases. The acidification of lumen leads to protonation of the PsbS protein (Baker, 2008). By protonation of PsbS, Vx is activated. Violaxanthin de-epoxidase (VDE) converts Vx to Ax, where Vx loses one epoxide group. And by losing the second epoxide group, Ax is converted to Zx (Fig. 4). It is believed, that the conformational changes that are the consequence of Zx binding to PSII antenna proteins, induce quenching and heat dissipation

(Demmig-Adams and Adams, 1992). As the light intensity decreases, PsbS will be de-protonated and zeaxanthin epoxidase (ZEP) converts Zx back to Vx (Baker, 2008).

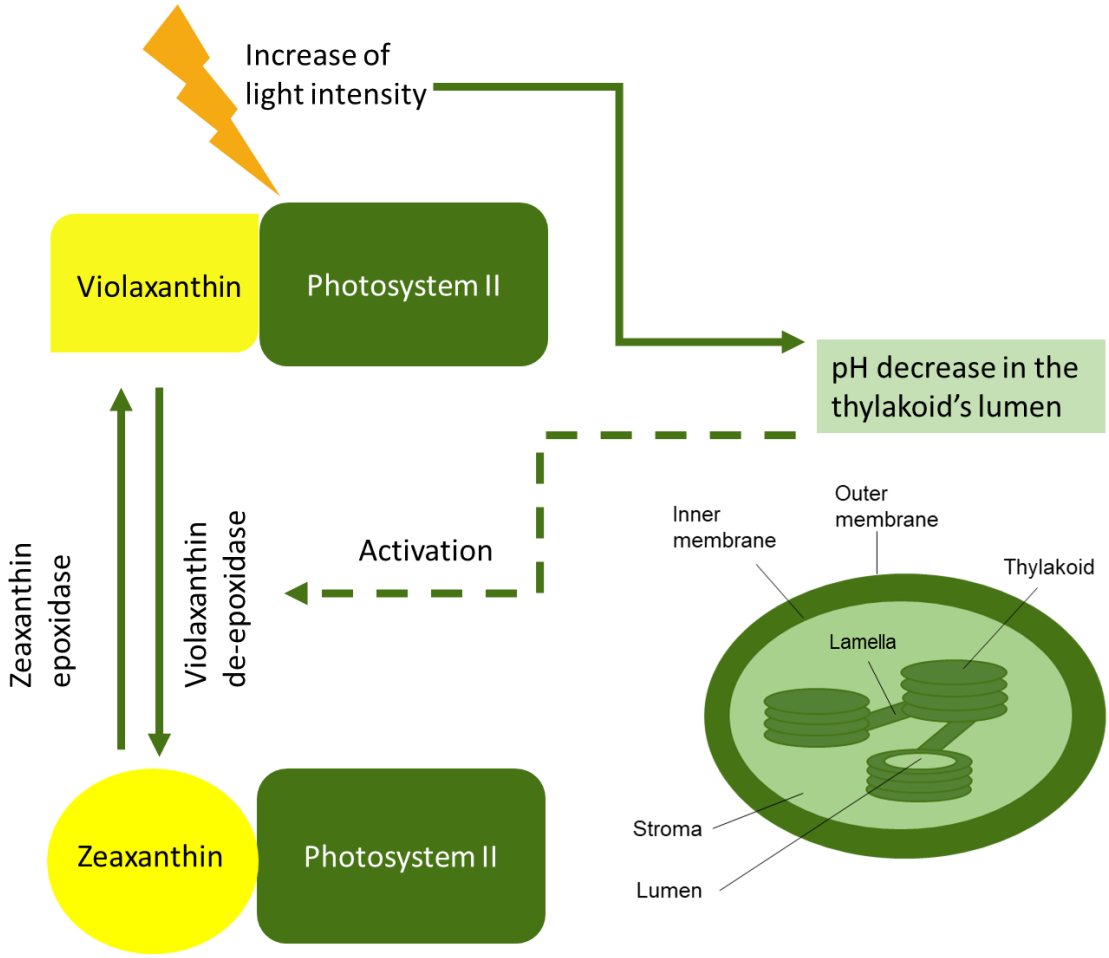

Fig. 4. Schematic illustration of the violaxanthin cycle. At high-light intensities, the pH in the thylakoid lumen decreases and activates violaxanthin. Firstly, violaxanthin is converted to antheraxanthin by violaxanthin de-epoxidase. When the light intensity decreases, zeaxanthin is converted back to violaxanthin by zeaxanthin epoxidase.

There is an antioxidative function described for Vx cycle where it binds as a non-protein xanthophyll to the lipid phase of thylakoidal membrane. The most important ROS compound in photooxidative damage under high-light stress is known to be the $^1O_2$. Photoprotective roles of Vx cycle are related either to reduction in $^1O_2$ formation and/or $^1O_2$ removal (Jahns and Holzwarth, 2012). Synergistic functions of Zx and tocopherol in membrane lipid protection against peroxidation have been studied (Havaux et al., 2007; Wrona et al., 2004). A study with tocopherol-deficient *Arabidopsis thaliana* mutants revealed a significant increase in VAZ-pool

size (Vx + Ax + Zx), where a significant sensitivity to high-light stress was observed in the double mutants (Zx and tocopherol-deficient; Havaux et al., 2005). Therefore, the synergistic functions of Zx and tocopherol lead to reduced lipid peroxidation, regardless of Zx binding to antenna proteins (Havaux et al., 2007). The effects of Mg deficiency on Vx-cycle however, have not been studied so far. In chapters 3 and 4 the impacts of Mg deficiency on antioxidative gene expression and xanthophyll pigments quantification in barley and spinach have been explained.

1.4 Objectives

Despite of Mg's essential roles in plants' physiology, and its vitality in biochemical processes including ribosomes assembly, enzyme activation, and activity, it has not been studied in details regarding its concentrations required for each specific reaction in crop nutrition studies. To address the optimal Mg concentrations required for efficient fertilization and optimal crop yield, it is important to understand the requirements of different plant species for Mg concentration in distinct physiological and biochemical processes.

In this thesis, we present our work at improving our understanding of how different plant species react to varying Mg concentrations. In the first study (Chapter 2) wheat (*Triticum aestivum* L.) and sunflower (*Helianthus annuus* L.), which were grown hydroponically in the greenhouse were used. Seven levels of Mg deficiency were induced and chlorophyll fluorescence and CO_2 assimilation measurements were performed to determine the critical Mg concentrations that influence photosynthetic performance. Moreover, the chlorophyll content reductions and the DM that is produced under the induced Mg concentrations was measured.

The second study (Chapter 3) focused on barley (*Hordeum vulgare* L.) grown hydroponically in the climate chamber. Three Mg deficiency treatments were induced to investigate the reductions in CO_2 assimilation, photosynthetic parameters including NPQ and F_v/F_m through chlorophyll fluorescence measurements, xanthophyll cycle's pigments quantifications, and analysis by quantitative real-time PCR (qPCR) of ROS scavenging enzymes.

The third study (Chapter 4) focused on spinach (*Spinacia oleracea*) grown hydroponically in the climate chamber. Three Mg deficiencies were induced to investigate the CO_2 assimilation, photosynthetic parameters such as NPQ and F_v/F_m, xanthophyll cycle's pigments quantifications, and analysis by quantitative real-time PCR (qPCR) of VDE, ZEP, and ROS scavenging enzymes.

Chapter 5 provides a general discussion of the results within chapter 2, 3, and 4 with respect to the related results from the literature.

The presented studies with various agronomic-relevant plants belonging to different plant genus, and their different physiological responses to Mg deficiency, helps us to better understand the importance of Mg availability in agricultural production and provide a broader knowledge, that can support efficient plant nutrition programs.

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Chapter 2

Minimum magnesium concentrations for photosynthetic efficiency in wheat and sunflower seedlings

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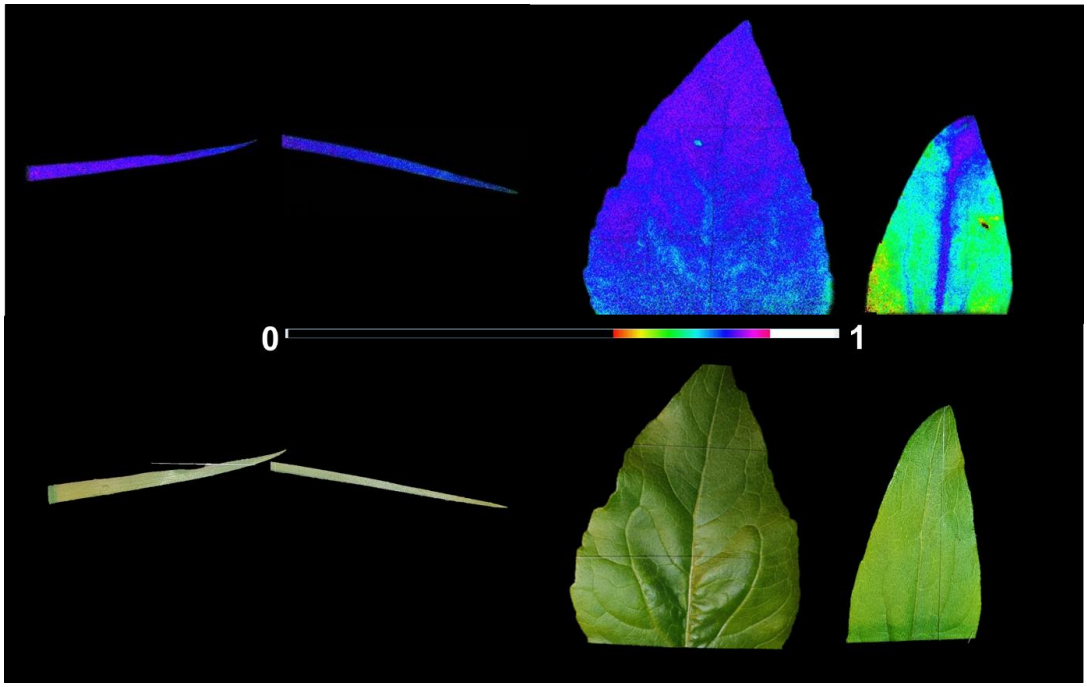

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Research article

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ABSTRACT

Photosynthetic processes in the chloroplast depend on the abundance of magnesium (Mg) in relatively high amounts; hence chloroplasts might react more sensitive to Mg-deficiency than other physiological processes within other organelles. Most authors suggest a critical Mg concentration to be 1.5 mg g^{-1} DM for biomass and yield formation. However, it is not yet elucidated whether this value also applies to photosynthetic processes. The present study focused on the response of photosynthetic processes to different Mg tissue concentrations. Wheat (*Triticum aestivum*) and sunflower (*Helianthus annuus*) plants were grown hydroponically for 10 days with 8 different levels of Mg supply (1.0, 0.5, 0.25, 0.1, 0.075, 0.05, 0.025, 0.01 mM Mg). Specific leaf mass, SPAD values, assimilation rate, F_v/F_m, electron transport rate and photochemical and non-photochemical quenching parameters were determined on youngest mature leaves.

Tissue Mg concentrations decreased with lowering Mg supply to lowest concentrations of 0.7 mg g^{-1} DM in wheat leaves, but photosynthetic capacity was not affected. In sunflower leaves, lowest Mg concentrations of 0.56 mg g^{-1} DM were achieved and a diminished photosynthetic capacity was observed. The study shows that a Mg tissue concentration of 1.5 mg g^{-1} DM did not induce a negative effect on the photosynthetic capacity of wheat and sunflower leaves under our experimental conditions and hence, the critical Mg concentration for photosynthetic processes might be lower than for biomass and yield formation.

1. Introduction

Magnesium (Mg), one of the 17 plant nutrients, is well known to play essential roles in numerous processes in plant metabolism. Particularly, in photosynthesis and related processes Mg is of major importance. Concentrations of Mg in plant cells are highest in chloroplasts (Karley and White, 2009), the cell organelle where photosynthesis takes place. 15–35% of total plant Mg is bound to chloroplasts (Chen et al., 2018) and Mg concentrations in the chloroplast are reported to reach 5 mM (Grzebisz, 2015). A large share of Mg, which can be up to 35%, is bound to chlorophyll molecules, depending on Mg status of the plant (Cakmak and Kirkby, 2008). Illumination was shown to influence Mg distribution in Mg-deficient poplar leaves (Dorenstouter et al., 1985). Leaves acclimated to lower light intensities had up to 57% of Mg bound to chlorophyll, whereas in high-light acclimated leaves, the proportion was only up to 37%. A frequently reported response to Mg deficiency is the reduction of chlorophyll concentrations (Mengutay et al., 2013; Faust and Schubert, 2016; Tränkner et al., 2016).

During illumination, Mg is transported from the thylakoid lumen to the stroma due to the H^+ transport across the thylakoid membrane (Ishijima et al., 2003). Hence, Mg concentrations in the stroma increase. In spinach chloroplasts, which were kept in dark, the internal Mg^{2+} concentration was estimated to be 0.50 mM, and illumination caused an increase in Mg^{2+} concentration to 2.0 mM in the stroma (Ishijima et al., 2003). Several enzymes in the stroma are activated by free Mg^{2+} concentrations such as fructose-1,6-bisphosphatase, which was shown to be activated by 1–2 mM free Mg^{2+} (Ashton, 1998). The activation of Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) might require a considerable part of stromal Mg as this enzyme is present in very high concentrations (Dorenstouter et al., 1985). Low Mg concentrations induced an increase in the rate of deactivation of purified spinach Rubisco (Kim and Portis, 2006) and in citrus, Rubisco activity was decreased under Mg deficiency (Tang et al., 2012). Similarly, carbon dioxide (CO_2) assimilation rates were reported to decrease under deficient Mg supply (Lasa et al., 2000; Jezek et al., 2015; Tränkner et al., 2016). Limited CO_2 fixation is associated with carbohydrate accumulation in Mg-deficient source leaves due to impaired phloem loading (Cakmak and Kirkby, 2008). Accumulation of soluble sugars (sucrose, fructose, glucose) was observed in *Sulla carnosa* plants

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Abbreviations

Abs	Absorptivity
A_n	CO_2 net assimilation rate
CO_2	Carbon dioxide
DM	Dry matter
ETR	Electron transport rate
F_0	Minimum fluorescence of dark adaptation
$F_{0'}$	Minimal fluorescence yield of illumination
F_m	Maximum fluorescence of dark adaptation
$F_{m'}$	Maximum fluorescence yield of illumination
F_t	Fluorescence yield of light adaptation
Φ_{NO}	Quantum yield of nonregulated energy dissipation
Φ_{NPQ}	Quantum yield of regulated energy dissipation
Φ_{PSII}	Effective PSII quantum yield
F_v/F_m	Maximum PSII quantum efficiency
LA	Leaf area
LHCII	Light harvesting complex of photosystem II
Mg	Magnesium
NPQ	Non-photochemical quenching
PPFD	Photosynthetic photon flux density
PSII	Photosystem II
qL	Fraction of open PSII reaction centres
qP	Coefficient of photochemical quenching
RC	Reaction centre
ROS	Reactive Oxygen Species
Rubisco	Ribulose-1, 5-bisphosphate carboxylase/oxygenase
SLM	Specific leaf mass

when plants were supplied with 0.01 mM or 0 mM Mg (Farhat et al., 2014). Besides sugars, starch accumulation in the chloroplast is well documented by electron microscopy images, iodine staining or by chemical analysis (Vesk et al., 1966; Hall et al., 1972; Hermans et al., 2005; Farhat et al., 2014). The accumulation of non-structural carbohydrates, particularly starch, can increase specific leaf mass (SLM) by affecting leaf cell density (Britz and Adamse, 1994). In cucumber, the accumulation of starch was suggested to be the cause for increased SLM because subtracting the non-structural carbohydrate component and thereby expressing SLM on the residual dry matter (DM) basis, the increase in SLM was reduced (Britz and Adamse, 1994).

Reduced energy consumption in the light-independent processes of photosynthesis is suggested to lead to an over-reduction of the electron acceptors in the light-dependent reactions of photosynthesis (Cakmak and Kirkby, 2008). This leads to increased production of reactive oxygen species (ROS), which damage proteins, in particular the D1 protein of photosystem II (PSII). If the rate of damage exceeds the rate of repair of D1 protein, photoinhibition occurs. In combination with qI, photoinhibition can be assessed by the chlorophyll fluorescence parameter maximum PSII quantum efficiency (F_v/F_m), which was shown to be decreased under Mg-deficiency in citrus (Yang et al., 2012), sugar beet (Hermans et al., 2004) and *Sulla carnosa* (Farhat et al., 2015). Besides F_v/F_m, chlorophyll fluorescence allows to study the quantum efficiency of PSII, which gives insight about the redox state of PSII reaction centres. Light energy reaching the thylakoid membranes can either be used in biochemical reactions of photosynthesis (described by the chlorophyll fluorescence parameter Φ_{PSII}) or non-photochemically quenched and dissipated as heat (Φ_{NPQ}). Hence, assessing both photochemical and non-photochemical quenching provides an estimate of photosynthetic efficiency at PSII.

Typically studies on Mg deficiency either apply very low Mg concentrations or even no Mg, known to certainly induce Mg deficiency, and/or results are not related to the tissue Mg concentration. Furthermore, the concept of critical concentration is rarely applied to Mg studies. The critical nutrient concentration is defined as the "single point within the bend of the curve where the plant nutrient status shifts from deficient to adequate" (Dow and Roberts, 1982), and other definitions are mostly relating critical concentration to growth or yield formation (Dow and Roberts, 1982). For Mg it was reported that concentrations of 0.7 mg Mg g^{-1} leaf DM may be required to achieve 90% of maximum yield (Smith et al., 1985). It is commonly suggested that critical Mg tissue concentration is < 1.5 mg g^{-1} DM (Cakmak and Kirkby, 2008), but critical concentrations seem to vary among physiological processes. Recently, critical leaf Mg concentrations for CO_2 net assimilation were suggested to be higher than those for dry weight production (Hauer-Jákli and Tränkner, 2019). Hence, chloroplastic processes might react more sensitive to Mg concentrations. Furthermore, a meta-analysis reported on lower critical Mg concentrations for CO_2 net assimilation in monocots than in dicots (Hauer-Jákli and Tränkner, 2019). In the present study, eight different Mg supply levels were chosen to induce a gradient in Mg tissue concentrations in sunflower (dicot) and wheat (monocot) and photosynthetic processes were analysed. The objective of this study was to identify the Mg concentration at which photosynthesis and related processes are affected and to determine whether the analysed processes respond differently at different concentrations.

2. Materials and methods

2.1. Plant culture

Plant growth was performed in the greenhouse with a day/night light cycle of 14/10 h and a photosynthetic photon flux density of approx. 350 µmol m^{-2} s^{-1} at canopy height. Seeds of wheat (*Triticum aestivum* L. cv. Cornetto) and sunflower (*Helianthus annuus* L. cv. Delfie) were germinated in paper rolls in 1 mM $CaSO_4$. The germination solution for sunflower seeds also contained 20 µM H_3BO_3. After germination, seedlings were transferred into 5 L pots (2 plants per pot) containing a nutrient solution. Seedlings were grown for four days in half-strength nutrient solution, then 26 days in 100% of full-strength nutrient solution. The full-strength nutrient solution of control treatments contained: 1.75 mM $Ca(NO_3)_2$*4 H_2O, 1 mM K_2SO_4, 1 mM $MgSO_4$*7 H_2O, 0.25 mM NH_4NO_3, 0.2 mM $Ca(H_2PO_4)_2$* H_2O, 0.05 mM $CaCl_2$*2 H_2O, 0.1 mM $C_{10}H_{12}FeN_2NaO_8$, 1 µM or 20 µM H_3BO_3 for wheat and sunflower, respectively, 1 µM $ZnSO_4$*7 H_2O, 1 µM $MnSO_4$*H_2O, 0.2 µM $CuSO_4$*5 H_2O, 0.1 µM $H_{24}Mo_7N_6O_{24}$*4 H_2O. Seven different treatments of Mg-deficiency were established at the day of transplanting seedlings into nutrient solution (= 0 days after treatment start (DAT)). The concentrations of the Mg-treatments were 0.5 mM, 0.25 mM, 0.1 mM, 0.075 mM, 0.05 mM, 0.025 mM and 0.1 mM $MgSO_4$*7 H_2O. Nutrient solutions were constantly aerated and exchanged every three to four days depending on plant water consumption. Each treatment was replicated four times. Measurements were performed on youngest fully expanded leaves. For the purpose of increasing comparability, all measurements were done on the same leaf of the plant at 10 DAT.

2.2. Leaf SPAD measurements

Relative leaf chlorophyll concentrations were estimated *in vivo* using a SPAD-502 (Konica-Minolta, Japan) prior to measurements of chlorophyll *a* fluorescence on the same leaves; hence four replicates per treatment were measured. One replicate consisted of three measurements per leaf which were averaged directly by device option. Values are expressed as SPAD units.

2.3. Chlorophyll a fluorescence

Chlorophyll *a* fluorescence was determined using a PAM-

fluorometer (Imaging-PAM Maxi, Heinz Walz GmbH, Germany). Leaves were dark-adapted for 20 min prior to measurements by placing the whole plant in a large box (1.8 m^3) which allowed a photosynthetic photon flux density (PPFD) of only 10 µmol m^{-2} s^{-1}. Maximum PSII quantum yield [F_v/F_m = (F_m–F_o)/F_m] (Maxwell and Johnson, 2000) was measured and then, actinic light was switched on at a PPFD of 461 µmol m^{-2} s^{-1} and once per minute a saturation light pulse was applied at a PPFD of 2700 µmol m^{-2} s^{-1} for 800 ms. The following quenching parameters were determined after 15 min: effective PSII quantum yield [Φ_{PSII} = (F_m'–F_t)/F_m'] (Genty et al., 1989), quantum yield of regulated energy dissipation [Φ_{NPQ} = 1/Φ_{PSII} –1/NPQ + 1 + qL (F_m/F_o–1))], quantum yield of non-regulated energy dissipation [Φ_{NO} = 1/NPQ + 1 + qL (F_m/F_o–1))], coefficient of photochemical quenching (qP = (F_m'–F)/(F_m'–F_o') and NPQ (NPQ = (F_m–F_m')/F_m') (Kramer et al., 2004). F_m and F_o denote, respectively, the maximum and minimum fluorescence of dark-adapted samples. F_t is the fluorescence yield of light-adapted samples and F_m' the maximum fluorescence yield in the light following a saturation pulse, and $F_o' = F_o/(F_v/F_m + F_o/F_m')$ (Oxborough and Baker, 1997). ETR was determined by calculating ETR = Φ_{PSII} *0.5*Absorptivity*461 µmol m^{-2} s^{-1}. Absorptivity is calculated as Abs. = 1-NR/NIR, where R is remission at 660 nm and NIR is remission at 780 nm. A circular and a rectangular area of interest were selected for sunflower and wheat leaves, respectively, avoiding the edges of the leaves and considering the centre of the leaf the most representative leaf area. Fluorescence values of all pixels within this area were averaged automatically by the devices software ImagingWin v2.41a (Heinz Walz GmbH, Germany). Measurements were performed with four replications per treatment.

2.4. Measurement of CO_2 net assimilation rates

CO_2 net assimilation rates (A_n) were determined by measuring leaf gas exchange (GFS-3000, Heinz Walz GmbH, Germany) on 4 cm^2 of non-chlorotic leaf area. Cuvette conditions were set as follows: 22 °C, 55% relative humidity, 380 ppm CO_2, PPFD of 1000 µmol m^{-2} s^{-1}. After A_n had stabilized, values were averaged over 5 min. Measurements were performed between 9 a.m. and 5 p.m. during the experimental period with four replications per treatment.

2.5. Calculating specific leaf mass and determination of Mg concentrations

To calculate specific leaf mass, leaves were cut after measurements and were photographed 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). Leaves were dried at 60 °C to weight constancy and dry weight was determined. Specific leaf mass (SLM) was calculated by dividing dry weight per obtained leaf area.

Determination of magnesium concentrations was performed with modifiations as in Hansen et al. (2009). Using a high-accuracy balance, 100 mg of dried and powdered leaf material was transferred to a Teflon digestion tube. The digestion medium consisted of 4 ml concentrated HNO_3 and 2 ml 30% H_2O_2 and micowave-digestion was performed at 200 °C at 15 bar for 75 min (Ethos.lab, MLS, Germany). After digestion, samples were diluted in 25 ml double-distilled H_2O. In each batch of microwave digestion, a certified reference material (apple leaf, SRM 1515, National Insitute of Standards and Technology, USA) was also digested. Magnesium concentrations were measured at 279.078 nm by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Vista RL, CCD simultaneous ICP-OES, Varian Inc., USA) equipped with a Quarz Torch Low Flow with a 1.4 mm injector and a Sea Spray nebulizer with sample uptake of 2 ml min^{-1}. Calibration was achieved by a multielement standard solution purchased from Bernd Kraft, Germany. After approx. each 20 samples, measurement of the certified reference material is included to ensure accuracy of measurements. Both, SLM and Mg concentrations were analysed in four eplications per treatment.

2.6. Statistical analyses

Data and statistical analyses were performed using the software RStudio (R Core Team, 2017) and the R packages *agricolae* (de Mendiburu, 2017), *plyr* (Wickham, 2011), *fasttime* (Urbanek, 2016), *matrixStats* (Bengtsson, 2017), *data. table* (Dowle and Srinivasan, 2017), *tidyr* (Wickham and Henry, 2018), and *reshape* (Wickham, 2007). 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 (α = 0.05) where ANOVA indicated a significance. To determine significant differences in the decrease of Mg concentrations between control and Mg concentrations of the respective treatment, a *t*-test was performed (α = 0.05). Non-linear regressions were fitted with the *nls* function implemented in R, using the model equation of $y = ax^{-1} + b$, which fitted best to the data set. Adjusted R^2 for non-linear regressions was obtained by the package *soilphysics* (da Silva and de Lima, 2017) and according to Spiess and Neumeyer (2010).

3. Results

3.1. Mg concentrations in leaves

Reducing the Mg supply significantly reduced the Mg concentrations both in wheat and sunflower (Fig. 1 and Table 1). Leaves of wheat that were supplied with 1 mM Mg (control) contained 2.7 ± 0.2 mg Mg g^{-1} DM. Tissue concentrations below the critical value of 1.5 mg Mg g^{-1} DM were achieved with a supply of 0.05 (1.2 mg ± 0.1 Mg g^{-1}

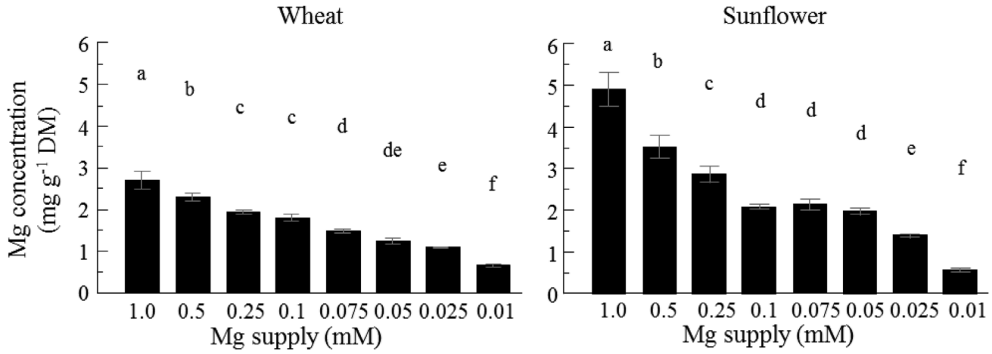

Fig. 1. Magnesium concentrations in latest fully expanded leaves of wheat and sunflower supplied with different magnesium concentrations. Mean values ± SE are shown (n = 4). Different letters indicate significant differences within the plant species (p ≤ 0.05).

Table 1
Percentage of Mg concentrations in latest fully expanded leaves of wheat and sunflower supplied with different magnesium concentrations compared to control leaves (100%), which were supplied with 1 mM Mg. '*', '**', '***' indicate significance levels at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively, ns is non-significant compared to control.

Mg supply	Wheat		Sunflower	
mM	%		%	
0.5	86.6 ± 8.0	ns	72.1 ± 3.3	**
0.25	72.9 ± 4.6	**	58.7 ± 1.1	***
0.1	67.9 ± 5.8	*	43.3 ± 3.6	***
0.075	56.2 ± 5.6	**	43.8 ± 1.6	***
0.05	46.2 ± 4.4	**	40.9 ± 1.8	***
0.025	40.8 ± 3.1	***	29.1 ± 3.0	***
0.01	24.9 ± 0.9	***	11.8 ± 1.7	***

DM), 0.025 (1.1 mg ± 0.0 Mg g^{-1} DM) and 0.01 mM Mg (0.7 ± 0.0 mg Mg g^{-1} DM). In wheat leaves, the Mg concentrations reached 24.9 ± 0.9% of the control concentration when supplied with 0.01 mM (Table 1). Leaves of sunflower which were supplied with 1 mM Mg contained 4.9 ± 0.4 mg Mg g^{-1} DM. Low Mg supply of 0.025 and 0.01 mM induced tissue concentrations of 1.4 ± 0.1 and 0.56 ± 0.05 mg Mg g^{-1} DM, respectively, hence below the critical value. Under the latter supply, Mg concentrations decreased to 11.8% of that in control plants (Table 1).

3.2. Leaf area and specific leaf mass

The leaf area (LA) of the single wheat leaf was not affected by Mg supply (Fig. 2). The leaf area averaged over all treatments was 5.27 ± 0.20 cm^2, with control treatment showing the highest LA of 6.02 ± 0.31 cm^2 and a supply of 0.075 mM the lowest LA of 4.15 ± 0.24 cm^2. The LA of the single sunflower leaf was significantly reduced with decreasing Mg concentrations (Fig. 2). Significant reduction of LA compared to control was observed at a Mg supply of 0.05 and 0.025 mM Mg (49.95 ± 5.40 and 49.26 ± 5.50 cm^2, respectively). Lowest LA of 30.75 ± 1.06 cm^2 was obtained at lowest Mg concentrations. The specific leaf mass (SLM) of both wheat and sunflower was not affected by Mg tissue concentrations (Fig. 3). The mean SLM of wheat ranged from 4.35 ± 0.60 to 5.5 ± 0.26 mg cm^{-2} and that of sunflower from 3.53 ± 0.74 to 4.5 ± 0.32 mg cm^{-2}.

3.3. SPAD values and net assimilation rates

SPAD values as an estimate for relative chlorophyll concentrations were not affected by Mg concentrations in wheat leaves (Fig. 4). The mean SPAD value was 50 ± 0.57. In sunflower leaves, a curvilinear relationship was found between SPAD values and Mg concentrations (Fig. 4). High SPAD values of 39.88 ± 1.45 were observed at highest Mg concentrations. Low Mg concentrations of < 1 mg g^{-1} DM reduced SPAD values to 27.95 ± 1.54.

In wheat leaves, net assimilation rates (A_n) were not affected by Mg concentrations in the leaf tissue (Fig. 5). The mean A_n was 29.54 ± 6.69 µmol CO_2 cm^{-2} s^{-1}. In contrast, in sunflower leaves a strong positive curvilinear relationship between A_n and Mg concentrations was found (Fig. 5). The lowest Mg concentrations of 0.46–0.70 significantly reduced A_n. Both Mg-deficient wheat and sunflower leaves showed A_n comparable to control plants, though Mg concentrations fell below the critical threshold of 1.5 mg Mg g^{-1} DM.

3.4. Chlorophyll fluorescence

Measurement chlorophyll fluorescence was used to obtain the maximum quantum yield (F_v/F_m), the electron transport rate (ETR), the effective quantum yield (Φ_{PSII}), the quantum yield of regulated energy dissipation (Φ_{NPQ}) and the quantum yield of non-regulated energy dissipation (Φ_{NO}). Mg deficiency did not affect F_v/F_m in wheat where the mean F_v/F_m was 0.79 ± 0.00 (Fig. 6). In sunflower leaves, only the lowest Mg concentration reduced F_v/F_m by 12% (0.708 ± 0.02) compared to the control (Fig. 6). Pictures obtained during measurement of F_v/F_m of sunflower illustrate the lower F_v/F_m of leaves supplied with 0.01 mM by more greenish colours (i.e. lower range on the false-colour scale) (Fig. 10). The heterogeneity of the leaf regarding F_v/F_m is clearly visible as interveinal areas show lower F_v/F_m than the major veins. In contrast, leaves of the other treatments show a homogenous distribution of F_v/F_m.

The ETR in wheat did not respond to decreasing Mg concentrations and was in average 70 ± 2 µmol m^{-2} s^{-1} (Fig. 7) whereas in sunflower, lowest Mg concentrations significantly reduced ETR by 23 ± 8% compared to control. In order to identify the proportions of light energy that are used to drive photochemistry (Φ_{PSII}), dissipated as heat (Φ_{NPQ}) and dissipated non-regulatory (Φ_{NO}), the quantum yields are displayed as a sum of 1 (Fig. 8 and Fig. 9). In wheat plants, the proportion of each quantum yield did not differ between the treatments. From Fig. 9, it is clearly visible that wheat plants were not affected by reducing Mg concentrations. The proportion of Φ_{PSII} and Φ_{NPQ} were almost equal, thus the lowest proportion constituted Φ_{NO}. Mean Φ_{PSII} was 0.388 ± 0.004, mean Φ_{NPQ} 0.387 ± 0.005 and mean Φ_{NO} 0.225 ± 0.001. In sunflower, lowest Mg concentrations reduced Φ_{PSII} and increased Φ_{NPQ}, whereas Φ_{NO} remained unaffected. The decrease of Φ_{PSII} was by 24 ± 7% and the increase of Φ_{NPQ} by 27 ± 7%. Pictures obtained during measurement of Φ_{NPQ} of sunflower demonstrate higher Φ_{NPQ} of leaves supplied with 0.01 mM by more blueish-purple colours (i.e. higher range on the false-colour scale) (Fig. 10). Similarly to F_v/F_m, the leaves display heterogeneity in Φ_{NPQ} distribution over the leaf area whereas leaves of the other treatments show a homogenous

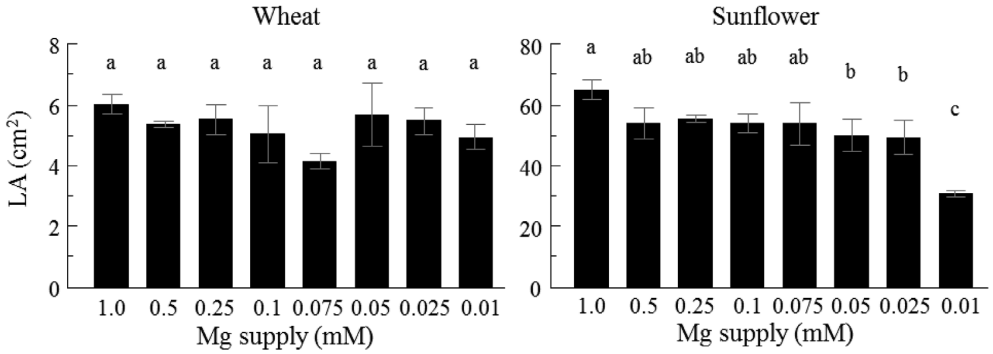

Fig. 2. Leaf area (LA) of latest fully expanded leaves of wheat and sunflower supplied with different magnesium concentrations. Mean values ± SE are shown (n = 4). Different letters indicate significant differences within the plant species ($p \leq 0.05$).

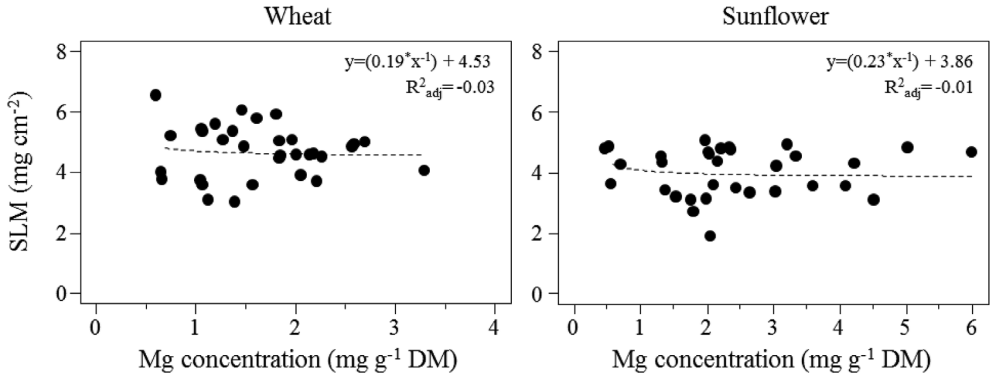

Fig. 3. Specific leaf mass (SLM) of latest fully expanded leaves of wheat and sunflower in dependence on different magnesium concentrations in the leaves.

distribution. Non-photochemical quenching, reflecting heat dissipation of excitation energy in the PSII antenna system was not affected by Mg supply in wheat leaves (Fig. 11 A). The mean NPQ was 1.72 ± 0.04. In sunflower leaves, NPQ was increased by 21 ± 5% under lowest Mg concentrations (2.298 ± 0.071). In both wheat and sunflower leaves, Mg concentrations did not affect the parameter qP that is an estimate for the fraction of PSII centres. The mean qP was 0.663 ± 0.005 and 0.781 ± 0.009 in wheat and sunflower, respectively.

4. Discussion

In the present study, the 8 different decreasing Mg supply levels induced a gradient of Mg concentrations youngest fully expanded leaves. Reducing the Mg supply reduced Mg concentrations of both wheat and sunflower. Assuming a Mg concentration of at least 1.5 mg g^{-1} DM optimal, Mg supply of less than 0.075 mM in wheat and less than 0.025 mM in sunflower induced Mg concentrations which fell below the optimal range and induced deficient concentrations. The magnitude of decrease in Mg concentration was larger in sunflower. Despite having much higher Mg concentrations under 1 mM Mg supply, sunflower leaves had lower concentrations than wheat leaves when supplied with only 0.01 mM Mg. Hence, sunflower reacts more sensitive than wheat to decreasing Mg concentrations.

SLM can be considered a measure to reflect relative carbon accumulation (Witkowski and Lamont, 1991) as it describes the mass per unit leaf area. In tobacco, nitrogen deficiency decreased the specific leaf area (SLA; the inverse of SLM), thus increased SLM (Senbayram et al., 2015). The authors contribute this effect to increases in non-structural carbohydrates such as starch which had higher concentrations in nitrogen deficient plants. An accumulation of photosynthates under Mg deficiency was previously often described in numerous crops such as sugar beet (Hermans et al., 2005), bean (Cakmak et al., 1994), and maize (Mengutay et al., 2013). The proposed mechanism underlying a carbohydrate accumulation is based on limited sucrose loading into phloem cells in source leaves due to reduced plasma membrane H^+-ATPase activity which establishes an H^+-gradient necessary to for the sucrose co-transporters to transport sucrose from the apoplast to the cytosol of phloem cells. In the present study, SLM was unaffected by decreasing Mg concentrations, which does not indicate accumulation of non-structural carbohydrates. As leaf area in sunflower leaves was decreased under deficient Mg concentrations, dry matter must have been decreased proportionally to result in unaltered SLM. This is in line with a study on potato where Mg deficiency decreased leaf area (Cao and Tibbitts, 1992) and a study in birch seedlings where SLA was not affected by decreasing Mg supply (Ericsson and Kähr, 1995). However, Riga and Anza (2003) observed a decrease of SLA under Mg deficiency in pepper plants. This study shows that decreased Mg concentrations affect growth of sunflower (decreased LA), but does not affect the weight of a unit leaf area (SLM) both in wheat and sunflower leaves.

In order to determine the Mg concentration which exerts a limitation on photosynthesis and related processes, SPAD, A_n and chlorophyll fluorescence were measured. One of the first steps in photosynthesis is the harvest of light energy by antenna complexes. The antenna complexes transfer the absorbed light energy to the reaction centres (RC) of the PS where photochemical reactions take place. The majority of the pigments in the antenna complexes are chlorophylls. The relative concentration of chlorophyll can be estimated by SPAD readings using a non-linear relationship as presented by Netto et al. (2005) and Uddling et al. (2007). In the present study, SPAD values and thus relative chlorophyll concentrations were reduced when Mg concentrations were approx. 0.6 mg g^{-1} DM in sunflower leaves. The decrease of chlorophyll concentrations under Mg deficiency are well known and were previously described in numerous reports (Hermans et al., 2004; da Silva et al., 2014; Faust and Schubert, 2016). Similarly to SPAD, the A_n

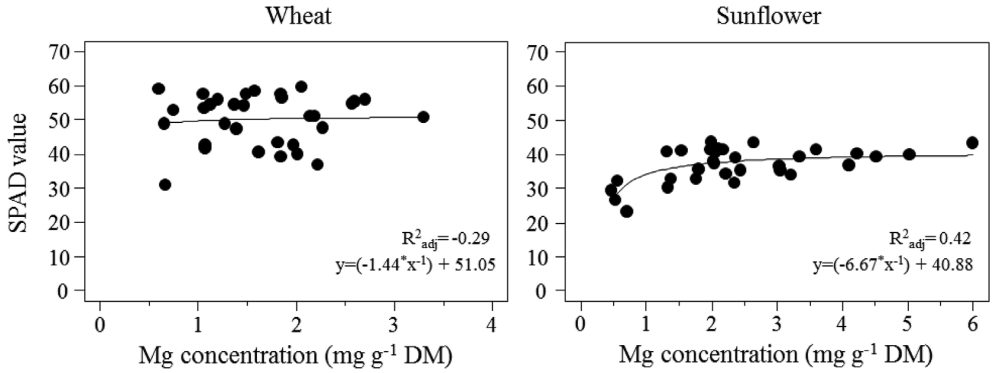

Fig. 4. SPAD values of latest fully expanded leaves of wheat and sunflower in dependence on different magnesium concentrations in the leaves.

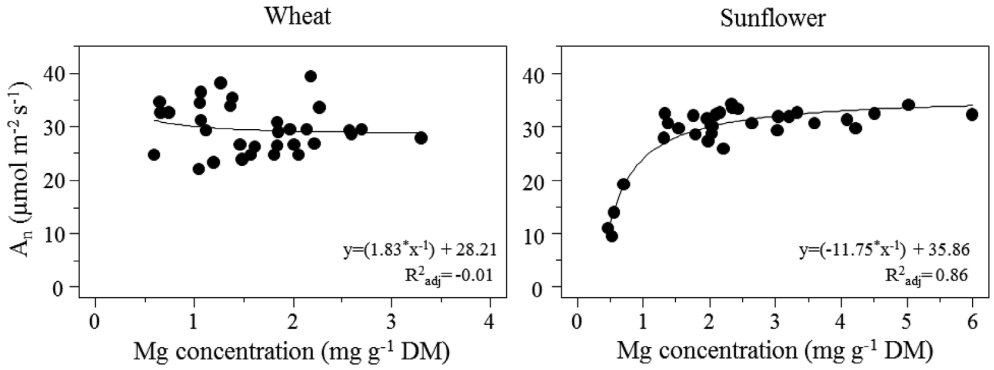

Fig. 5. Net assimilation rates (A_n) of latest fully expanded leaves of wheat and sunflower in dependence on different magnesium concentrations in leaves.

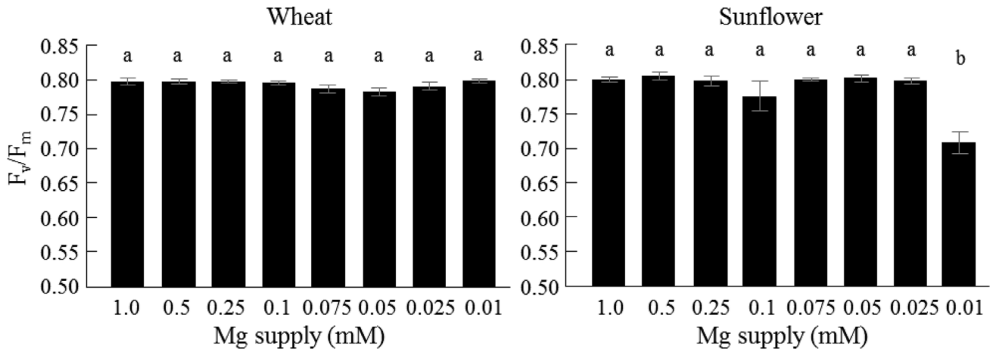

Fig. 6. Maximum PSII quantum yield (F_v/F_m) of wheat and sunflower leaves supplied with different magnesium concentrations. Latest fully expanded leaves were dark adapted for 20 min prior to measurements. Mean values ± SE are shown (n = 4). Different letters indicate significant differences within the plant species ($p \leq 0.05$).

was decreased when Mg concentrations in sunflower leaves were reduced to 0.6 mg g^{-1} DM. Reduced A_n under Mg deficiency is commonly associated with decreased activities of enzymes involved in CO_2 fixation such as Rubisco, and accumulation of carbohydrates triggering a negative feedback on Rubisco, whereas the former and the latter reactions are primary and secondary effects, respectively (Tränkner et al., 2018). A decline in assimilation rates under low Mg supply was observed in numerous species such as sugar beet (Terry and Ulrich, 1974), maize (Jezek et al., 2015), broad bean (Hariadi and Shabala, 2004) and *Sulla carnosa* (Farhat et al., 2015). However, in the present study only the lowest Mg supply level induced decreased SPAD and A_n, though Mg tissue concentrations were reduced already at higher Mg supply levels. Hence, the present study shows that the critical Mg concentration of 1.5 mg g^{-1} DM can be lower in sunflower leaves under these experimental conditions. In wheat plants, neither SPAD nor A_n were affected by Mg concentrations, although Mg tissue concentrations fell below the critical value. Mengutay et al. (2013) induced comparable Mg concentrations in 22-days-old wheat seedlings, but they could observe a decrease in SPAD values and increases in specific dry weights and antioxidant enzyme activities. In another study on wheat, low Mg supply did not affect vegetative biomass formation, but grain yield when Mg concentrations in leaves were approx. only 0.3 mg g^{-1} DW (Ceylan et al., 2016). Mg concentrations were determined after 148 days, hence in fully mature plants and might have been higher in young growth stages when total demand is lower. According to Dow and Roberts (1982), the growth stage of the plant at the time of sampling must

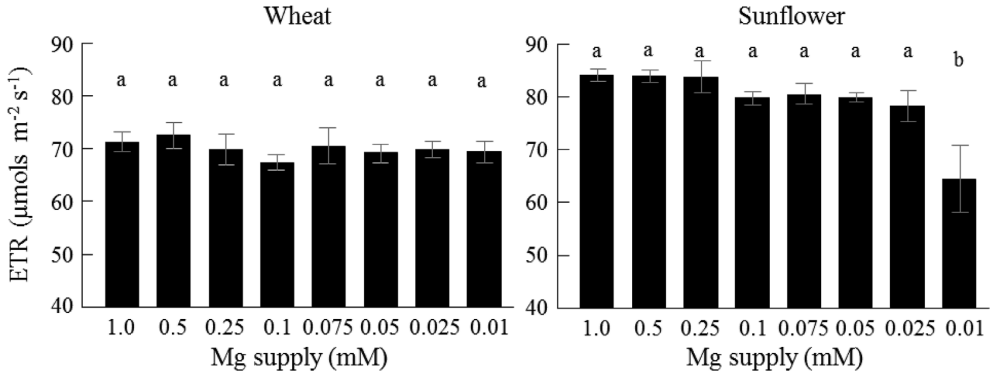

Fig. 7. Electron transport rate (ETR) of wheat and sunflower leaves supplied with different magnesium concentrations. Latest fully expanded leaves were taken for measurements. Mean values ± SE are shown (n = 4). Different letters indicate significant differences within the plant species ($p \leq 0.05$).

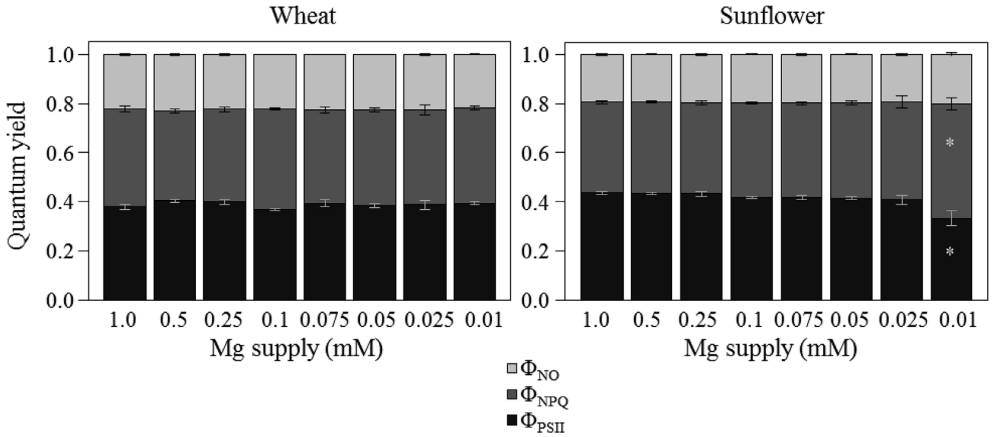

Fig. 8. Effective PS II quantum yield (Φ_{PSII}), non-photochemical quenching (Φ_{NPQ}) and non-regulated energy dissipation (Φ_{NO}) of wheat and sunflower leaves supplied with different magnesium concentrations. Mean values ± SE are shown (n = 4). Asterisks indicate significant differences within the plant species ($p \leq 0.05$).

always be noted when critical nutrient concentrations are considered. They showed that rapid changes in nutrient concentration occur from one growth stage to another in potato petioles. Moreover, photosynthetic performance was shown to be affected by leaf and plant age in a study on Arabidopsis (Bielczynski et al., 2017). However, in the present study all measurements were performed on the identical leaf, thus we can exclude differences due to different development stages.

Measurements of A_n provide insight about photosynthetic processes on a broader spatial scale, whereas chlorophyll fluorescence measurements allow to assess the redox state of PSII, and thus provide detailed understandings on biochemical processes on a narrower scale. The chlorophyll fluorescence parameters Φ_{PSII}, Φ_{NPQ} and Φ_{NO} were assessed to determine changes in the proportion of the fate of absorbed light induced by Mg deficiency. In wheat leaves, the proportion of light used for driving photosynthetic electron transport and the proportion of light which is dissipated as heat are similar irrespective of Mg concentrations. This indicates that the photosynthetic performance is not restricted and is in line with the results of A_n and ETR. In sunflower, the proportion of light which is dissipated as heat (Φ_{NPQ}) is increased under lowest Mg supply, whereas the proportion of light used for driving photosynthetic electron transport is decreased, but the proportion of non-regulated energy dissipation remained unaltered. Φ_{NO} is composed of chlorophyll fluorescence internal changes through the triplet state of chlorophyll, which leads to the formation of singlet oxygen (Moustaka et al., 2015). The constant Φ_{NO} and the increase of Φ_{NPQ} indicates that in sunflower leaves, which had a concentration of 0.56 mg Mg g^{-1} DM, light is excessive and Φ_{NPQ} serves as a photoprotective mechanism to prevent photoinhibition of PSII (Niyogi, 1999). This can be confirmed by increased NPQ. The site for NPQ is the LHCII antenna and the proton gradient (ΔpH) across the thylakoid membrane serves as a trigger for LHCII antenna aggregation required to induce the NPQ state (Ruban, 2016). Over 75% of absorbed photons can be eliminated by NPQ (Niyogi, 1999), hence NPQ can be a substantial regulatory valve for dissipation of excess energy. However, if light energy cannot be sufficiently quenched, loss of PSII activity occurs and photoinhibition takes place (Edreva, 2005). In contrast to NPQ, qP was not affected by low Mg concentrations. The parameter qP can be considered a measure of the fraction of oxidized PSII centres, thus serving as an indicator for the redox state of primary quinone electron acceptor (Q_A). The maintenance of a constant Q_A redox state might be a result of down-regulated ETR and increased energy dissipation as heat (NPQ) (Moustaka and Moustakas, 2014). Thus, in Mg-deficient sunflower leaves photoprotection was efficiently regulated.

In order to assess the PSII functionality under varying Mg supply, F_v/F_m was studied which gives insight about the maximal proportion of absorbed light that can be used to drive photosynthesis. Optimal values of F_v/F_m were reported to be approx. 0.83 (Maxwell and Johnson, 2000), but they might differ between different plant species and

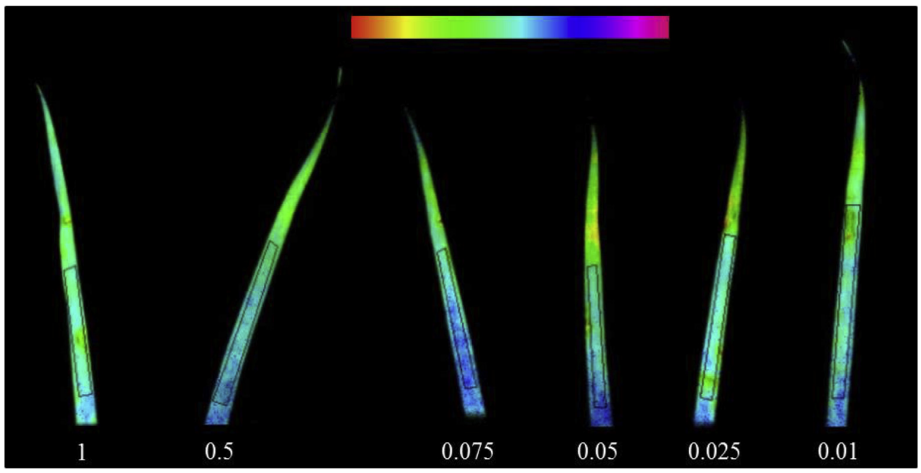

Fig. 9. Representative false colour images of chlorophyll *a* fluorescence showing effective PSII quantum use efficiency (Φ_{PSII}) of wheat leaves. Each image shows leaves of wheat grown under a different Mg supply (mM) as indicated next to the leaves. Imaging was performed 10 days after onset of Mg treatments. Rectangles indicate the area used for calculation of Φ_{PSII}. The colour scale depicted at the top represents a range of 0.133 (red) to 0.647 (pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

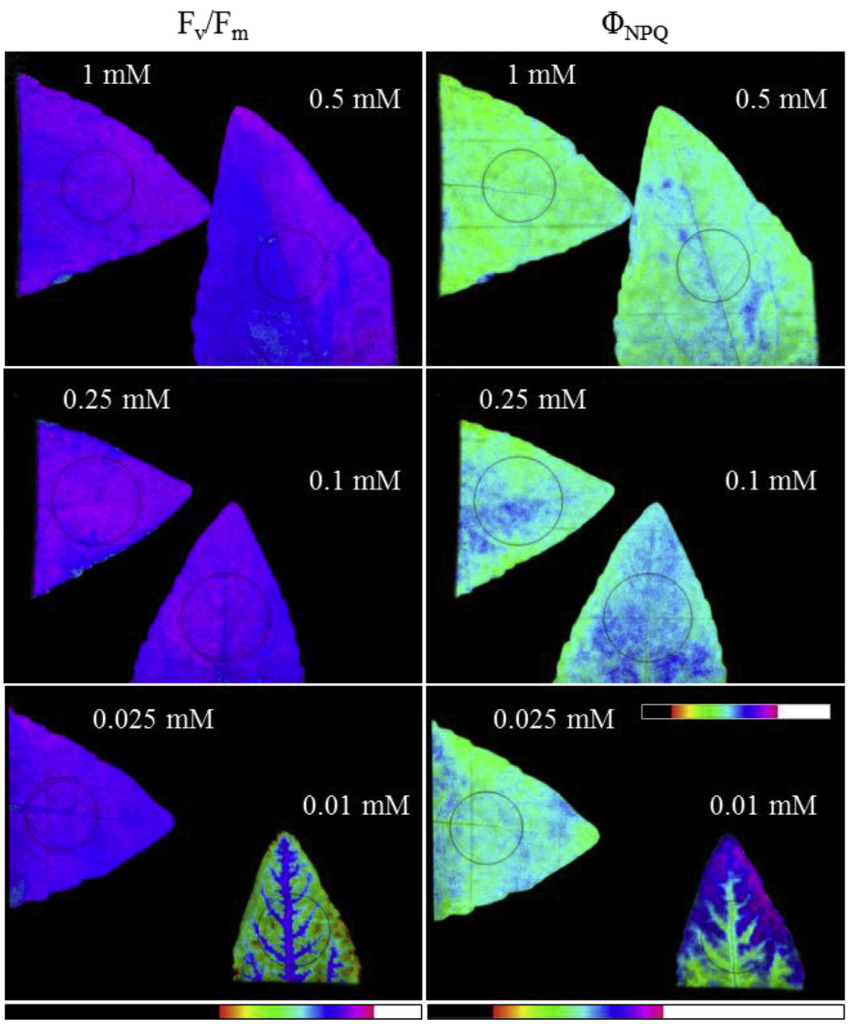

Fig. 10. Representative false colour images of chlorophyll *a* fluorescence showing maximum PSII quantum use efficiency (F_v/F_m, left column) and non-photochemical quenching (Φ_{NPQ}, right column) of sunflower leaves. Each image shows leaves of sunflower grown under a different Mg supply as indicated next to the leaves. Imaging was performed 10 days after onset of Mg treatments. Circles indicate the area used for calculation of F_v/F_m and Φ_{NPQ}. The colour scale depicted at the bottom of images represents a range of 0.514 (red) to 0.878 (pink) for F_v/F_m and 0.157 (red) to 0.561 (pink) for Φ_{NPQ}. Note that the range of the colour scale depicted in the lower right image is broader in order to display higher Φ_{NPQ} in 0.01 mM leaf (0.157 (red) to 0.718 (pink)). It only refers to the 0.01 mM leaf. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

environmental growth conditions. In the present study, the F_v/F_m of wheat and sunflower of control treatment was 0.797 and 0.800, respectively. In a phenotyping study on more than 1000 wheat cultivars, the mean F_v/F_m was 0.802 (Sharma et al., 2012), thus substantially lower than 0.83. Another study on young wheat plants reported F_v/F_m values of control plants of 0.784 (Zlatev, 2009). Hence, there is considerable variation of F_v/F_m among studies. In the present study, F_v/F_m values in Mg-deficient wheat leaves did not differ from those adequately supplied indicating that quinone A, the primary electron acceptor, is in a reduced state and that PSII functionality is not affected under Mg deficiency. However, F_v/F_m of sunflower leaves was decreased under lowest Mg supply, i.e. a Mg concentration of 0.56 mg g^{-1} DM. In two different citrus seedlings, F_v/F_m was decreased under Mg deficiency (Yang et al., 2012). Interestingly, the decrease in F_v/F_m was only observable when Mg leaf concentrations were below 1 mg g^{-1} DM, similarly to our observations in the present study. A decreased F_v/F_m points at a disturbance in or damage at the photosynthetic apparatus (Lichtenthaler et al., 2005). The decrease in F_v/F_m might be due to photoinhibition or have other causes. For determining photoinhibition, measurements of relaxation kinetics to obtain the photoinhibitory quench qI are necessary (Lichtenthaler et al., 2005). Photoinhibition may occur when plants suffer from unfavourable growth conditions like nutrient deficiency or drought (Aro et al., 1993). It is suggested that photoinhibition is induced by an overreduction of the acceptor side of PSII, formation of triplet chlorophyll and production of singlet oxygen (Vass et al., 1992; Telfer et al., 1994; Lindahl et al., 2000). Singlet oxygen is the most important species responsible for degradation of the D1 protein in the reaction centre of PSII (Krieger-Liszkay, 2005). According to Ruban (2016), besides measurement of F_v/F_m, assessment of D1 protein degradation is commonly used to analyse photoinhibition. Hermans et al. (2004) showed that D1 protein (encoded by the gene *PsbA*) content in sugar beets is not affected by Mg deficiency. However, an unchanged Y(NO) indicates no enhanced singlet oxygen production, hence, a decrease in F_v/F_m might have other causes, such as leaf optical properties as indicated by (Murchie and Lawson, 2013). Detailed studies are needed to reveal the reasons for this phenomenon.

In sunflower leaves which were exposed to lowest Mg concentrations, the chlorophyll fluorescence parameters, SPAD values and A_n were affected by Mg deficiency. However, A_n responded with the largest magnitude. The decrease of A_n under lowest Mg supply compared to control was 68%, whereas SPAD values and Φ_{PSII} decreased only by 26% and 24%. Hence, we suggest that from all measured parameters A_n reacts most sensitive to decreasing tissue Mg concentrations. Numerous enzymes involved in CO_2 fixation are activated by chloroplastic free Mg^{2+} concentrations (Wolosiuk et al., 1993). Using a divalent ionophore stromal Mg^{2+} concentration of illuminated spinach chloroplasts were lowered and consequently, sedoheptulose 1,7-bisphosphate and fructose 1,6-bisphosphate levels increased, and this reaction was reversible when Mg^{2+} was added again. The authors suggest that CO_2 fixation is limited due to inhibited activity of the bisphosphatases and

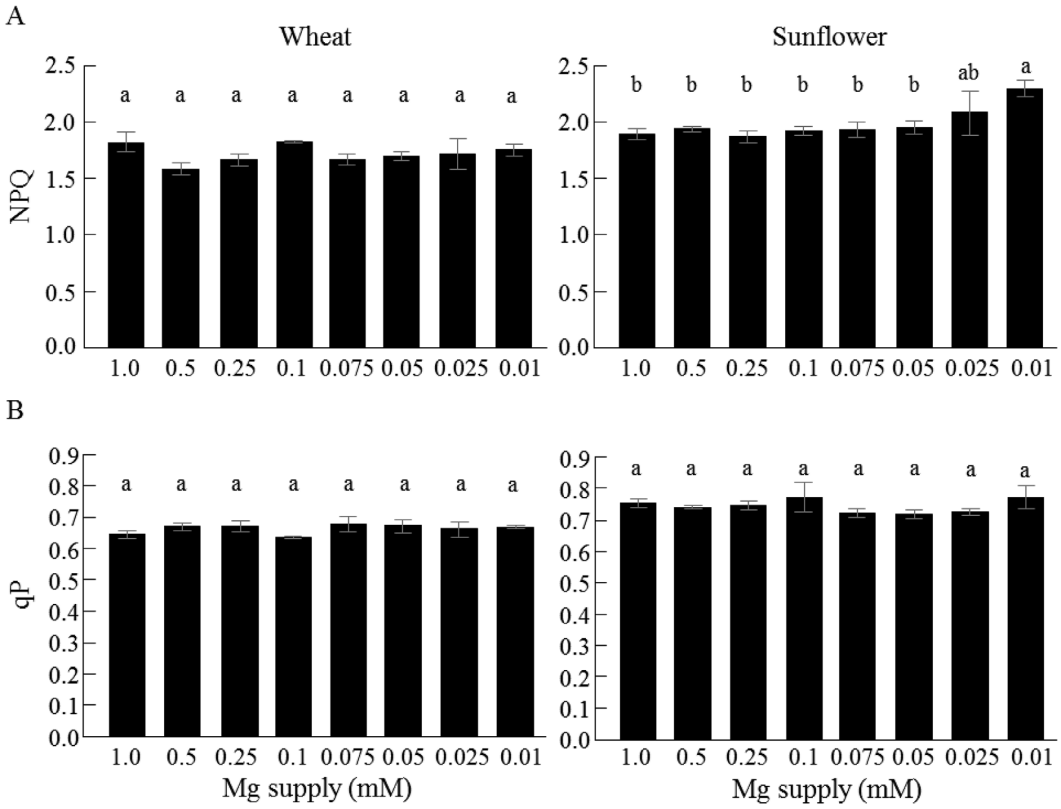

Fig. 11. Non-photochemical quenching (NPQ) (A) and coefficient of photochemical quenching (qP) (B) of wheat and sunflower leaves supplied with different magnesium concentrations. Latest fully expanded leaves were taken for measurements. Mean values ± SE are shown (n = 4). Different letters indicate significant differences within the plant species ($p \leq 0.05$).

this conclusion was confirmed by Ishijima et al. (2003). A higher sensitivity of A_n over Φ_{PSII} was also observed in a study by Farhat et al. (2015) where *Sulla carnosa* plants showed decreased A_n at higher Mg-deficient supply levels, whereas Φ_{PSII} and F_v/F_m where decreased only when Mg was absent. However, how the Mg tissue concentrations observed in the present study, affect stromal Mg concentrations and whether this might affect the activity of bisphosphatases requires further studies.

In summary, in wheat leaves photosynthetic capacity was not decreased though Mg concentrations were considerably reduced. In sunflower leaves, photosynthetic capacity was diminished when leaves reached a Mg concentration of 0.56 mg Mg g^{-1} DM. Hence, critical concentrations were much lower than the reported value of 1.5 mg g^{-1} DM under our experimental conditions. We propose that critical concentrations should be precisely linked to a physiological process, e.g. yield formation, and sampled tissue age as sensitivity to the abundance of Mg might not be equal among several physiological processes.

Contributions

S. Jamali Jaghdani performed the experiment and contributed to data analysis. M. Tränkner analysed the data and wrote the manuscript.

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Chapter 3

Mg deficiency induces photo-oxidative stress primarily by limiting CO_2 assimilation and not by limiting photosynthetic light utilization

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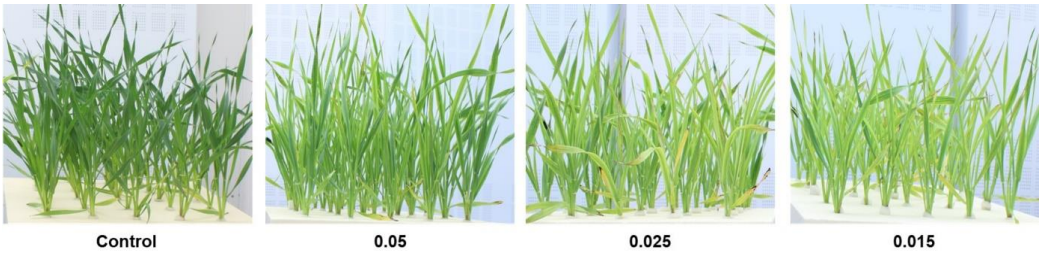

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ABSTRACT

Photosynthetic processes within chloroplasts require substantial amounts of magnesium (Mg). It is suggested that the minimum Mg concentration for yield and dry matter (DM) formation is 1.5 mg g^{-1} DM. Yet, it was never clarified whether this amount is required for photosynthetic processes as well. The aim of this study was to determine how varying Mg concentrations affect the photosynthetic efficiency and photoprotective responses. Barley (*Hordeum vulgare* L.) was grown under four different Mg supplies (1, 0.05, 0.025 and 0.015 mM Mg) for 21 days to investigate the photosynthetic and photoprotective responses to Mg deficiency. Leaf Mg concentrations, CO_2 assimilation, photosystem II efficiency, electron transport rate, photochemical and non-photochemical quenching, expression of reactive oxygen species (ROS) scavengers, and the pigment composition were analyzed. Our data indicate that CO_2 assimilation is more sensitive to the reduction of tissue Mg concentrations than photosynthetic light reactions. Moreover, supply with the two lowest Mg concentrations induced photo-oxidative stress, as could be derived from increased expression of ROS scavengers and an increased pool size of the xanthophyll cycle pigments. We hypothesize, that the reduction of CO_2 assimilation is a critical determinant for the increase of photo-oxidative stress under Mg deficiency.

1. Introduction

The constant increase in the world population poses great challenges on agricultural production. A significant increase in the yield of major crop plants will be essential in order to meet the food supply requirements by the estimated world population by 2050 [1]. It is essential to provide sufficient supplies of nutrients for the growing plants in order to meet the expected yields. In this context, magnesium (Mg) is one of the most crucial plant nutrients which is known to have several key functions in various biochemical and metabolic processes [2,3]. Since it is one of the key elements in the chlorophyll molecule, one of the major roles of Mg is the formation of chlorophyll in photosynthetic organisms [4]. It is been reported that up to 35 % of the total Mg content within plants is located in chloroplasts [2]. Expectedly, decreases of the total chlorophyll concentration under Mg deficiency have been reported in various plant species [5–10] Mg has essential functions within the photosynthetic apparatus. It is directly involved in the activity of numerous enzymes such as ATPases and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) [10,11] as it is needed for CO_2 fixation. Mg is required for protein synthesis which indirectly affects CO_2 fixation via Rubisco protein abundance [13]. It is required for a well-structured organization of grana and stroma lamellae to support chloroplast integrity and efficient light absorption. Therefore, Mg deficiency can cause changes in chloroplast organization, disruption of grana structure and un-stacked membranes [14]. Consequently, a robust inhibitory effect on photosynthetic capacity and net CO_2 assimilation (A_n) has been reported as a typical impact of Mg deficiency in various plant species [14–18,7,9,19,20,22] Impairment of photosynthetic

Abbreviations: A_n, Net CO_2 assimilation; Ax, Antheraxanthin; cAPX, Cytosolic ascorbic peroxidase; CAT, Catalase; Chl, Chlorophyll; Cu/Zn-SOD, Superoxide dismutase; DEPS, De-epoxidation state of the VAZ pigments; ETR, Electron transport rate; F_o, Minimum fluorescence of dark-adapted leaves; $F_{o'}$, Minimal fluorescence yield of light-adapted leaves; F_m, Maximum fluorescence of dark-adapted leaves; $F_{m'}$, Maximum fluorescence yield of light-adapted leaves; Φ_{PSII}, Effective PSII quantum yield; F_v/F_m, Maximum PSII quantum efficiency; GR, Glutathione reductase; NPQ, Non-photochemical quenching; qE, Energy-dependent quenching; qP, Coefficient of photochemical quenching; qZ, Zeaxanthin-dependent quenching; VAZ, Sum of xanthophyll cycle pigments (Vx+ Ax+ Zx); Vx, Violaxanthin; ZxE, Zeaxanthin epoxidase; Zx, Zeaxanthin.

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electron transport rate (ETR) under low availability of Mg have been reported [6,7,21] and it is suggested to be an important factor in reduced CO_2 assimilation [23]. Studies using chlorophyll fluorescence have described considerable decrease in PSII photochemistry under Mg deficiency in different plant species [6,9,22,20].

Under Mg deficiency, where A_n is limited, the absorbed light is excessive to the photosynthetic capacity which leads to an enhanced production of reactive oxygen species (ROS) and plants suffer from oxidative damage [23,24]. Singlet oxygen ($^1O_2$), superoxide (O^-_2), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) are the ROS compounds known to cause oxidative damage which leads to peroxidation of lipids, oxidation of proteins, inhibition of enzymes, and damage to DNA/RNA [26]. ROS detoxification is done by different enzymes such as superoxide dismutase (SOD), ascorbic peroxidase (APX), glutathione reductase (GR) and catalase (CAT) [25,12]. Elevated ROS levels and ROS scavenging enzymes activity under low Mg availability in different plant species have been reported [28]. Absorption of excessive light energy leads to excessive excitation of chlorophyll molecules and results in generation of more triple states (^{3}Chl) and $^1O_2$ [27]. The deactivation of ^{3}Chl and $^1O_2$ in antenna proteins and reaction centers (RC) is provided by firmly bound carotenoids [29]. The non-photochemical quenching (NPQ) of the excitation energy includes the involvement of the xanthophylls in the light harvesting antenna proteins. It allows a reversible switch of light harvesting complexes (LHC) between light-harvesting state under low-light and dissipative state under high-light [29].

One of the xanthophyll cycles described for land plants is known as the violaxanthin (Vx) cycle where Vx is converted to zeaxanthin (Zx) via antheraxanthin (Ax) [27]. The xanthophyll cycle pigments are described to have crucial functions in photoprotection. Ax and Zx in particular are known for heat dissipation of excessive light energy (non-photochemical quenching; NPQ) [30]. Thermal dissipation of excessive absorbed energy by NPQ is known as one of the major protective mechanisms against damages caused by photoinhibition [31–34]. NPQ involves the activity of Vx de-epoxidase converting Vx to Zx which is induced by lumen acidification occurring under illumination.

It is described that the critical Mg tissue concentration is 1.5 mg g^{-1} dry matter [35]. However, the critical Mg tissue concentration seems to vary among different physiological processes. Hauer-Jákli and Tränkner [36] have reported that critical leaf Mg concentrations for A_n have been proposed to be higher than dry matter production. It can be considered that photosynthetic processes might react more sensitive to Mg deficiency. Understanding how Mg nutrition impairs photosynthetic efficiency and at which extent protection against excess radiation is limited, can contribute to improving productivity. Under optimal management practices and in the absence of biotic and abiotic stresses, the efficiency of light interception and energy conversion efficiency are determinants for the yield potential [37], thus suboptimal Mg nutrition might exacerbate limitations in these factors. The aim of this study was to investigate how varying Mg concentrations affect the photosynthetic efficiency of PSII and photoprotective mechanisms.

2. Materials and methods

2.1. Experimental setup

The seeds of barley (*Hordeum vulgare* L. variety Quench) were germinated on paper rolls in 1 mM $CaSO_4$. After one week, the plants were transplanted into hydroponic plant culture. Each 30 L container included 25 plants. Nutrient solution composition was modified from Jákli et al. (2017) [38], hence the final nutrient solutions contained macro- and micronutrients as follows: 1 mM K_2SO_4, 0.25 mM NH_4NO_3, 1.75 mM $Ca(NO_3)_2.4\ H_2O$, 0.05 mM $CaCl_2.2\ H_2O$, 1 mM $MgSO_4.7\ H_2O$, 0.2 mM $Ca(H_2PO_4)_2.H_2O$, 0.1 mM Fe-EDTA, 10 µM H_3BO_3, 1 µM $MnSO_4.H_2O$, 1 µM $ZnSO_4.7\ H_2O$, 0.2 µM $CuSO_4.5\ H_2O$, 0.14 µM $(NH_4)_6Mo_7O_{24}.4\ H_2O$.

The experiment contained three different magnesium (Mg) deficiency treatments which were established at transplanting: 0.05, 0.025, 0.015 mM $MgSO_4.7\ H_2O$. The control treatment was 1 mM. Each Mg treatment was replicated four times. Nutrient solution was refreshed every three to four days based on the plant development. $CaCO_3$ was used to keep the pH at ≈ 6.7. The solutions were provided with constant injection of air in order to supply oxygen.

The plants were cultivated in a climate chamber with day/night light cycle of 16/8 h and a photosynthetic photon flux density (PPFD) of approx. 400 µmol m^{-2} s^{-1} (Valoya®, B-series) at canopy height, 20 °C and 56 % relative humidity. The experimental set up was completely randomized.

2.2. Harvest and leaf Mg concentration

After 21 days after transplanting (DAT), fully expanded leaves were harvested between 9–12 in the morning and dried at 60 °C. Determination of Mg concentrations were performed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) and as described in [21].

2.3. Measurement of net CO_2 assimilation rates

The measurements were performed 17 DAT. Net CO_2 assimilation rates (A_n) were determined by measuring leaf gas exchange (GFS-3000, Heinz Walz GmbH, Germany) on 4×1 cm^2 cuvette size, where the definite area of the measured leaf was determined and corrected if leaves were too small to fill the complete cuvette area. In these cases, the leaf area was determined by measuring the leaf width at both sides of the chamber using the formula for trapeze area. Cuvette conditions were set as follows: 22 °C, 55 % relative humidity, 380 ppm CO_2, PPFD of 1000 µmol m^{-2} s^{-1}. After A_n had stabilized, values were averaged over 5 min. Measurements were performed between 9 a.m. and 5 p.m. with four replications per treatment.

2.4. Chlorophyll a fluorescence

The measurements were performed 17 DAT. Chlorophyll fluorescence measurements were done by using a PAM-fluorometer (IMAGING-PAM Maxi, Heinz Walz GmbH, Germany). Prior to the measurements, leaves were dark adapted for 20 min. The leaf of interest was placed under the device where it was covered by a black cloth and it allowed a photosynthetic photon flux density (PPFD) of 10 µmol m^{-2} s^{-1}. First the maximum PSII quantum yield was measured via [$F_v/F_m = (F_m–F_o)/F_m$] [39]. Then the actinic light was switched on at a PPFD of 461 µmol m^{-2} s^{-1}. Every minute a saturation light pulse was applied with a PPFD of 2700 µmol m^{-2} s^{-1} for 800 ms. Effective PSII quantum yield was calculated as [$\Phi_{PSII} = (F_{m'}–F_t)/F_{m'}$] [40]. F_m is an indicator of the maximum fluorescence of dark-adapted samples. $F_{m'}$ indicates the maximum fluorescence yield in the light following a saturation pulse, where $F_{o'} = F_o/(F_v/F_m + F_o/F_{m'})$ [41]. ETR was determined by calculating ETR=Φ_{PSII}*0.5*Absorptivity*461 µmol m^{-2} s^{-1}. Absorptivity was calculated as Abs. = 1-NR/NIR, where R is remission at 660 nm and NIR is remission at 780 nm. Coefficient of photochemical quenching ($q_P=(F_{m'}–F)/(F_{m'}–F_{o'})$ and NPQ ($NPQ=(F_m–F_{m'})/F_{m'}$) [42] were determined. The area of interest was selected in a rectangular shape for barley leaves, respectively, where the edges of the leaf were avoided, and the center of the leaf was considered as the most representative of the leaf area. The fluorescence values of all pixels within the representative area were averaged automatically by the device's software ImagingWin v2.41a (Heinz Walz GmbH, Germany). Measurements were performed with four replications per treatment.

2.5. Quantitative real-time PCR

After 21 DAT the fully expanded leaves were harvested, frozen in

liquid nitrogen and kept at −80 °C until the RNA extraction. Total RNA was extracted from 100 mg leaf samples using the RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer protocol. The extracted RNA was reverse transcribed into cDNA using cDNA Synthesis Kit (QuantiTect Reverse Transcription Kit, QIAGEN, Hilden, Germany) with prior elimination of genomic DNA and according to the manufacturer protocol. Candidate genes were chloroplast copper/zinc superoxide dismutase (Cu/Zn-SOD), cytosolic ascorbic peroxidase (cAPX), chloroplastic glutathione reductase (GR), peroxisomal catalase1 (CAT1), and the housekeeping gene was actin. Information on primer sequences are given in Table 1. Each qPCR reaction was performed in a total volume of 20 µL using 1 µL of cDNA, 1.6 µL of forward and reverse primers according to the manufacturer protocol (qPCRBIO SyGreen Mix Lo-ROX, PCR Biosystems Ltd., London, UK). qPCR was performed on a CFX96 cycler (BioRad Laboratories, Hercules, CA) with the following cycling conditions: 95 °C for 2 min; 44 cycles of 95 °C for 10 s, 60 °C for 30 s. The ΔCT values were determined via the Bio-Rad CFX Manager 3.1 software (©2012 Bio-Rad Laboratories). The equation $2^{-\Delta\Delta CT}$ was used to estimate expression levels. The expression was normalized to the housekeeping gene and control samples gene expression was calculated as 1.

2.6. Pigment analysis via HPLC

After 21 DAT the fully expanded leaves were harvested between 9–12 in the morning, frozen in liquid nitrogen and kept at −80 °C until pigment extraction. For pigment extraction, leaf samples were homogenized in pre-cooled mortar and pestle. 20 mg of the homogenized leaf material was filled into a 1 mL microtube, 1 mL pure ethanol was added and the sample was homogenized by vortexing until a light green mixture was observed. The samples were incubated in the dark at 4 °C over night until the precipitate was white the next morning. The samples were centrifuged for 5 min at 13,000 x *g*. The supernatant was filtrated through a 0.2 µm filter (Corning®, 15 mm syringe filter, RC membrane). The HPLC analysis was done according to [43]. De-epoxidation state (DEPS) was expressed as 100 * (0.5 antheraxanthin + zeaxanthin) / (violaxanthin + antheraxanthin + zeaxanthin).

2.7. Statistical analysis

Statistical analyses were performed using the software RStudio (R Core Team, 2019) and the R packages agricolae [44], plyr [45], fasttime [46], ggplot2 [47]. Analysis of variance (ANOVA) was performed to determine whether effects of treatments on the respective factor were significant, followed by Tukey's post-hoc test ($\alpha = 0.05$) where ANOVA indicated a significance. To determine significant differences in the decrease of Mg concentrations between control and treatments, a *t*-test was performed ($\alpha = 0.05$).

Table 1
Nucleotide sequence of primers used in quantitative real-time PCR.

Gene	Sequence	Gene description
Cu/Zn-SOD	5' GACTGGCCCTAATGCAGTTG 3'	Chloroplastic copper/zinc superoxide dismutase
	5' TGGCGTCGTTACAGGTATGA 3'	
cAPX	5' CACGGAGCTTTTGAGTGGTG 3'	Cytosolic ascorbic peroxidase
	5' CTGGTCGCGCATAGTAGCAG 3'	
GR	5' TACCGAGGAGCAGGCTATTG 3'	Chloroplastic glutathione reductase
	5' TCTTGCTTTGTCAACCCAGC 3'	
CAT1	5' GCGGAAAATGAACAGCTTGC 3'	Peroxisomal catalase
	5' CATTCACGGGGAGCATCAAG 3'	
Actin	5' AAGTACAGTGTCTGGATTGGAGGG 3'	Housekeeping gene
	5' TCGCAACTTAGAAGCACTTCCG 3'	

3. Results

3.1. Mg concentration in leaves

Reduction in the Mg supply significantly reduced the Mg concentration in the latest fully expanded leaves (Fig. 1-A) which also resulted in typical phenotypic yellowing among barley leaves (Fig. 1-B). Leaves of control plants which were supplied with 1 mM Mg contained 2.41 ± 0.28 mg Mg g^{-1} DM. Leaves of plants supplied with 0.05 mM Mg contained 0.59 ± 0.05 mg Mg g^{-1} DM, hence 25.19 % of the control. The plants which were supplied with 0.025 and 0.015 mM Mg contained 0.36 ± 0.03 and 0.28 ± 0.02 mg Mg g^{-1} DM, respectively. Their Mg concentrations were only 15.59 % and 12.12 %, respectively of the control concentrations.

3.2. Net assimilation rates

Net assimilation rates (A_n) were significantly reduced in all Mg deficiency treatments in comparison with the control (Fig. 2). In control leaves, A_n was 26.32 ± 1.73 but dropped with increasing Mg deficiency to 11.90 ± 2.08 (45.24 % of the control), 6.52 ± 1.04 (24.78 % of the control) and 5.61 ± 0.18 (21.30 % of the control). The reduction in A_n levels thus correlated with the reduced Mg accumulation in *Hordeum vulgare* leaves.

3.3. Chlorophyll a fluorescence

3.3.1. Maximum quantum yield

Decreasing Mg supply induced a slight, but significant decrease in F_v/F_m. Compared to controls (0.79 ± 0.00), however, reduction in F_v/F_m levels was observed in 0.05 mM Mg treated *Hordeum vulgare* plants (0.72 ± 0.01). In contrast, F_v/F_m values determined for plants treated with 0.025 and 0.015 mM Mg were significantly lower with 0.67 ± 0.02 and 0.65 ± 0.01, respectively (Fig. 3), corresponding to a reduction of F_v/F_m to about 83 % of the control value at the lowest Mg concentration. The impact of reduced Mg supply on the maximum PSII efficiency was thus much less pronounced compared to net assimilation rates, indicating that less, but nearly fully functional photosynthetic units accumulated in response to Mg deficiency.

3.3.2. Effective PS II quantum yield (Φ_{PSII}), electron transport rate (ETR), coefficient of photochemical quenching (qP) and non-photochemical quenching (NPQ)

Effective PS II quantum yield (Φ_{PSII}) and ETR continuously decreased in response to decreasing Mg supply (Fig. 4-A and B). Φ_{PSII} at 0.025 and 0.015 mM Mg supply was 0.3 ± 0.01 and 0.27 ± 0.00, corresponding to 81.08 and 72.97 % of control values, respectively. A similar reduction was determined for ETR, which decreased from 79.08 ± 2.28 in controls to 51.40 ± 3.95 µmol m^{-2} s^{-1} and 45.35 ± 2.08 µmol m^{-2} s^{-1} in plants treated with 0.025 mM Mg and 0.015 mM Mg, respectively. Both Φ_{PSII} and ETR were not affected by Mg supply of 0.05 mM. In contrast, qP which is an estimate of the open fraction of PSII centers and NPQ were not significantly affected by Mg supply (Fig. 4-C and D). These findings again support the view that the photosynthetic units accumulating upon Mg deficiency are fully functional.

3.4. Gene expression of ROS scavenging enzymes

Mg deficiency increased the expression of chloroplastic *Cu/Zn-SOD* (Fig. 5-A). At 0.05 mM Mg supply, the expression value was 2.43-fold higher than the control. Highest expression values were observed at 0.025 mM and 0.015 mM Mg supply with 4.97-fold and 3.98-fold higher levels than the control, respectively.

The expression of cytosolic *APX* was decreased significantly at 0.05 and 0.025 mM Mg supply with expression values of 0.27-fold and 0.3-fold of the control, respectively. At 0.015 mM Mg supply, the

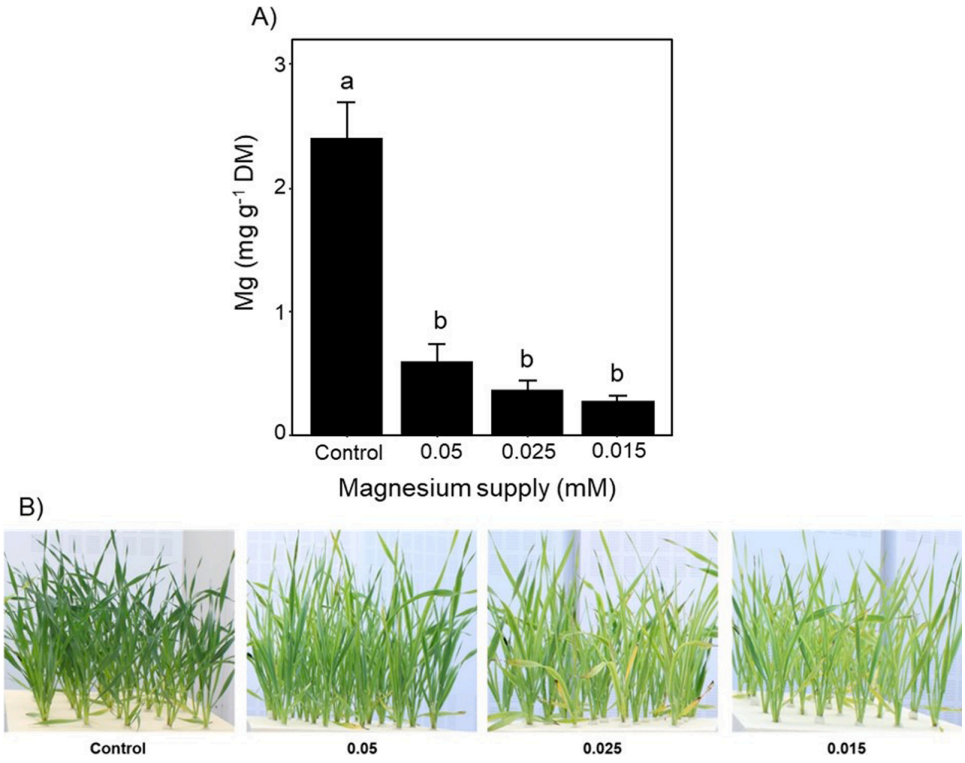

Fig. 1. A) Magnesium (Mg) concentrations in latest fully expanded leaves of *Hordeum vulgare* supplied with different magnesium concentrations. The control plants were supplied with 1 mM Mg. The mean values ± SE are shown (n = 4). Different letters indicate significant differences among the treatments ($p \leq 0.05$). DM = dry matter. B) Barley plants (*Hordeum vulgare*) under different magnesium (Mg) treatments. From left to the right control (1 mM), 0.05, 0.025 and 0.015 mM Mg treatments are shown.

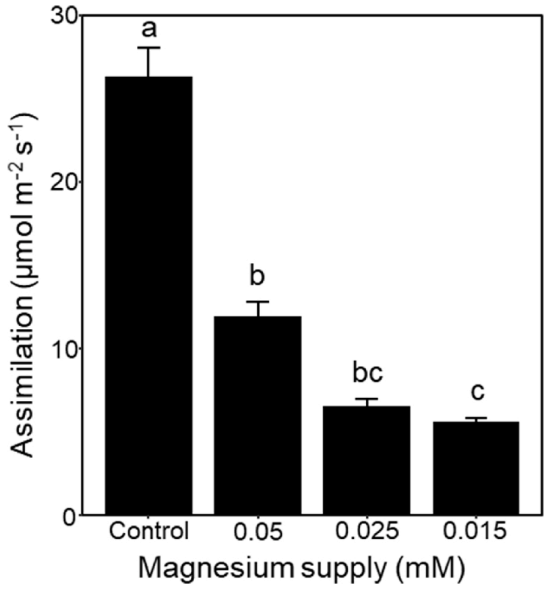

Fig. 2. Net assimilation rates (A_n) of latest fully expanded leaves of *Hordeum vulgare* supplied with different magnesium concentrations at 17 days after transplanting. The control plants were supplied with 1 mM Mg. Different letters indicate significant differences among the treatments ($p \leq 0.05$, n = 4).

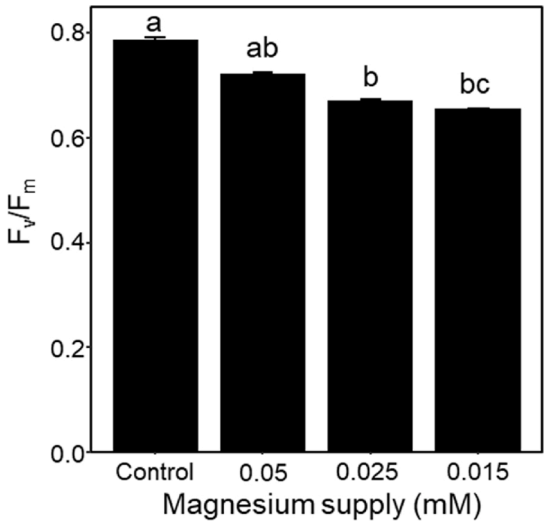

Fig. 3. Maximum PSII quantum yield (F_v/F_m) of *Hordeum vulgare* leaves supplied with different magnesium concentrations 17 days after transplanting. Latest fully expanded leaves were dark adapted for 20 min prior to measurements. The control plants were supplied with 1 mM Mg. Mean values ± SE are shown (n = 4). Different letters indicate significant differences among the treatments ($p \leq 0.05$).

expression of 0.65-fold of the control was observed (Fig. 5-B).

The expression of chloroplastic *GR* was increased under Mg deficiency except for the 0.05 mM Mg treatment. At 0.025 mM and 0.015 mM Mg supply, Mg deficiency induced an expression increase of *GR* by 1.58-fold and 1.55-fold compared to control, respectively (Fig. 5-C).

Peroxisomal *CAT1* expression was not affected at 0.05 mM Mg supply, but increased at to 3.46-fold and 10.52-fold higher values than the control at 0.025 mM Mg and 0.015 mM Mg supply, respectively (Fig. 6-D). In summary, these results indicate that ROS scavenging enzymes are upregulated upon Mg deficiency.

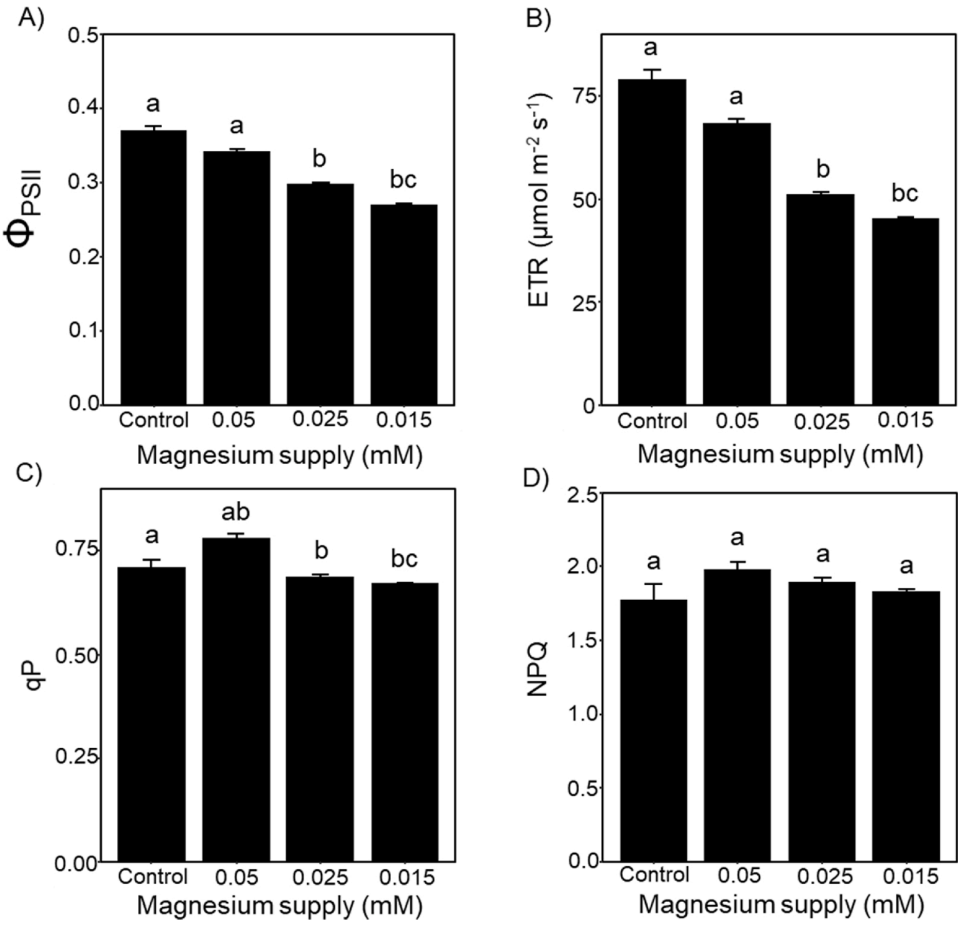

Fig. 4. Effective PS II quantum yield ($\Phi_{PSII,}$) (A), electron transport rate (ETR) (B), coefficient of photochemical quenching (qP) (C) and non-photochemical quenching (NPQ) (D) of *Hordeum vulgare* leaves supplied with different magnesium concentrations at 17 days after transplanting. Latest fully expanded leaves were taken for measurements. Mean values ± SE are shown (n = 4). Different letters indicate significant differences among the treatments ($p \leq 0.05$).

3.5. Pigment composition

Mg deficiency led to pronounced reduction of total pigment content, but to a relative increase of the amount of xanthophyll cycle pigments Vx, Ax and Zx (VAZ pool) (Table 2). Compared to control plants, the chlorophyll (Chl) content on fresh weight (FW) basis was reduced to 34 %, 11 % and 7% in plants treated with 0.05 mM, 0.025 mM and 0.015 mM Mg, respectively. This relative reduction of the Chl content is thus very similar to that observed for the Mg concentration (Fig. 1). Whereas the content of most carotenoids was similarly reduced as the Chl content, the reduction of Ax and Zx was much less pronounced, resulting in a strong increase of the VAZ pool size (on Chl basis) in plants treated with 0.025 mM and 0.015 mM Mg compared to controls (Fig. 6, Table 2). The de-epoxidation state (DEPS) of the VAZ pigments was low in control plants (7.25) and plants treated with 0.05 mM Mg (11), but increased strongly after treatment with 0.025 mM Mg (51.75) and 0.015 mM Mg (69.75), resulting in a strong accumulation of Zx under these two conditions. Moreover, the Chl a/b ratio increased from 3.15 in control plants up to 3.73 with increasing Mg deficiency. The relative increase of Chl a likely reflects a reduction of the antenna size of photosynthetic reaction centers. This interpretation is supported by the relative increase of β-carotene, which is exclusively bound by reaction centers but not by antenna proteins. To our knowledge, this is the first study to investigate xanthophyll pigments compositions under Mg deficiency.

4. Discussion

Critical Mg concentrations are reported to be at 1.5 mg g^{-1} DM [35]. In the present study, the four decreasing Mg supplies induced leaf Mg concentrations below 1 mg g^{-1} DM, hence all treatments decreased leaf Mg concentrations to levels far below the critical Mg concentrations and thus induced Mg deficiency. Nutrient deficiencies can adversely affect the photosynthetic apparatus structure and functions, specially photosystem II (PSII) photochemistry [48]. Reductions in A_n under Mg deficiency have been reported in studies with *Phaseolus vulgaris* [49], *Pinus radiate* [50], *Vicia faba* [19] and *Zea mays* [51]. In the present study, A_n was reduced even at the mildest Mg deficiency treatment (0.05 mM). Mg concentrations in the leaf tissue were decreased by 75 %, whereas the reduction of A_n was less pronounced (by 54 %) indicating an increasing photosynthetic Mg use efficiency at the level of CO_2 assimilation. Similarly, in *Triticum aestivum* and *Helianthus annuus* assimilation rates did not respond proportionally to decreasing Mg concentrations whereas leaf area was negatively affected pointing at a preferential allocation of Mg to photosynthetic processes under low Mg leaf concentrations [21]. However, low Mg concentrations induced a decline in A_n which can be associated with a reduction in chlorophyll content [52], and lower activity of enzymes involved in photosynthesis such as Rubisco [23]. Recently, Li et al. 2020 [22] could show that in rice plants *in-vivo* Rubisco activity in light correlated with reduced chloroplastic Mg concentrations due to diel Mg fluctuations and Mg depletion. In contrast to reduced Rubisco activity, the Rubisco protein quantity was not affected

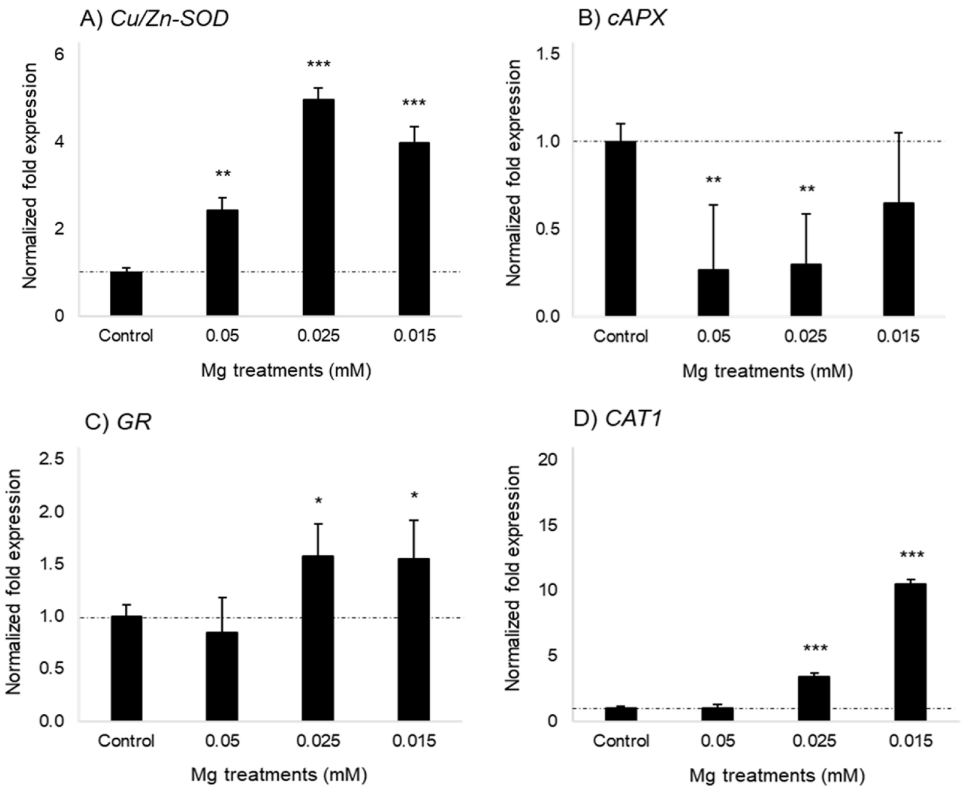

Fig. 5. Normalized gene expression of A. superoxide dismutase (*Cu/Zn-SOD*), B. cytosolic ascorbic peroxidase (*cAPX*), C. glutathione reductase (*GR*) and D. catalase (*CAT1*) in fully expanded *Hordeum vulgare* leaves. The expression level is normalized to the house-keeping gene and the control samples expression is calculated as 1. The dashed horizontal line shows the expression level of the control samples with 1 mM Mg supply. The bars represent the fold changes in different treatments. The asterisks show the significant differences in each treatment in comparison with the control ('*', '**', '***' indicate significance levels at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively, $n = 8$).

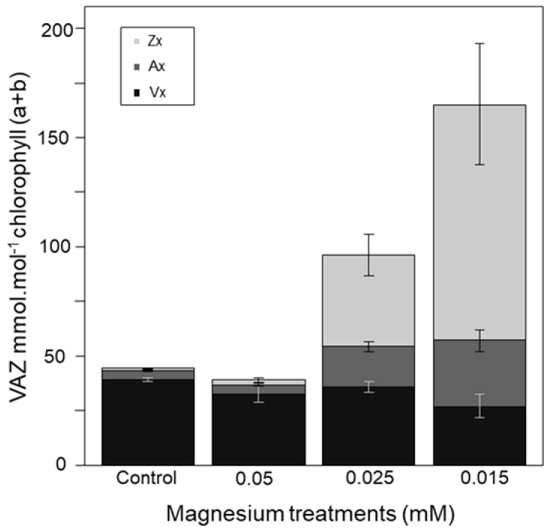

Fig. 6. Sum of violaxanthin (Vx), antheraxanthin (Ax) and zeaxanthin (Zx) (VAZ) in latest fully expanded leaves of *Hordeum vulgare* supplied with different magnesium concentrations for 21 days after transplanting. Mean values ± SE are shown (n = 4).

by lower Mg supply [22]. Furthermore, accumulation of photo-assimilates in source organs due to restricted photoassimilate translocation under Mg deficiency has an adverse effect on the formation and activation of Rubisco [53]. The limitations in CO_2 assimilation decrease the consumption of photochemical energy (ATP) and reducing power (NADPH), thereby exerting excitation pressure and limiting utilization of light energy. Under this situation the absorbed light energy becomes

Table 2
Pigment composition, chlorophyll *a/b* ratio and de-epoxidation state of the VAZ pigments (Vx, Ax, Zx) (DEPS) in fully expanded leaves of *Hordeum vulgare* supplied with different magnesium concentrations for 21 days after transplanting. The control plants were supplied with 1 mM Mg. The data are normalized to 1000 Chl (a + b). Mean values ± SE are shown (n = 4). Car = carotenoid.

Mg treatments (mM)	per 1000 Chl (a + b)			µmol g^{-1} FW
	Car	DEPS	Chl a/b	Chl (a + b)
Control	62.75 ± 3.88	7.25 ± 0.95	3.15 ± 0.10	4.26 ± 1.10
0.05	57.5 ± 0.96	11 ± 2.00	2.75 ± 0.33	1.45 ± 0.27
0.025	83.5 ± 5.87	51.75 ± 5.39	3.34 ± 0.29	0.49 ± 0.06
0.015	110.25 ± 9.80	69.75 ± 9.14	3.73 ± 0.21	0.30 ± 0.07

excessive and an overreduction of the electron transport chain, leading to a production of reactive oxygen species (ROS) such as H_2O_2 [54]. Increased concentrations of H_2O_2 under Mg deficiency were described for numerous crops, such as barley [28], maize [55] and mulberry [56]. A meta-analysis showed that levels of ROS were increased by 31 % under Mg deficiency compared to control treatments [57]. To assess the level of oxidative stress in the chloroplast and mitochondria in response to the different Mg concentrations, transcript abundance of ROS scavenging enzymes was measured. An upregulation of three of the four tested genes was observed under Mg deficiency, indicating oxidative stress. The expression levels of chloroplastic *Cu/Zn-SOD*, responsible for the detoxification of -O_2 to H_2O_2 [58], were increased. Increased activity of *SOD* under Mg deficiency in *Hordeum vulgare* [28] and *Morus alba* [54] have been reported which is in line with our findings on increased expression levels. Tsang et al. [59] have reported that under different environmental stresses such as high light, changes in *SOD* mRNA levels were observed. Similarly, expression of chloroplastic *GR* was increased. Our findings are in line with the studies on *GR* activity under Mg deficiency reported in literature [23,28,56]. Increased *GR* activity is

reported to confer stress tolerance with the ability to alter the redox state of important components of the electron transport chain [60]. *GR* is a component of the ascorbate-glutathione pathway where it reduces oxidized glutathione which then serves as a reductant for other detoxification processes [61]. In contrast to the upregulation of *Cu/Zn-SOD* and *GR*, the expression of cytosolic *APX* in Mg deficient treatments was lower than in control plants. Regarding *APX* enzyme activity, in contrast to our findings, enhanced activities of *APX* in *Hordeum vulgare* [28], *Citrus reticulata* [23] and *Morus alba* [56] have been reported. However, in those studies *APX* extraction was not cell organelle specific. Hence, plastidic *APX* might have been responsible for the high activity levels, indicating increased oxidative stress in the chloroplast upon Mg deficiency. It has been suggested that H_2O_2 has the ability to cross membranes via aquaporins [62] and to diffuse to the cytosol with relative ease [63]. In our study, however, lower expression levels of *cAPX* indicate a lower need for cytosolic H_2O_2 detoxification.

CAT is responsible for the detoxification of H_2O_2 in peroxisomes. The high level of *CAT* expression might be related to increased photorespiration rates. Increased photorespiration might be due to altered specificity of Rubisco, changed CO_2 concentrations at the site of carboxylation or the extent of either Mg^{2+} or Mn^{2+} binding by Rubisco [64,65]. Altered Rubisco specificity was observed in spinach under high growth temperatures [66], and was suggested to indicate an acclimation of Rubisco kinetics to the environment [64]. CO_2 concentrations at the site of carboxylation can be determined by resistances against diffusion of CO_2 from the intercellular air space to the site of carboxylation. Increased mesophyll resistance was shown for potassium deficient sunflower [38] and winter oilseed rape [67]. However, to which extent Mg deficiency induces alterations in the mesophyll resistance and thereby limits CO_2 supply to Rubisco, needs further research. The binding of Mn^{2+} to Rubisco induces similar rates of carboxylation and oxidation as opposed to when Mg^{2+} binds to Rubisco (carboxylation ratio is higher at ambient O_2 and CO_2 concentrations). The activity of the photorespiratory enzyme 2-PG phosphatase which converts 2-PG to glycolate in the chloroplast depends on Mg^{2+} [68], hence its activity might be restricted under Mg deficiency and thereby also affecting photorespiratory processes. Concluding from our results, it is conceivable that Mg deficiency induces oxidative stress in the chloroplast and enhances the need for H_2O_2 detoxification in the peroxisome.

The limitation of CO_2 fixation and the resulting photo-oxidative stress due to Mg deficiency is expected to negatively affect PSII performance. In order to investigate the efficiency of light utilization by PSII, the photosynthetic efficiency of PSII was assessed by Chl fluorescence analyses. The operating efficiency at PSII (Φ_{PSII}) indicates the proportion of absorbed light that is used in PSII photochemistry [40] and in combination with estimates of light absorptance by the leaf and photosystems, electron transport rates through PSII can be assessed. Mg deficiency decreased both Φ_{PSII} and ETR. In accordance, the level of photochemical quenching of PSII (qP), which indicates the proportion of oxidized reaction centers, was reduced under Mg deficiency. Hence, Mg deficiency induces limitations in the photosynthetic quantum conversion in the light-adapted state of the photosynthetic apparatus. However, the impact on PSII photochemistry was much less pronounced compared to the reduction of the assimilation rate, indicating a more severe effect of Mg deficiency on CO_2 assimilation compared to photosynthetic light utilization.

To estimate whether a damage to the photosynthetic apparatus has occurred, the maximum quantum efficiency of PS II photochemistry, F_v/F_m, is frequently applied [69]. The ratio F_v/F_m describes the maximum efficiency at which light absorbed by PSII is used for reduction of the primary quinone acceptor Q_A in dark adapted plants [70]. In control plants, F_v/F_m was 0.79, which is close to the optimal value of about 0.83 in unstressed plants reported by Björkman and Demmig [71] and Maxwell and Johnson [39], which may vary among different species and under different environmental conditions. A decrease in F_v/F_m levels under Mg deficiency have been reported in *Beta vulgaris* [6], *Citrus reticulata* [23] and *Helianthus annuus* [21]. However, in a study on *Pinus radiata* no reduction on F_v/F_m ratio was observed although the A_n was decreased [50]. In the present study, Mg deficiency induced a slight but significant decrease of F_v/F_m. Hence, Mg deficiency results in a reduction of PSII photochemistry in *Hordeum vulgare*, but to less extent than the reduction of CO_2 assimilation. The decline in F_v/F_m might be due to photoinhibition of PSII, as a result of photo-oxidative stress.

The upregulation of ROS scavenging enzymes in response to Mg deficiency suggests that limited Mg availability results in photo-oxidative stress. Thus, we investigated other photoprotective responses that are known to be activated in high-light acclimated plants. In fact, our pigment analysis revealed an increase of the Chl a/b ratio and a strong increase of the VAZ pool size, which both represent typical high-light acclimation responses [72]. Whereas the increase of the Chl a/b ratio is indicative of a reduction of light-harvesting antenna proteins and thus of the functional antenna size of the reaction centers, the strongly increased VAZ pool size reflects a high photoprotective demand. In particular, the xanthophyll Zx, which is formed from Vx under high light, is known to serve a broad range of photoprotective functions. Zx is not only involved in different NPQ mechanisms, such as qE and qZ [73], but is also important for the protection of damaged PSII during the photoinhibition repair cycle [74]. Moreover, Zx is supposed to serve photoprotective functions in the thylakoid membrane independent of NPQ [75], possibly by affecting the thylakoid membrane fluidity and/or stability [76]. The observed up to 4-fold increase of the VAZ pool size under Mg deficiency further implies, that the additional pool of VAZ pigments is predominantly present as non-protein bound pool in the lipid phase of the thylakoid membrane. This can be derived from the fact that each antenna protein can bind only up to 1 xanthophyll cycle pigment [77]. This suggests, that the additional VAZ pool serves predominantly NPQ-independent functions. In agreement with this assumption, we did not find an increased NPQ capacity of Mg deficient plants (Fig. 4-D), in line with former work on *Sulla carnosa* [24].

Another striking observation was that the DEPS of the VAZ pool was strongly increased in Mg deficient plants, but low in control plants (Fig. 6). Vx to Zx conversion is catalyzed by the Vx de-epoxidase. This enzyme is localized in the thylakoid lumen and activated when the lumen pH drops below a value of about 6.2 [29], ensuring that Zx is synthesized when photosynthetic electron transport becomes saturated. The presence of high amounts of Zx in Mg deficient plants, but not in control plants, indicates that Mg deficiency leads to a lower lumen pH compared to control plants, when grown at a light intensity of approx. 400 μmol photons m^{-2} s^{-1}. This is likely a consequence of the strongly reduced assimilation rate, which results in reduced ATP consumption and in turn to reduced proton flux through the ATP-synthase. The high DEPS under Mg deficiency is thus likely a secondary effect caused by reduction of the assimilation rate.

In this context it is noteworthy that a reduction of the Mg concentration by 75 % in the leaves (induced by the mildest Mg deficiency treatment, i.e. supply of 0.05 mM Mg), which is far below the described critical Mg concentration, led to a pronounced decrease of assimilation rates, but did not affect the VAZ pool, DEPS, F_v/F_m and Φ_{PSII}. This supports the hypothesis that CO_2 assimilation is more sensitive to Mg deficiency than photosynthetic electron transport.

5. Conclusions

Magnesium deficiency has various consequences on photosynthesis. In this study with *H. vulgare*, particularly the CO_2 assimilation rate was found to be significantly reduced by the mildest Mg deficiency treatment. We thus conclude that A_n rate and Rubisco activity are most sensitive to Mg deficiency. Notably, Chl fluorescence analyses revealed that photosynthetic light reactions are less severely affected than A_n rates under Mg scarcity. Nevertheless, the upregulation of the expression of ROS scavenging enzymes and the strong increase of the VAZ pool size indicated photo-oxidative stress and/or damage. However, the

increased accumulation of Zx did not affect the NPQ properties, indicating that the increase of the VAZ pool size under Mg deficiency serves NPQ-independent photoprotective functions. We thus conclude that Mg limitations have more severe impact on A_n, but moderate limitation of CO_2 fixation, as determined here in response to the mildest Mg deficiency treatment, is not necessarily sufficient to induce photo-oxidative stress and hence the activation of photoprotective mechanisms.

CRediT authorship contribution statement

Setareh Jamali Jaghdani: Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing. **Peter Jahns:** Resources, Writing - review & editing. **Merle Tränkner:** Conceptualization, Writing - review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Chapter 4

The impact of magnesium deficiency on photosynthesis and photoprotection in *Spinacia oleracea*

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ABSTRACT

Limited magnesium (Mg) supply adversely affects photosynthesis. This is particularly related to the high demand for Mg of key enzymes in the chloroplast, such as the photosystems, the ATP synthase and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). The accepted critical Mg concentrations for yield and dry matter (DM) are 1.5–3.5 mg Mg g^{-1} DM. Earlier studies on Mg deficiency indicated that carbon fixation by Rubisco is severely affected in various plant species, whereas the impact of Mg scarcity on light reactions and photoprotective mechanisms is quite variable. The latter could be related to species-specific differences in the general high light-sensitivity of photosynthetic light reactions. To test this hypothesis, we studied the impact of Mg deficiency in spinach (*Spinacia oleracea*) plants, which are known to be rather high light resistant. *S. oleracea* seeds were grown hydroponically under four Mg treatments (1 (control), 0.05, 0.025 and 0.015 mM) and the impact of Mg deficiency on CO_2 assimilation, photosynthetic light reactions and photoprotection was determined. Our results show that the photosynthetic efficiency and the overall light stress response were not altered under Mg deficiency, whereas the CO_2 assimilation as well as leaf and root Mg concentrations were significantly reduced.

1. Introduction

Due to the growing world population and the increase in food demand, agricultural cropping systems are constantly challenged by efficient, fast and high-quality production. Therefore, it is vital to sufficiently provide nutrients for the crops not to limit yields and to meet the expected demands. Magnesium (Mg) is one of the most abundant elements on earth which is often named as "the forgotten element" (Cakmak and Yazici, 2010). It is one of the most vital elements of plant nutrition which is needed for growth and development (Gransee and Führs, 2013), and which is involved in numerous biochemical processes (Verbruggen and Hermans, 2013). It is the core element of chlorophyll (Chl) molecule which is located in chloroplasts. White and Broadley (2009) have reported that about 75% of the Mg in leaves is involved in protein synthesis and about 15–20% is associated with Chl pigments. Therefore, reductions in Chl concentration have been reported in literature under Mg deficiency (Ceppi et al., 2012; Huang et al., 2019). Besides its function in light harvesting, Mg has crucial functions within the photosynthetic machinery in the chloroplast. It is involved in the activation of numerous enzymes, among those adenosine triphosphate (ATP)-ases and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Li et al., 2001; Shaul, 2002) which is needed for carbon fixation. Mg is further required for grana stacking and hence for efficient energy transfer between the photosystems. As a result, Mg deficiency

; ***Abbreviations:*** A_n, Net CO_2 assimilation; Ax, Antheraxanthin; APX, Cytosolic ascorbate peroxidase; ATP, Adenosine triphosphate; CAT, Catalase; Chl, Chlorophyll; Cu/Zn-SOD, Superoxide dismutase; DAT, Days after transplanting; DEPS, De-epoxidation state of the VAZ pigments; DM, Dry matter; DW, Dry weight; ETR, Electron transport rate; F_o, Minimum fluorescence of dark-adapted leaves; F_o', Minimal fluorescence yield of light-adapted leaves; F_m, Maximum fluorescence of dark-adapted leaves; F_m', Maximum fluorescence yield of light-adapted leaves; Φ_{PSII}, Effective PSII quantum yield; F_v/F_m, Maximum PSII quantum efficiency; FW, Fresh weight; GR, Glutathione reductase; LHCII, Light harvesting complex II; Mg, Magnesium; NPQ, Non-photochemical quenching; PSII, Photosystem II; qE, Energy-dependent quenching; qP, Coefficient of photochemical quenching; qZ, Zeaxanthin-dependent quenching; RC, Reaction center; ROS, Reactive oxygen species; Rubisco, Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); VAZ, Sum of xanthophyll cycle pigments (Vx+ Ax+ Zx); VDE, Violaxanthin de-epoxidase; Vx, Violaxanthin; ZEP, Zeaxanthin epoxidase; Zx, Zeaxanthin.

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can cause disruption in grana structure and membranes (Izawa and Good, 1966). A large number of studies on different plant species have reported a substantial decrease in net CO_2 assimilation (A_n) under Mg deficiency (Farhat et al., 2013; Jamali Jaghdani et al., 2020; Ridolfi and Garrec, 2000; Terry and Ulrich, 1974; Tränkner and Jamali Jaghdani, 2019). Mg deficiency has been reported to induce impairment of electron transport rate (ETR) (Hermans and Verbruggen, 2005; Tang et al., 2012). Tang et al. (2012) suggested the impairment of ETR to be one of the main factors for low A_n under Mg deficiency. Various studies have reported extensive decreases in photosystem II (PSII) photochemistry under Mg deficiency (Farhat et al., 2013; Li et al., 2020). The electron transport chain becomes over-reduced under Mg deficiency, when the absorbed light energy is not utilized in CO_2 assimilation and leads to the production of reactive oxygen species (ROS). The ROS compounds such as singlet oxygen ($^1O_2$), superoxide (O^-_2), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2) cause oxidative damage that leads to the oxidation of proteins, peroxidation of lipids, damage to RNA/DNA and inhibition of enzymes (Hasanuzzaman et al., 2017). ROS can be detoxified by various enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalase (CAT) (Apel and Hirt, 2004). Under high light intensities or limited photosynthetic light utilization as under Mg deficient conditions, light absorption exceeds the capacity of photosynthetic electron transport. As a consequence, the excessively absorbed light energy leads to over-excitation of Chl molecules, which increases the probability of triplet chlorophyll (^{3}Chl) and hence ROS formation (Bhatla and Lal, 2018). Carotenoids are known to contribute to the deactivation of excited ^{3}Chl and $^1O_2$ in antenna proteins (lutein and zeaxanthin) and reaction centers (β-carotene) (Jahns and Holzwarth, 2012). In antenna proteins, lutein acts as efficient quencher of ^{3}Chl (Mathis et al., 1979; Mozzo et al., 2008), whereas zeaxanthin (Zx), which is formed from violaxanthin (Vx) through the intermediate antheraxanthin (Ax) in the xanthophyll cycle (Yamamoto et al., 1962; Jahns et al., 2009), is involved in the deactivation of ^{1}Chl in the dissipation of excessive light energy as heat (non-photochemical quenching; NPQ) (Demmig-Adams and Adams, 1992; Jahns and Holzwarth, 2012; Ruban et al., 2012). NPQ is composed of several components which are activated/deactivated at different time scale and thus contribute to photoprotection in the short- and long-term (Nilkens et al., 2010; Malnoë, 2018). The most rapidly switchable NPQ component is the pH-regulated qE component of NPQ, which is strictly regulated by the thylakoid lumen pH (active at pH $<$ 6.0) and modulated by Zx (Ruban et al., 2012). While qE is essential under rapidly fluctuating light conditions, long-term high light conditions lead to the activation of the qZ, qI and qH components of NPQ which contribute to sustained down-regulation of PSII (Demmig-Adams and Adams, 1992; Nilkens et al., 2010; Verhoeven, 2014; Malnoë, 2018).

It is accepted that the general critical Mg concentration within plants should be between 1.5–3.5 mg Mg g^{-1} dry matter (DM). However, different species differ in their critical concentrations (Hauer-Jákli and Tränkner, 2019). Critical Mg concentrations for spinach are reported to be higher than the commonly accepted concentration; being at 3.5–8 mg g^{-1} DM in latest fully developed leaves (Bergmann, 1993). Tränkner and Jamali Jaghdani (2019) and Jamali Jaghdani et al. (2020) observed that different Mg deficiency treatments affected CO_2 assimilation rates more adversely than photosynthetic light reactions among various crop species. Therefore, it could be suggested that plant Mg concentrations differentially affect physiological and biochemical processes. There is relatively sufficient knowledge about Mg deficiency and its impact on photosynthates transportation disruption from source to sink and DM reduction. However, the knowledge on how Mg nutrition influences photosynthetic efficiency and photoprotection is limited. Better understanding of the impact of Mg deficiency on these processes may help to improve yield production. This study was focused on the influence of different Mg concentrations on photosynthesis and photoprotective responses in spinach.

2. Materials and methods

2.1. Experimental setup

Seeds of spinach (*Spinacia oleracea* variety "Woodpecker", Rijk Zwaan Zaadteelt en Zaadhandel B.V.) were germinated on filter paper rolls in 1 mM $CaSO_4$. Plants were transplanted into 5 L hydroponic plant culture after eight days. Two plants were included in each 5 L pot. The nutrient solution contained macro- and micronutrients as follows, modified after Jákli et al. (2017): 1 mM K_2SO_4, 0.25 mM NH_4NO_3, 1.75 mM $Ca(NO_3)_2.4\ H_2O$, 0.05 mM $CaCl_2.2\ H_2O$, 0.2 mM $Ca(H_2PO_4)_2.H_2O$, 0.1 mM Fe-EDTA, 10 μM H_3BO_3, 1 μM $MnSO_4.H_2O$, 1 μM $ZnSO_4.7\ H_2O$, 0.2 μM $CuSO_4.5\ H_2O$, 0.14 μM $(NH_4)_6Mo_7O_{24}.4\ H_2O$. Three different Mg deficiency treatments were induced at the day of transplanting: 0.05, 0.025, 0.015 mM $MgSO_4.7\ H_2O$. Control plants received 1 mM $MgSO_4.7\ H_2O$.

Each Mg treatment was replicated four times (four pots per Mg treatment). The nutrient solution was refreshed every three to four days based on the plant development. $CaCO_3$ was used to buffer the pH at ≈ 6.5. Constant injection of air was provided to nutrient solutions in order to provide oxygen.

Plants were cultivated in a climate chamber with day/night light cycle of 16/8 h and a photosynthetic photon flux density (PPFD) of approx. 200 μmol m^{-2} s^{-1} (Valoya®, B-series) at canopy height, day/night temperature of 21/17 °C and 56% relative humidity. The experimental set up was completely randomized.

2.2. Measurement of net CO_2 assimilation rates

The measurements were performed 37 days after transplanting (DAT). The net CO_2 assimilation rates (A_n) were determined by measuring leaf gas exchange (GFS-3000, Heinz Walz GmbH, Germany) on 4 × 1 cm cuvette size on the new fully expanded leaf. Cuvette conditions were set as follows: 22 °C, 55% relative humidity, 400 ppm CO_2, PPFD of 1000 μmol m^{-2} s^{-1}. After stabilization of A_n, values were averaged over 5 min. Measurements were performed between 9 a.m. and 5 p.m. with four replications per treatment.

2.3. Chlorophyll a fluorescence

Fluorescence measurements were performed with a PAM-fluorometer (IMAGING-PAM Maxi, Heinz Walz GmbH, Germany) at 37 DAT on the same leaf used for CO_2 assimilation measurements. Prior to all measurements, leaves were dark adapted for 20 min. The leaf was placed under the device where it was covered by a black cloth that allowed a photosynthetic photon flux density (PPFD) of ~10 μmol m^{-2} s^{-1}. After determination of the maximum PSII quantum efficiency [$F_v/F_m = (F_m–F_o)/F_m$] (Maxwell and Johnson, 2000), actinic light was switched on at a PPFD of 335 μmol m^{-2} s^{-1}, and every minute a saturation light pulse (2700 μmol m^{-2} s^{-1}, duration 800 ms) was applied to determine the effective quantum yield $\Phi_{PSII} = (F_{m'}–F_t)/F_{m'}$ (Genty et al., 1989) and NPQ = $(F_m–F_{m'})/F_{m'}$ (Kramer et al., 2004). Absorptivity (Abs) was determined by calculating Abs = 1-NR/NIR, where NR is remission at 660 nm and NIR is remission at 780 nm. ETR was calculated as ETR = Φ_{PSII}*0.5*Abs*335 μmol m^{-2} s^{-1}. The coefficient of photochemical quenching (qP) was determined as $q_P = (F_{m'}–F)/(F_{m'}–F_{o'})$. The area of interest (AOI) that was used for the analyses was selected in a circular shape in the center of the leaves. Within this area the fluorescence values of all pixels were averaged automatically by the device's software ImagingWin v2.41a (Heinz Walz GmbH, Germany). Measurements were performed with four biological replicates per treatment.

2.4. Quantitative real-time PCR

The leaves used for the measurements of CO_2 assimilation and chlorophyll fluorescence were harvested at 38 DAT, frozen in liquid

nitrogen and stored at −80 °C until RNA extraction. Total RNA was extracted from 100 mg of the frozen leaf tissue with RNeasy® Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer protocol. cDNA was produced via reverse transcription of the extracted RNA using the cDNA synthesis kit (QuantiTect Reverse Transcription Kit, QIAGEN, Hilden, Germany) with prior elimination of genomic DNA according to the manufacturer protocol. The candidate genes for xanthophyll cycle enzymes included Vx de-epoxidase (VDE) and Zx epoxidase (ZEP). Candidate genes for ROS scavenging enzymes included chloroplastic copper/zinc superoxide dismutase (Cu/Zn-SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and peroxisomal catalase1 (CAT1). Actin was chosen as housekeeping gene. Information on primer sequences are given in Table 1. Each qPCR reaction was performed in a total volume of 20 µL using 1 µL of cDNA, 1.6 µL of forward and reverse primers according to the manufacturer protocol (qPCRBIO SyGreen Mix Lo-ROX, PCR Biosystems Ltd., London, UK). qPCR was performed on a CFX96 cycler (BioRad Laboratories, Hercules, CA) and the cycling conditions were as follows: 95 °C for 3 min; 44 cycles of 95 °C for 30 s, 60 °C for 40 s. ΔCT values were determined with the Bio-Rad CFX Manager 3.1 software (Bio-Rad Laboratories). The expression levels were estimated with the Eq. $(2)^{-\Delta\Delta CT}$. The control samples gene expression was calculated as 1 and expression was normalized to the housekeeping gene. For each Mg treatment, four biological and two technical replicates were analyzed.

2.5. Pigment analysis via HPLC

The xanthophyll cycle pigments zeaxanthin, antheraxanthin and violaxanthin, and chlorophyll a and b were analyzed. For their determination, fully expanded leaves were harvested at 38 DAT between 3 h after illumination onset (6 a.m.), frozen in liquid nitrogen, and stored at −80 °C. For pigment extraction, leaf material was homogenized in a precooled mortar and 20 mg of the leaf material was transferred to a 1 mL microtube. After addition of 1 mL ethanol, samples were homogenized by vortexing until a light green mixture was observed. Samples were incubated at 4 °C in the dark overnight to allow full extraction of pigments and precipitation of unsolved leaf material. Subsequently, samples were centrifuged at 13,000 x *g* for 5 min and the supernatant was filtrated through a 0.2 µm filter (Corning, 15 mm syringe filter, RC membrane). HPLC analysis was done according to Färber et al. (1997). The de-epoxidation state (DEPS) of the xanthophyll cycle pigments was expressed as 100 * (0.5 Ax+ Zx) / (Vx + Ax + Zx).

2.6. Harvest and Mg concentration

Whole plants (i.e. leaves, shoots and roots) were harvested at 38 DAT 3 h after illumination onset (6 a.m.) and dried at 60 °C. Determination of Mg concentrations were performed by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) as described in Tränkner and Jamali Jaghdani (2019). The dry weight (DW) of shoot and root samples was determined after five days incubation at 60 °C.

2.7. Statistical analysis

Analysis of variance (ANOVA) was applied to determine whether the impact of treatments on different factors were significant. Tukey's post-hoc test ($\alpha = 0.05$) was followed where ANOVA indicated a significant difference. A t-test was performed ($\alpha = 0.05$) to determine significant differences regarding the decrease in Mg concentrations between the control and the other treatments. Statistical analyses were performed using the software RStudio (R Core Team, 2019) and the R packages agricolae (Mendiburu, 2020), plyr (Wickham, 2011), fasttime (Urbanek, 2016) and ggplot2 (Wickham, 2008).

3. Results

3.1. Mg concentrations

Reduction in Mg supply resulted in decreased Mg concentrations in fully expanded leaves at 38 DAT. Control plants were supplied with 1 mM Mg and contained 12.04 ± 0.37 mg Mg g^{-1} DM (Fig. 1-A). Leaves of plants supplied with 0.05 mM Mg accumulated 2.04 ± 0.08 mg Mg g^{-1} DM, hence 16.9% of controls (Fig. 1-A). Further reduction of the Mg supply to 0.025 and 0.015 mM Mg resulted in accumulation of 1.01 ± 0.05 and 0.79 ± 0.06 mg Mg g^{-1} DM, respectively (Fig. 1-A), corresponding to only 8.4% and 6.6%, respectively, of controls.

In both shoots and roots, Mg deficiency treatments induced a significant decrease in the tissue Mg concentration. Control plants supplied with 1 mM Mg contained 11.97 ± 1.15 and 14.31 ± 1.01 mg Mg g^{-1} DM in shoots and roots, respectively (Fig. 1-B and C). Shoots of plants supplied with 0.05 mM Mg contained 1.39 ± 0.09 mg Mg g^{-1} DM (= 11.6% of controls), whereas reduction of Mg supply to 0.025 and 0.015 mM reduced the Mg content to 0.86 ± 0.07 (7.2% of controls) and 0.70 ± 0.03 (5.8% of controls) mg Mg g^{-1} DM, respectively (Fig. 1-B). In roots, reduction of Mg concentrations to 25.2, 18.6 and 17.3% of the control concentrations were determined for the deficiency treatments of 0.05, 0.025 and 0.015 mM Mg, respectively (Fig. 1-C). Obviously, the reduction of Mg concentrations in roots was less pronounced compared

Table 1
Nucleotide sequence of primers used in quantitative real-time PCR for xanthophyll cycle pigments and reactive oxygen species (ROS) scavenging enzymes.

Gene	Sequence	Gene description
Xanthophyll cycle pigments		
VDE	5′ CTGATTTCGTGTGGGTGGGA 3′ 5′ AGCTTGTGCCGAAGACATGA 3′	Violaxanthin de-epoxidase
ZEP	5′ ACTCGGTACACGCTATGCAG 3′ 5′ TTCAACGCTCTTTTGCCACG 3′	Zeaxanthin epoxidase
Actin	5′ CGTTTGGATATTTTGCCTGCCT 3′ 5′ GCAAGGTCGAGACGAAGGAT 3′	Housekeeping gene
Reactive oxygen species (ROS) scavenging enzymes		
Cu/Zn-SOD	5′ TAAGCCCAACCACCCAATCC 3′ 5′ GCAGCAACAATGGTGAGTGG 3′	Chloroplastic copper/zinc superoxide dismutase
APX	5′ TTCGCTTAGGTTGGCACGAT 3′ 5′ TTGGCTCCACCTCTTTGTGG 3′	Chloroplastic ascorbate peroxidase
GR	5′ TCAACGATGGCGTCGAGAAA 3′ 5′ CGGCGTCATCTTCGACAGTA 3′	Chloroplastic glutathione reductase
CAT1	5′ CCAGTTGGCCGTATGGTCTT 3′ 5′ CTGAGTAGTGGACCCCAGGA 3′	Peroxisomal catalase
Actin	5′ ACGTTTGGATATTTTGCCTGCC 3′ 5′ GTAGTCTGTCAGGTCACGCC 3′	Housekeeping gene

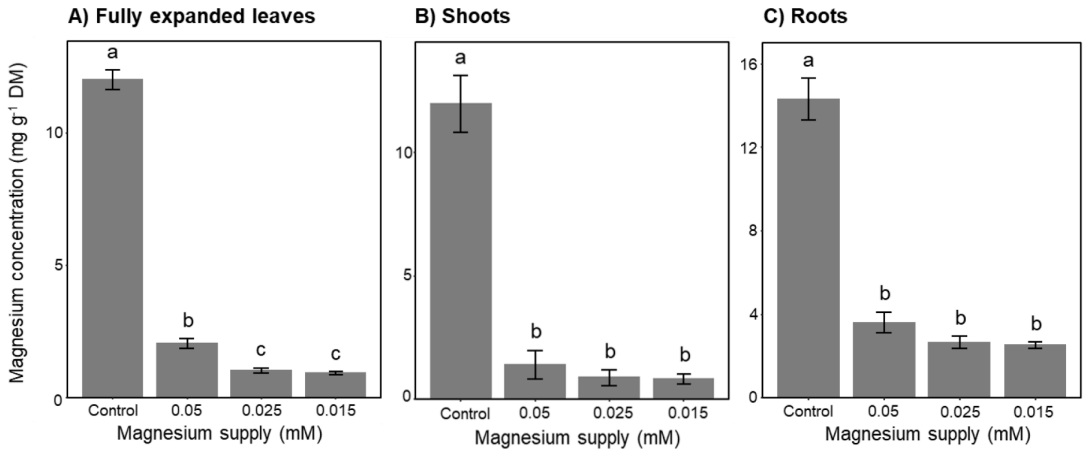

Fig. 1. Accumulation of Mg in different tissues. (A) Latest fully expanded leaves, (B) shoots and (C) roots of *S. oleracea* supplied with different magnesium concentrations, as indicated. Control plants were supplied with 1 mM Mg. Mean values ± SE are shown ($n = 4$). Different letters indicate significant differences among the treatments ($p \leq 0.05$). DM = dry matter.

Fig. 2. Spinach (*Spinacia oleracea*) plants under different Mg treatments 37 DAT. The treatments include control (1 mM), 0.05 mM, 0.025 mM, and 0.015 mM respectively.

to shoots.

3.2. Shoot and root fresh and dry weight

The spinach plants at the whole level under the induced Mg deficiency treatments 38 DAT is presented in Fig. 2. Determination of the shoot fresh weight (FW) indicated significant decreases in response to Mg deficiency (Fig. 3-A). Control plants, which were supplied with 1 mM Mg showed shoot FW of 148.74 ± 14.95 g pot $^{-1}$, reduction to 136.63 ± 9.57 (91.86%), 123.92 ± 22.84 (83.31%) and 71.23 ± 16.72 (47.89%) g pot^{-1} were found after 0.05, 0.025 and 0.015 mM Mg treatments, respectively (Fig. 3-A). In control plants the FW of roots (Fig. 3-B) indicated 110 ± 11.62 g pot $^{-1}$ which was followed by 93.22 ± 3.85 (84.75%), 91.96 ± 14.14 (83.6%), and 59.59 ± 13.82 (54.17%) in 0.05, 0.025 and 0.015 mM Mg treatments, respectively (Fig. 3-B). The shoot dry weight (DW) indicated slight, but not significant reductions in response to Mg deficiency treatment (Fig. 3-C). While control plants, which were supplied with 1 mM Mg showed shoot DW of 12.35 ± 1.20 g pot $^{-1}$, reduction to 10.42 ± 0.78 (84.37%), 10.33 ± 1.57 (83.64%) and 7.95 ± 2.16 (64,37%) g pot^{-1} were found after 0.05, 0.025 and 0.015 mM Mg treatments, respectively (Fig. 3-C). In contrast, a significant reduction in root DW was observed in response to Mg deficiency (Fig. 3-D). The DW of controls (5.32 ± 0.56 g pot^{-1}) was reduced to 4.45 ± 0.26 (16.35%), 3.72 ± 0.56 (30.08%) and 2.25 ± 0.48 (57.71%) g pot^{-1} in 0.05, 0.025 and 0.015 mM Mg treated plants, respectively (Fig. 3-D). The root/shoot ratio is presented in the supplementary data (Fig. S1).

3.3. Net assimilation rates and chlorophyll content

Net assimilation rate (A_n) was significantly reduced in the lowest Mg

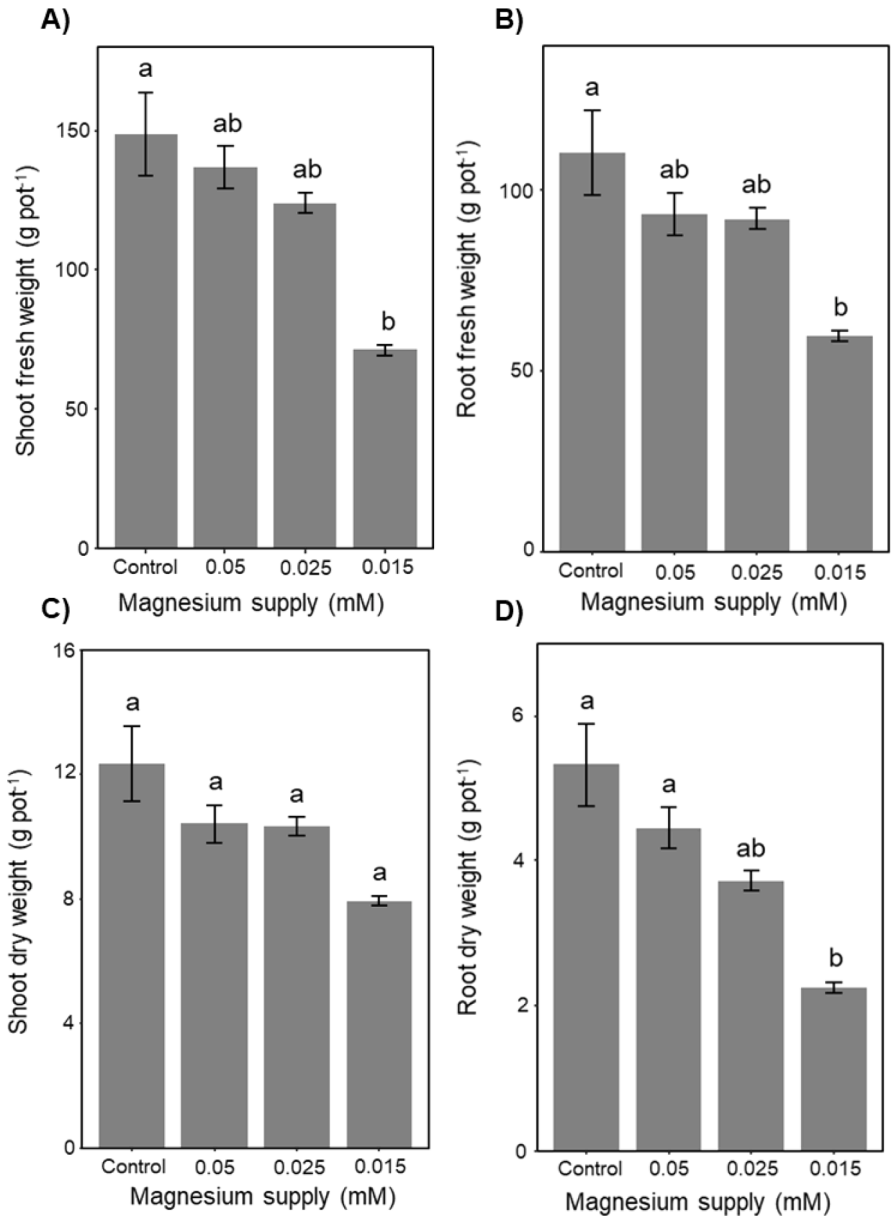

Fig. 3. Biomass accumulation of *S. oleracea* plants in response to Mg deficiency. (A), Fresh weight (FW) of shoots, (B) FW of roots, (C) DW of shoots, and (D) DW of roots at 38 DAT. Control plants were supplied with 1 mM Mg, other concentrations as indicated. Mean values ± SE are shown ($n = 4$). Different letters indicate significant differences among the treatments ($p \leq 0.05$).

deficiency treatment in comparison with the control (Fig. 4-A). In control leaves, A_n was 29.10 ± 1.91 and was followed by 28.57 ± 0.82 in 0.05 mM Mg treated plants (98.2% of the control). A_n achieved after 0.025 and 0.015 mM Mg treatments were 21.87 ± 1.76 (75.2% of the control) and 17.97 ± 4.56 (61.8% of the control), respectively. The Chl concentration observed in control plants was 1444 ± 127 nmol g^{-1} FW (Fig. 4-B), whereas it was reduced to 844 ± 172 (58.44% of the control), 1009 ± 91 (69.88%) and 1019 ± 51 (70.57%) in plants treated with 0.05, 0.025 and 0.015 mM Mg, respectively.

3.4. Photosynthetic light utilization

Chlorophyll fluorescence analyses were applied to determine changes of the photosynthetic performance in response to Mg deficiency. The maximum quantum yield of PSII in the dark-adapted state was derived from the F_v/F_m ratio (Fig. 5-A). Compared to control plants (0.812 ± 0.01) a slight reduction of F_v/F_m was found upon increasing Mg deficiency, which was only significant for the lowest supplied Mg concentration of 0.015 mM (0.777 ± 0.010). This indicates that PSII activity remains nearly unchanged in spinach plants upon Mg deficiency treatments. This view was supported by determination of the ETR (Fig. 5-B) and the photochemical quenching parameter qP (Fig. 5-C), which both reflect the PSII activity in the light-adapted state. In fact, no significant changes compared to controls (ETR = 75.05 ± 1.35 μmol electrons m^{-2} s^{-1} and qP = 0.79 ± 0.01) were detectable for both parameters under all Mg deficiency treatments. Only the NPQ value decreased continuously from 1.58 ± 0.01 (controls) to 1.57 ± 0.06 (0.05 mM Mg), 1.34 ± 0.15 (0.025 mM Mg) and 1.25 ± 0.06 (0.015 mM

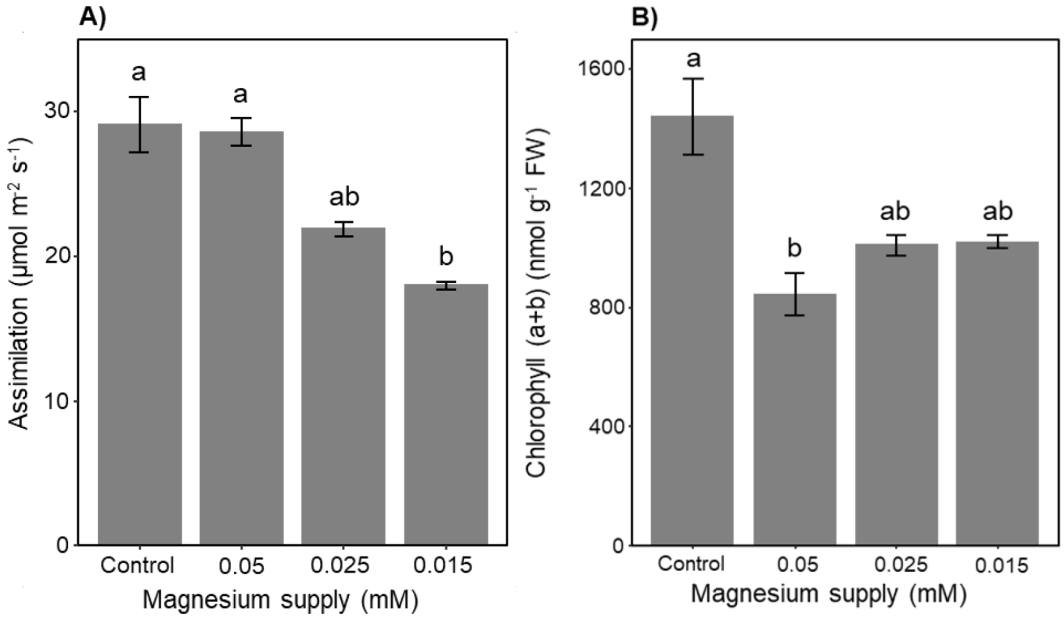

Fig. 4. Net assimilation rates (A) at 37 DAT and chlorophyll concentrations (B) at 38 DAT of *S. oleracea* in latest fully expanded leaves. Control plants were supplied with 1 mM Mg, other concentrations as indicated. Mean values ± SE are shown ($n = 4$). Different letters indicate significant differences among the treatments ($p \leq 0.05$).

Mg) (Fig. 5-D). However, these differences were statistically not significant. Reduction of NPQ could be understood as the consequence of decreased lumen acidification. Since ETR was unchanged in all Mg deficient plants, this might be related to increased ATP-synthase activity.

3.5. Gene expression of ROS scavenging enzymes

Magnesium deficiency induced significant reduction of gene expression of ROS scavenging enzymes (Fig. 6). Compared to controls, for which the expression of different genes was normalized to 1, the expression of plastidic *Cu/Zn-SOD* was reduced to 0.51, 0.16 and 0.25-fold in plants treated with 0.05, 0.025 and 0.015 mM Mg, respectively (Fig. 6-A). Similar patterns of reduction in expression in response to Mg deficiency were observed for the other analyzed enzymes: Expression of plastidic *APX* was reduced to 0.36, 0.20 and 0.40-fold (Fig. 6-B), expression of plastidic *GR* to 0.33, 0.26 and 0.25-fold (Fig. 6-C), and expression of peroxisomal *CAT1* was reduced to 0.57, 0.25 and 0.51-fold (Fig. 6-D), each in 0.05, 0.025 and 0.015 mM Mg treated plants, respectively. This reduction in ROS scavenging enzymes' gene expression supports the view that Mg deficiency did not induce oxidative stress in spinach plants under the chosen experimental conditions.

3.6. Gene expression of xanthophyll cycle enzymes

To further elucidate the response to Mg deficiency of the expression of genes related to photo-oxidative stress, we determined the changes of the expression of the genes of the two xanthophyll cycle enzymes, Vx de-epoxidase (VDE) and Zx epoxidase (ZEP). Compared to the control, *VDE* expression was found to be reduced to 0.62, 0.22 and 0.34-fold levels (Fig. 7-A) and *ZEP* expression to 0.51, 0.15 and 0.19-fold levels (Fig. 7-B) in 0.05, 0.025 and 0.015 mM Mg treated plants, respectively. Thus, both genes showed similar behavior than those coding for ROS scavenging enzymes (Fig. 6). In line with analyses of photosynthetic light utilization, these results indicate that Mg deficiency did not induce activation of the photoprotective system in *S. oleracea* fully expanded leaves.

3.7. Pigment composition

The total VAZ pool size, i.e. the total amount of the xanthophyll cycle pigments Vx, Ax, and Zx, and the de-epoxidation state (DEPS) of the VAZ pool was used as further indicator of possible photo-oxidative stress in response to Mg deficiency. Compared to the control, the VAZ pool size in leaves of Mg deficient plants was either slightly lower or higher, but none of these differences were statistically significant (Table 2). The same was observed for the DEPS. Both the unchanged VAZ pool size and the unchanged DEPS indicate that Mg deficiency does not lead to increased photo-oxidative stress under the given experimental conditions. Moreover, also the Chl a/b ratio was not altered in response to Mg deficiency (Table 2). This indicates that the photosynthetic complexes and the photosystem stoichiometries remained unchanged upon reduced Mg supply, supporting again the view that no oxidative stress was induced by Mg deficiency.

4. Discussion

The three Mg deficiency treatments in the present study induced the decrease of Mg concentration in fully expanded leaves, shoots and roots (Fig. 1). In leaves of control plants of spinach that were treated with 1 mM Mg, higher concentrations of Mg were determined than in leaves of wheat, sunflower and barley (Jamali Jaghdani et al., 2020; Tränkner and Jamali Jaghdani, 2019) under similar experimental conditions. The higher Mg concentration in spinach leaves indicates a more efficient Mg uptake and/or a higher demand of spinach for Mg. It has been repeatedly reported that Mg availability is critical for DM production. There have been several studies in various plant species including sugar beet (Hermans et al., 2004), barley (Tränkner et al., 2016) and Arabidopsis (Hermans and Verbruggen, 2005) that report DM reduction under Mg deficiency. Cakmak and Kirkby (2008) suggested that Mg concentrations below 2 mg g^{-1} DM are critical for most plants, albeit critical concentrations are also dependent on the species and the growth light intensities. In our experiment with spinach, the shoot DW was not significantly reduced whereas root DW was substantially affected under Mg deficiency (Fig. 3). Phloem loading impairment is one of the impacts

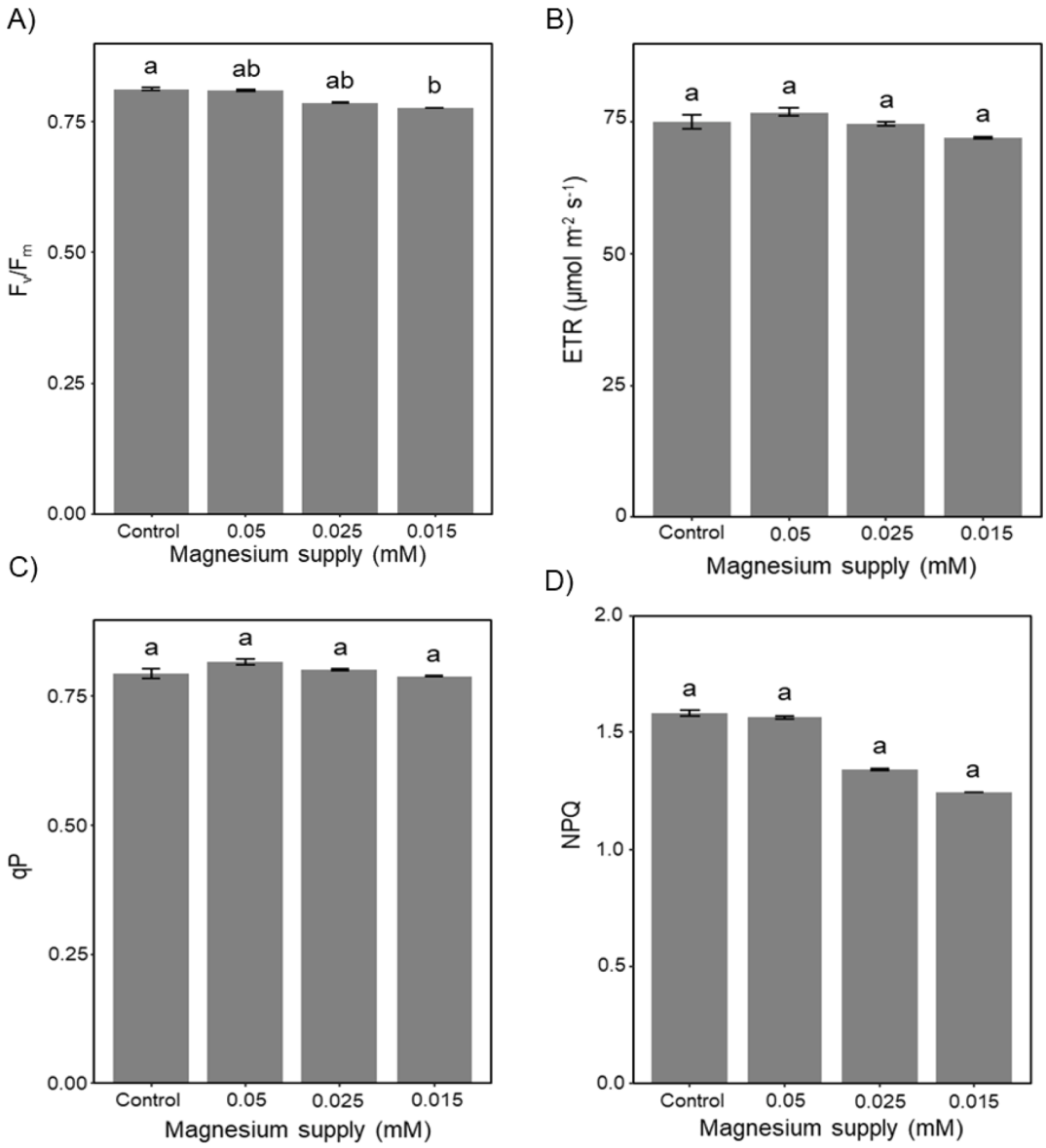

Fig. 5. Photosynthetic light utilization in response to Mg deficiency. (A), Maximum PSII quantum efficiency (F_v/F_m), (B) Electron transport rate (ETR), (C) Coefficient of photochemical quenching (qP), and (D) Non-photochemical quenching (NPQ) was determined in *S. oleracea* leaves at 37 DAT. Latest fully expanded leaves were used. Control plants were supplied with 1 mM Mg, other concentrations as indicated. Mean values ± SE are shown ($n = 4$). Different letters indicate significant differences among the treatments ($p \leq 0.05$).

of Mg deficiency (Cakmak et al., 1994), thus limiting photosynthate transport from source to sink organs, and by that reducing root growth, flowers and fruits. Furthermore, reductions in root growth are observed much earlier than the reductions in shoot growth (Cakmak and Kirkby, 2008). Therefore, the reduction in DW of spinach can be related to phloem loading impairment. However, A_n remained unaffected in latest fully expanded leaves of plants treated with 0.05 mM Mg, which accumulated a Mg concentration of 2 mg g^{-1} DM (Fig. 1-A). This slightly higher Mg concentration compared to the total shoot Mg concentration (1.39 mg Mg g^{-1} DM, Fig. 1-B) can be explained by Mg translocation from older to younger leaves. We can conclude that a leaf concentration of 2 mg Mg g^{-1} DM is sufficient to maintain A_n rates, but lower concentrations are critical for photosynthetic CO_2 assimilation. Indeed, critical Mg deficiency concentrations influencing photosynthetic mechanisms adversely, especially A_n and photosystem II (PSII) photochemistry (Kalaji et al., 2014), have been estimated with about 2 mg Mg g^{-1} DM (Cakmak and Kirkby, 2008), in line with our results.

In accordance with our findings, reductions in A_n under Mg deficiency have been reported in different plants such as *Sulla carnosa* (Farhat et al., 2015), *Vicia faba* (Hariadi and Shabala, 2004), *Hordeum vulgare* (Jamali Jaghdani et al., 2020), *Zea mays* (Jezek et al., 2015), *Triticum aestivum* and *Helianthus annuus* (Tränkner and Jamali Jaghdani, 2019), and *Citrus sinensis* (Ye et al., 2019). Reduction in A_n rate is frequently assigned to reduced activities of enzymes involved in CO_2 fixation such as Rubisco and Rubisco activase or to the reduction in Chl content (Fig. 4; Mengutay et al., 2013). Applying a proteomic approach, Peng et al. (2015) showed for *Citrus sinensis* that under Mg deficiency the abundance of Rubisco and Rubisco-activase was decreased concomitant with decreased CO_2 assimilation, indicating that the Rubisco content is limiting A_n under Mg deficiency. These findings can easily be explained

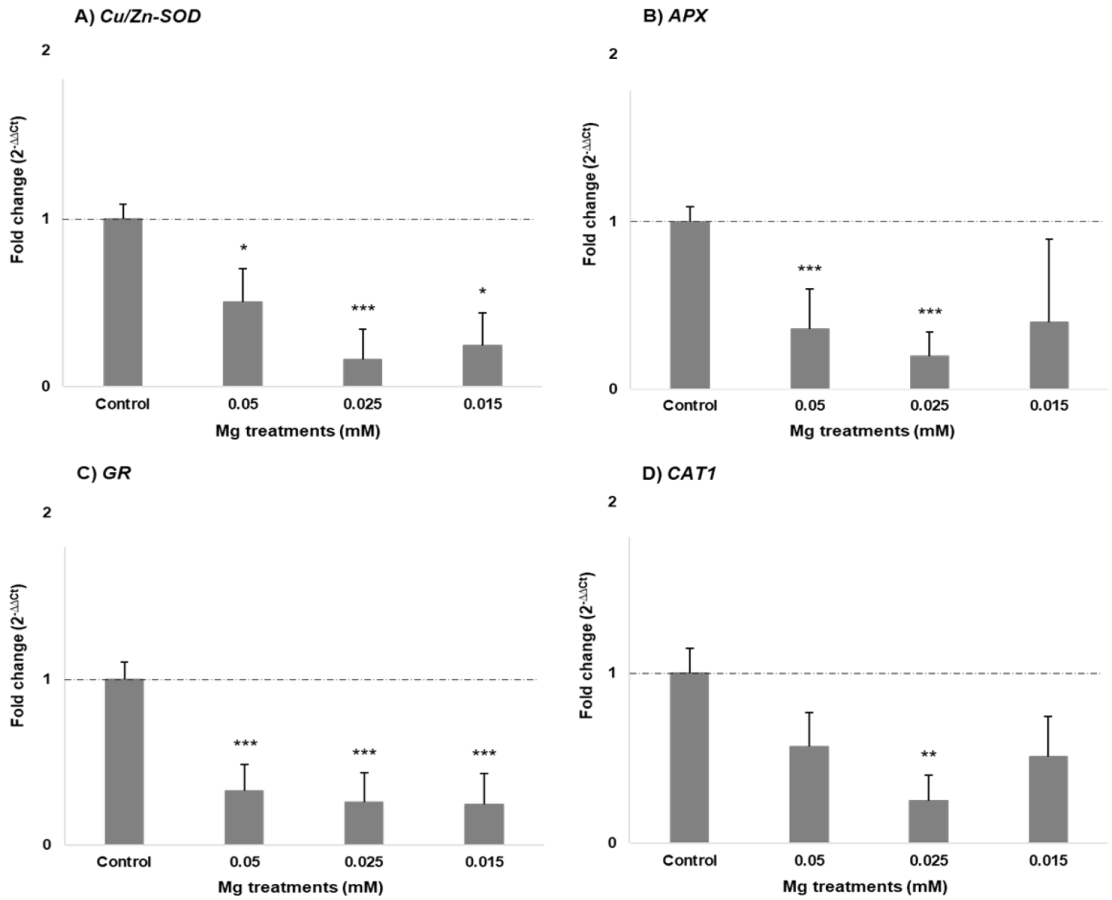

Fig. 6. Expression of genes coding for ROS scavenging enzymes in response to Mg deficiency. (A) Plastidic superoxide dismutase (*Cu/Zn-SOD*), (B) Plastidic ascorbate peroxidase (*APX*), (C) Plastidic gluthatione reductase (*GR*) and (D) Peroxisomal catalase (*CAT1*). Fully expanded *S. oleracea* leaves at 38 DAT were used for RNA extraction. Expression levels were normalized to the housekeeping gene *ACTIN* and the expression in control samples was set to 1. The dashed horizontal line shows the expression level of the control samples with 1 mM Mg supply. Other Mg concentrations as indicated. The bars represent the fold changes in different treatments in relation the control. Asterisks indicate significant differences in each treatment compared to the control (*, **, *** refer to significance levels of $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively, $n = 8$).

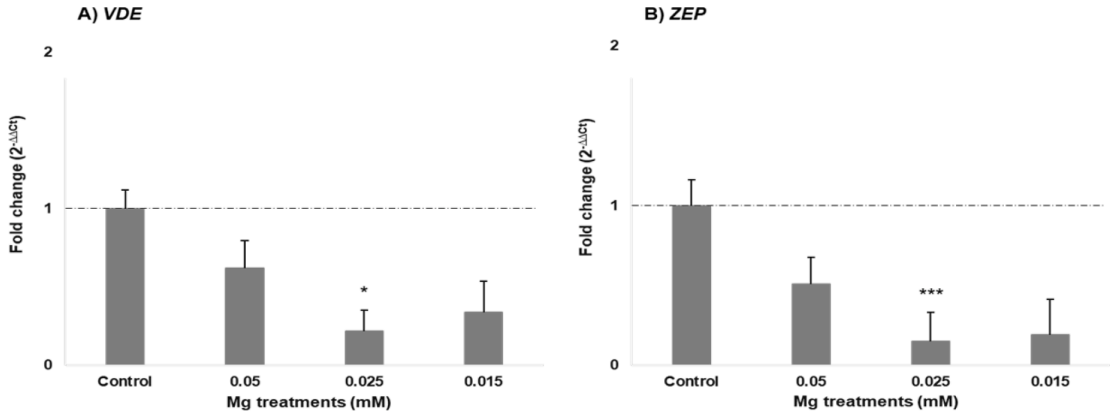

Fig. 7. Expression of genes coding for xanthophyll cycle enzymes in response to Mg deficiency. (A) Vx de-epoxidase (*VDE*) and (B) Zx epoxidase (*ZEP*). Fully expanded *S. oleracea* leaves at 38 DAT were used for RNA extraction. Expression levels were normalized to the housekeeping gene *ACTIN* and the expression in control samples was set to 1. The dashed horizontal line shows the expression level of the control samples with 1 mM Mg supply. Other Mg concentrations as indicated. The bars represent the fold changes in different treatments. Asterisks indicate significant differences in each treatment compared to the control (*, **, *** refer to significance levels at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ respectively, $n = 8$).

Table 2
Xanthophyll cycle pigments and Chl a/b ratio. Pigments were extracted from fully expanded leaves of *S. oleracea* at 38 DAT. Control plants were supplied with 1 mM Mg. The VAZ pool size is normalized to 1000 Chl ($a + b$) and the DEPS is given in%. Mean values ± SE are shown ($n = 4$).

Mg treatments (mM)	VAZ pool per 1000 Chl ($a + b$)	DEPS	Chl a/b
Control	81 ± 5	5.75 ± 0.85	2.83 ± 0.02
0.05	70 ± 3	4.67 ± 1.20	3.06 ± 0.14
0.025	79 ± 4	6.25 ± 1.31	2.84 ± 0.05
0.015	86 ± 6	9.33 ± 4.37	2.82 ± 0.13

by a direct effect of Mg on Rubisco activity, since both activation of Rubisco and Rubisco activity are known to require Mg (Portis and Heldt, 1976). In agreement with this, Yuguan et al. (2009) reported in a study with spinach plants, that both the expression and the activity of Rubisco and Rubisco activase were reduced under Mg deficiency. Moreover, Li et al. (2020) observed that Rubisco activity correlated with reduced chloroplastic Mg concentrations in response to diel Mg fluctuations and Mg depletion in rice plants. Hence, These studies consistently indicate that the Rubisco content and Rubisco activity are critical determinants for the limitation of A_n under Mg deficiency. This assumption can also explain the reduced assimilation rate under Mg deficiency (Fig. 4-A) independently of any significant effect on photosynthetic light utilization (Fig. 5), determined in the present study with spinach, Moreover, reduced translocation of photosynthates to sink organs under Mg deficiency, adversely influences formation and activity of Rubisco (Krapp et al., 1991). Based on the reduction in root DW of spinach plants concomitant with a slight decrease in shoot DW observed in our experiments, we can assume that Mg deficiency adversely affected the Rubisco activity.

However, not only Rubisco activity *per se*, but also the availability of ATP and NADPH, which are both provided by the light reactions, may limit CO_2 assimilation. Conversely, decreased consumption of ATP and NADPH due to reduced CO_2 fixation may negatively affect photosynthetic electron transport. The activity of PSII is a reliable indicator for changes in the functionality of electron transport in response to Mg deficiency. In fact, reduction of PSII efficiency (Φ_{PSII}) in response to Mg deficiency has been reported in *Sulla carnosa* (Farhat et al., 2015), barley (Jamali Jaghdani et al., 2020) and wheat (Tränkner and Jamali Jaghdani, 2019). Moreover, reduction of the maximum quantum yield of PSII, F_v/F_m, under Mg deficiency has been observed in different plant species such as *Beta vulgaris* (Hermans et al., 2004), *Citrus reticulata* (Tang et al., 2012), *Hordeum vulgare* (Jamali Jaghdani et al., 2020) and *Helianthus annuus* (Tränkner and Jamali Jaghdani, 2019). Surprisingly, however, neither PSII-driven ETR (which reflect the PSII efficiency) nor the maximum PSII quantum yield (F_v/F_m) were found to be affected in the present study with spinach under Mg deficient conditions (Fig. 5). This indicates that the reduction of A_n under Mg deficiency in spinach is not related to reduced ATP/NADPH supply from the light reactions, and conversely, that the reduced A_n does not adversely affect photosynthetic electron transport and PSII activity.

Limitations in CO_2 assimilation might further lead to increased photooxidative stress and hence to enhanced ROS production due to an increased excitation pressure (Cakmak and Kirkby, 2008). However, the unchanged PSII activity and ETR, as well as the slightly reduced NPQ (Fig. 5) do not indicate an increased excitation pressure under our experimental conditions. In line with these results, gene expression levels of ROS scavenging enzymes such as *Cu/Zn-SOD, APX, GR* and *CAT* were decreased in spinach plants under our experimental conditions (Fig. 6). Similarly, Jamali Jaghdani et al. (2020) observed reduced gene expression of cytosolic *APX* in barley, but increased expression of peroxisomal *CAT*, plastidic *GR* and *Cu/Zn-SOD*. The activity of ROS scavenging enzymes has been reported to be reduced in response to Mg deficiency. Ze et al. (2009) have shown that the activity of SOD was decreased due to an accumulation of H_2O_2 when spinach chloroplasts were cultivated in a media without Mg, whereas other studies found decreased activity of CAT under Mg deficiency (Tewari et al., 2006; Ze et al., 2009; Tang et al., 2012; Mengutay et al., 2013). Lower CAT activity was assigned to sensitivity of CAT to photoinactivation (Tang et al., al.,2012). Photoinactivation of CAT is suggested to be a result of photooxidative damage originating from chloroplasts by absorption of light by the heme moieties of the enzyme itself (Shang and Feierabend, 1999). Mengutay et al. (2013) assumed that CAT may be easily photo-inactivated under abiotic stress conditions, possibly related to the similarity of CAT to the D1 protein of PSII, which is known to be prone to photooxidative damage, However, increased expression and activity of ROS scavenging enzymes have been reported under abiotic stress conditions in different plant species (Abu-Romman and Shatnawi, 2011; Cakmak and Marschner, 1992; Tränkner et al., 2016; Tsang et al., 1991). In this context, it is important to consider the various isoforms of the genes encoding ROS scavenging enzymes, as well as the presence of different isoforms of ROS scavenging enzymes in different compartments. For example, Han et al. (2020) reported both increased and decreased expression levels of different SOD isoforms in response to various abiotic stresses to *Salvia miltiorrhiza*. Likewise, Belin et al. (2015) found different expression levels of different glutaredoxin isoforms under stress in *A. thaliana*. Therefore, the interpretation of changes in the activity of ROS scavenging enzymes isolated from total protein extracts might be limited in significance regarding specific compartments, such as the chloroplast. The reduced expression of genes encoding ROS scavenging enzymes in chloroplasts suggests, that under our experimental conditions, the Mg deficiency treatment did not result in increased oxidative stress in chloroplast of spinach plants.

Another well-known acclimation response to high light and photooxidative stress is the increase of the VAZ pool size (Bailey et al., 2004; Demmig-Adams et al., 2012; Mishra et al., 2012; Schumann et al., 2017), which provides an increased capacity of Zx-dependent photoprotective mechanisms in thylakoid membranes. However, we did not find any evidence for changes of xanthophyll abundance or conversion in spinach plants in response to Mg deficiency. Both the VAZ pool size and the DEPS of the xanthophyll cycle pigments were not increased in Mg deficient plants (Table 2). Moreover, expression of the genes encoding the two xanthophyll cycle enzymes, VDE and ZEP, was nearly unchanged (Fig. 6). This is in line with the unchanged or only slightly reduced NPQ in response to Mg deficiency (Fig. 4-D). We can thus conclude, that also at the level of the xanthophyll cycle no indications for an increased photooxidative stress can be determined for spinach plants in response to Mg deficiency. In other studies, the response of NPQ to Mg deficiency varied among different species. Tränkner and Jamali Jaghdani (2019) observed increased NPQ values in sunflower but no changes in wheat under Mg deficiency. Jamali Jaghdani et al. (2020) reported slight increase of NPQ in barley whereas Farhat et al. (2015) did not observe any significant changes in NPQ in *Sulla carnosa* under Mg-deficient treatment. Hence, the photooxidative stress response induced by Mg deficiency is quite variable among different species. The same conclusion can be drawn from recent work by Jamali Jaghdani et al. (2020) showing substantially increased VAZ pool size in barley after Mg deficiency treatment, which is in contrast to the present study with spinach, where the VAZ pool size remained unchanged. Interestingly, the rather high VAZ pool size of spinach has recently been suggested to be a critical determinant for the higher resistance against high-light stress of spinach compared to Arabidopsis and tobacco plants (Bethmann et al., 2019). We therefore speculate that differences in the general high-light resistance may determine the variability of the photooxidative stress response to Mg deficiency among different species.

In conclusion, this study demonstrated that in spinach plants, Mg deficiency has pronounced negative impact on CO_2 assimilation and root growth but not on photosynthetic light reactions. Moreover, typical acclimation response to photooxidative stress are not induced by Mg deficiency in spinach. We hypothesize that high-light tolerant plants are generally less vulnerable to photo-oxidative stress induced by Mg

deficiency. Hence, increasing the high-light tolerance might be a promising strategy to improve the resistance of crop plants against Mg deficiency.

CRediT authorship contribution statement

Setareh Jamali Jaghdani: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Peter Jahns:** Resources, Writing – review & editing. **Merle Tränkner:** Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2021.100040.

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Chapter 5

General discussion

Magnesium (Mg) leaching from agricultural fields by rain, and no compensation through fertilization after crop harvest can lead to severe Mg deficiency in agricultural crop plants. Overlooking Mg nutrition in agricultural production within the past decades has led to Mg deficiency among people within the developed countries (Rosanoff et al., 2012 and Shaul, 2002). Mg is particularly important due to its vital roles in stabilization of nucleic acid, proteins cell membranes, and cell walls and its involvement in enzyme activity maintenance such as H^+-ATPase. Considering the association of 15-20% of the total Mg with chlorophyll pigment (White and Broadley, 2009), higher sensitivity of chloroplasts and photosynthetic mechanisms to Mg deficiency is expected. Chlorophyll degradation of older leaves is known as one of the consequences of low Mg nutrition in plants (Roy et al., 2007). Relative leaf chlorophyll concentrations were studied in wheat and sunflower (Chapter 2) under various Mg deficiency treatments. On one hand Mg deficiency did not enhance chlorophyll degradation in wheat plants, whereas sunflower was significantly affected under the strongest Mg deficient treatment (0.01 mM). Barley plants (Chapter 3) on the other hand, revealed significant reductions in chlorophyll content in comparison to control plants which were treated with 1 mM Mg. However, the chlorophyll degradation was not as severe in spinach (Chapter 4). Chlorophyll degradation is a process that allows recycling of nutrients and inhibits the accumulation of phytotoxic chlorophyll intermediates (Hörtensteiner, 2006). The first step of chlorophyll breakdown includes the elimination of the phytol tail (dephytylation) and the central Mg atom (Eckardt, 2009). Therefore, its breakdown will provide Mg for younger plant parts or sink organs such as younger leaves, roots, seeds and fruits. Bergmann (1992) has reported that chloroses occur due to the high phloem mobility capacity of Mg. However, the reduction in chlorophyll concentration has an impact on efficient light utilization and photosynthesis. Chlorophyll fluorescence measurement in wheat and sunflower (Chapter 2) was done 10 days after transplanting, on the fully expanded seedling leaves. The maximum photosystem II (PSII) quantum yield (F_v/F_m) in sunflower was significantly reduced at the lowest Mg treatment, while in wheat plants no reduction was observed. F_v/F_m was significantly decreased in barley (Chapter 3) and spinach (Chapter 4). The optimal F_v/F_m value is reported to be 0.83 (Björkman and Demmig, 1987). However, this value varies among different plant species under different growth conditions (Maxwell and Johnson, 2000). Aro et al. (1993) have reported that the decrease in F_v/F_m can be established upon diverse environmental stresses such as drought and nutrient deficiency which leads to photoinhibition. Excessive photosynthetic photon flux density in combination with environmental stresses, is reported to be as one of the causes for F_v/F_m reduction (Demmig-Adams and Adams, 1992). Overreduction of the acceptor side of the PSII leads to triplet chlorophyll (^{3}Chl) formation and forms singlet oxygen ($^1O_2$), which is one

of the main ROS responsible for D1 protein degradation in the reaction center (RC) of PSII. D1 protein is involved in the early stage of photoinhibition. The turnover of the D1 protein increases under high-light intensities and recovery from the photoinhibition requires *de novo* synthesis of it. The synthesis of D1 protein is exceptionally suppressed by elevated levels of $^1O_2$ and hydrogen peroxide (H_2O_2) (Nishiyama et al., 2001). Assessment of the D1 protein degradation is commonly studied to analyze photoinhibition besides F_v/F_m measurements (Ruban, 2016). Hermans et al. (2004) have proposed that the failure in PSII activity is due to the loss of PSII antenna. However, according to the study conducted by Farhat et al. (2015), under Mg deprivation in *Sulla carnosa*, only a mild drop in the abundance of D1 protein was observed. In *Arabidopsis thaliana* plants grown under Mg scarcity, the expression of gene *cab2* that encodes a chlorophyll a/b binding protein of PSII, was downregulated (Oswald et al., 2001). This downregulation affects chlorophyll content and PSII photochemical performance (Hermans and Verbruggen, 2005). Photochemical quenching (qP), which is represented by the redox state of quinone A (Q_A), was not affected by Mg deficiency in wheat and sunflower (Chapter 2). However, significant decreases were observed in barley (Chapter 3) and no decrease in spinach (Chapter 4). There are contrasting results reported from various studies under different nutrient deficiencies. Farhat et al. (2015) reported no decrease in qP values under Mg deprivation in *Sulla carnosa*. The reductions in qP under nutrient deficiencies are associated with an increase in the number of closed RC and the lack of capacity to transfer electrons to Q_A. According to Morales et al. (2000) the shifts in PSII efficiency are also attributed to the decrease in PSII that may indicate regulatory and/or protective mechanisms to avoid serious damage to photosynthetic machinery. The reduction in the operating efficiency at PSII (Φ_{PSII}) is reported to be associated with the reductions in qP (Elkhouni et al., 2017). Φ_{PSII} is an indicator for the proportion of absorbed light which is used in PSII photochemistry (Genty et al., 1989). Under Mg deficiency in sunflower (Chapter 2) significant reduction in Φ_{PSII} was observed. However, wheat did not show any decreases (Chapter 2). Whereas in barley (Chapter 3), significant decreases in Φ_{PSII} under Mg deficiency were observed. Usually, declines in Φ_{PSII} can result from an increase in thermal energy dissipation (non-photochemical-quenching; NPQ). NPQ is an essential regulatory valve and a fast process engaged to release the excitation pressure in the photosynthetic apparatus (Jahns and Holzwarth, 2012). Through NPQ, the efficiency of photochemical reactions is reduced by the decrease in electron transport rate (ETR) (Moustaka and Moustakas, 2014). An increase in NPQ under Mg deficiency was detected by chlorophyll fluorescence measurements (Sunflower; chapter 2) whereas wheat, barley and spinach (Chapter 2,3, and 4) were unaffected. The violaxanthin (Vx) cycle is the main mechanism involved in NPQ in land plants. Vx is reversibly converted to zeaxanthin (Zx) via antheraxanthin (Ax) (Jahns et al., 2009), where Zx is the main pigment involved in NPQ. The PsbS protein is a NPQ mediator that acts as a pH sensor of the lumen

(Li et al., 2004). Lumen pH regulates the activity of the violaxanthin de-epoxidase (VDE), which catalyzes the conversion of Vx to Zx. Hence Zx accumulation takes place under ETR saturation to avoid unnecessary NPQ under low light conditions (Kalituho et al., 2007). On one hand xanthophyll pigments quantification in barley (Chapter 3) revealed a significant increase in VAZ-pool (Vx + Ax + Zx) size under Mg deficiency. On the other hand, no significant increase of the VAZ-pool was observed in spinach plants (Chapter 4). Barley plants, which were grown under light intensity of approximately 400 µmol photons m^{-2} s^{-1} demonstrated an accumulation in Zx pigment, which is an indicator of ETR saturation and lumen pH reduction under Mg deficiency. However, spinach plants, which were grown under approximately 200 µmol m^{-2} s^{-1} did not accumulate Zx. Therefore, the light intensity of approximately 200 µmol m^{-2} s^{-1} did not induce any ETR saturation, which could lead to the decrease in lumen pH under the same Mg treatments as chapter 3. When plants are grown under higher light intensities, higher concentrations for xanthophyll pigments are expected (Jahns et al., 2009). Nevertheless, high levels of illumination, have other consequences such as reactive oxygen species (ROS) formation. As described in chapter 1, ROS are byproducts of photosynthesis. Depending on the environmental conditions including nutrient deficiency and excessive light intensity, their formation rate may increase. Cakmak and Marschner (1992) have reported that plants respond to elevated ROS concentrations induced under Mg deficiency, by increasing leaf antioxidant capacity. Numerous studies have reported antioxidant activity under Mg deficiency. In barley plants (Chapter 3), the gene expression of chloroplastic copper/zinc superoxide dismutase (*Cu/Zn-SOD*), chloroplastic glutathione reductase (*GR*) and peroxisomal catalase (*CAT1*) under Mg deficiency was upregulated. However, the cytosolic ascorbate peroxidase (*cAPX*) was downregulated. SOD is responsible for the detoxification of $^1O_2$, which is one of the most damaging ROS elements in photooxidative damage under high-light stress (Triantaphylidès et al., 2008). SOD is responsible for conversion of $^1O_2$ to H_2O_2. However, Zx enhanced concentration can be associated with $^1O_2$ removal or reduction. Therefore, an increase in VAZ-pool size in barley (Chapter 3) can be associated with NPQ-independent mechanisms under Mg deficiency and high-light intensity. H_2O_2 that is formed in chloroplasts, can cross membranes through aquaporins (Czarnocka and Karpiński, 2018). Hence, the upregulation in *CAT1* in peroxisomes was necessary (Chapter 3) for H_2O_2 detoxification. It can be concluded that under extreme light stress, Zx plays an antioxidant role in the lipid phase of the membranes to minimize the photooxidative damage that can be caused to membrane lipids (Jahns and Holzwarth, 2012). In spinach plants (Chapter 4) neither the gene expression of the ROS scavenging enzymes nor the VAZ-pool size was increased. It is noteworthy to keep in mind that various isoforms of genes encode ROS scavengers. Furthermore, the isoforms are located in different cell compartments. Studies with abiotic stresses have confirmed both decreased and increased expression levels of SOD and glutaredoxin isoforms in *Salvia miltiorrhiza* and

A. thaliana, respectively (Belin et al., 2015; Han et al., 2020). Consequently, analysis of total antioxidant activity based on total enzyme extraction is limited regarding being compartment specific. Ze et al. (2009), who grew spinach plants under 1200 µmol m^{-2} s^{-1} light intensity, reported that the ROS scavenging enzymes activities were decreased. Although they observed H_2O_2 accumulation in their experiment, the imbalance between ROS formation and antioxidant activity led to failure in ROS removal. Tewari et al. (2006) reported increased antioxidant activity under Mg deficiency in mulberry (*Morus alba* L.) plants that were grown under light intensity of 1175 –1355 µmol m^{-2} s^{-1}. Cakmak and Kirkby (2008) have demonstrated photooxidative damage of chlorophyll and membrane lipids under various light intensities and Mg deficiency treatments in bean (*Phaseolus vulgaris* L.) plants. In their study, plants were grown under high-light intensity (shaded or unshaded) and low-light intensity. Mg deficient plants exposed to high-light intensity developed chlorosis and necrosis symptoms as a consequence. However, when plants with the same Mg treatments were grown under lower light intensity, or when a part of the plant was shaded and another part was exposed, chlorosis and necrosis symptoms developed on the unshaded part. It can be concluded that the spinach plants described in chapter 4, which were grown under approximately 200 µmol m^{-2} s^{-1} light intensity and the same Mg treatments as described in chapter 3, did not require any upregulation of antioxidants gene expression or any increase in VAZ-pool size.

Limitations in net CO_2 assimilation (A_n) under Mg scarcity are one of the reasons for oxidative stress (Hauer-Jákli and Tränkner, 2019). The oversaturation of ETR leads to the formation of ROS instead of the use of electrons in photochemistry and CO_2 fixation. Under environmental stress conditions such as drought, cold, and nutrient deficiency, A_n decreases. Nevertheless, contrasting results have been reported in various studies under Mg deficiency (Tränkner and Jamali Jaghdani, 2019). As observed in chapter 2, A_n decreased in sunflower at the lowest Mg treatment, while wheat showed no restrictions. Barley (Chapter 3) and spinach (Chapter 4) showed significant decreases of A_n under Mg deficiency. There have been numerous studies which report reduced assimilation in response to decreased Mg supply (Hariadi and Shabala, 2004; Jezek et al., 2015; Tang et al., 2012; Terry and Ulrich, 1974). Mg is required for the activation of Rubisco and Rubisco activity for CO_2 fixation (Portis and Heldt, 1976). Hence, A_n is considerably affected by Mg scarcity. A study on proteomic analysis on *Citrus sinesis* under long term Mg deficiency revealed a decrease in the abundance of Rubisco and Rubisco activase (Peng et al., 2015). Proteomic analysis on spinach chloroplasts by Yuguan et al. (2009) confirmed the substantial reduction in expression of Rubisco activase subunit (rca), Rubisco large subunit (rbcL) and Rubisco small subunit (rbcS). Peng et al. (2015) have reported that studies on *Brassica campestris* and *Dimocarpus longana* suggest that the photosynthetic inhibition induced by Mg deficiency might be associated with the limited regeneration of ribulose-1,5-bisphosphate and decreases in carboxylation efficiency. In studies

with *Citrus sinesis* under Mg deficiency, a downregulation in the levels of protein which is responsible for photosynthetic electron transfer was observed (Peng et al., 2015). It can be concluded that Mg deficiency decreases the ETR by damaging the total ETC (electron transport chain) from PSII to PSI, hence decreasing A_n. The electrons are transferred between the single electron carrier ferredoxin and the double electron carrier NADPH by ferredoxin $NADP^+$ oxidoreductase (FNR) (Carrillo and Ceccarelli, 2003). According to Hajirezaei et al. (2002) decreased FNR activity can lead to a decrease in NADPH level and increases in Q_A reduction and the NADP level. Consequently, FNR is a limiting factor in photosynthesis and FNR's absence inhibits photosynthesis by deterioration of FNR-mediated electron transfer to NADPH from reduced ferredoxin. Likewise the study by Peng et al. (2015) showed a downregulation of FNR, which may be associated with the decrease in the activity of FNR under Mg deficiency and consequently to an increase in Q_A reduction, and damage in ETR. We can also conclude that the reduction in A_n by sunflower, barley, and spinach under Mg deficiency can be associated with Rubisco activase and Rubisco activity reduction. Furthermore, the overreduction of Q_A and impairment in ETR leads to decreases in A_n rate.

In conclusion, Mg scarcity in plants has several consequences in plant growth, physiological and biochemical mechanisms, as well as the yield measure. Mg is necessary for enzyme activation like Rubisco that drives the photosynthetic machinery. Accordingly, Mg is required for the plant's ability to overcome the photosynthetic stress conditions such as high-light irradiance and activation of the defense mechanisms. Hence, dissipating the excessive absorbed energy as heat and conversion of reactive oxygen species that are formed in higher concentrations. Mg deficiency has negative influences on assimilation rates in most of the plants. However, some plant species such as sunflower, barley, and spinach are more prone to be negatively affected under Mg deficiency. Our studies in wheat, sunflower, barley, and spinach delivered a broader view on the influences of Mg deficiency on photosynthesis, photoprotection and physiology of these agricultural crops that helps us to better understand the necessity of Mg fertilization. However, the experimental design can deliver contrasting results regarding photoprotective mechanisms under Mg deficiency treatments. Furthermore, field experiments are essential to investigate the influences of Mg deficiency under extreme and uncontrolled conditions.

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Summary

Intensive agricultural production without compensation of macro- and micro-nutrients that are taken up by the plants or leached out from the soil by rain, leads to nutrient deficiency in soils and plants. Subsequently, it leads to nutritional deficiencies in livestock and humans. Magnesium (Mg) is one of the most vital macro-nutrients that is taken up by the plants from the soil. Mg is the core element of chlorophyll pigments, which are in chloroplasts where photosynthesis takes place. Mg is required for several enzymes and enzymatic activities. One of the most important enzymes in photosynthesis that requires Mg for its activation is Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Photosynthates are then transported from source organs (leaves) to sink organs (younger leaves, fruits, and seeds). Therefore, Mg is required for adequate dry matter (DM) production. Due to the high requirement of Mg in chloroplasts, photosynthetic reactions can respond more sensitively to Mg scarcity. Moreover, under Mg deficiency, the level of reactive oxygen species (ROS) formation is enhanced. ROS are toxic and can lead to cell death. Nevertheless, plants have developed photoprotective mechanisms to overcome the excessive ROS production. Non-photochemical quenching (NPQ) is known as one of the photoprotective mechanisms, where excessive absorbed energy is dissipated as heat.

There have been several studies with various plants that investigate the influence of Mg deficiency on DM formation, shoot and root growth, ROS concentration, and ROS scavengers' activity. However, a critical Mg concentration that is determined to be influential on each of the mentioned parameters was not introduced.

In this Ph.D. project, the influence of Mg deficiency on photosynthetic parameters, photoprotection, and physiology of wheat, sunflower, barley, and spinach which were grown hydroponically, is studied. A narrow range of Mg deficiency, including seven deficiency treatments was induced in chapter 2, where the range was narrowed down to three in chapter 3 and 4.

The Mg deficiency had a significant influence on decreased CO_2 assimilation in all the mentioned crops except wheat. Reduced assimilation rate is frequently associated with reduced activities of enzymes involved in CO_2 fixation such as Rubisco and Rubisco activase. Moreover, the decreased level of photosynthates' translocation from source organs to sinks, and the photosynthates' accumulation in source organs, has a negative impact on Rubisco activity. In this regard, the measured shoot and root DM in spinach plants revealed the reduced translocation of photosynthates which included root growth reduction under Mg deficiency.

The chlorophyll fluorescence measurements provide a relatively good information about photosynthetic efficiency. Some of the parameters that were measured by this method are maximum quantum efficiency (F_v/F_m), photochemical quenching (qP), NPQ, and electron

transport rate (ETR) to name a few. qP was unaffected in sunflower, wheat, and spinach. Whereas it was significantly decreased in barley. qP is an indicator of the proportion of the open reaction centers. Hence, the reduction in its value reflects the increased number of closed reaction centers, and moreover a decrease in the capacity of electron transportation and subsequently a decrease in ETR. ETR was reduced substantially in sunflower and barley, whereas it was unaffected in wheat and spinach. The decrease in ETR damages the whole electron transport chain from photosystem II (PSII) to photosystem I and hence reducing the assimilation rate. Mg deficiency resulted in reductions in F_v/F_m in sunflower, barley, and wheat. Reductions in F_v/F_m can be an indicator of a damage in the photosynthetic apparatus. Moreover, a decline in F_v/F_m can indicate photoinhibition of PSII due to a photooxidative damage.

Photooxidative damage is a result of excessive ROS formation. In order to investigate the photoprotection efficiency and photooxidative damage levels, gene expression of ROS scavenging enzymes was analyzed in barley and spinach. In barley the expression levels of catalase, glutathione reductase, and superoxide dismutase were increased, whereas no increase in ascorbate peroxidase expression was observed. In spinach no increase in the expression level of any of the mentioned genes was observed. The level of ROS formation under Mg deficiency is also dependent on the light intensity available at the growth condition. Higher light intensities enhance the ROS formation under Mg deficiency.

Violaxanthin (Vx) cycle is known as one of the most important photoprotective mechanisms in high-light acclimated plants and contributes to the prevention of excessive ROS formation by NPQ. The pigments involved in violaxanthin (Vx) cycle were quantified to study the impacts of Mg deficiency on photoprotection in barley and spinach. Vx is converted to zeaxanthin (Zx) via antheraxanthin (Ax). The increase in the VAZ (Vx+ Ax+ Zx)-pool size under Mg deficiency in barley indicated a requirement for photoprotection against oxidative damage. However, the increase in VAZ-pool size was served as a NPQ-independent function. In line with this, no increase in the NPQ value by chlorophyll fluorescence measurements was observed. The Mg deficiency did not induce any increase in NPQ value or VAZ-pool size in spinach. Therefore, we could not confirm any photooxidative stress in spinach plants under Mg deficiency.

In overall, this study contributes to better understanding of influences of Mg deficiency on photosynthetic and photoprotective mechanisms. It provides insights and helps to close the knowledge gaps that exist regarding the critical Mg supply for certain physiological and biochemical mechanisms. Furthermore, it improves implementation of fertilization strategies.

Zusammenfassung

Intensive landwirtschaftliche Produktion ohne Ausgleich von Makro- und Mikro-Nährstoffen, die von den Pflanzen aufgenommen oder durch Regen aus dem Boden ausgewaschen werden, führt zu Nährstoffmangel in Böden und Pflanzen. In der Folge führt dies zu Nährstoffmängeln bei Nutztieren und Menschen. Magnesium (Mg) ist einer der wichtigsten Makronährstoffe, der von den Pflanzen aus dem Boden aufgenommen wird. Mg ist das Kernelement der Chlorophyll-Pigmente, die sich in den Chloroplasten befinden, wo die Photosynthese stattfindet. Mg wird für verschiedene Enzyme und enzymatische Aktivitäten benötigt. Eines der wichtigsten Enzyme der Photosynthese, das Mg für seine Aktivierung benötigt, ist die Ribulose-1,5-Bisphosphat-Carboxylase/Oxygenase (Rubisco). Assimilate werden dann von den Quellorganen (Blätter) zu den Senken-Organen (jüngere Blätter, Früchte und Samen) transportiert. Daher wird Mg für eine ausreichende Produktion von Trockenmasse (TM) benötigt. Aufgrund des hohen Mg-Bedarfs der Chloroplasten können photosynthetische Reaktionen empfindlich auf Mg-Knappheit reagieren. Außerdem wird unter Mg-Mangel die Produktion reaktiver Sauerstoffspezies (ROS) erhöht. ROS sind toxisch und können zum Zelltod führen. Daher haben Pflanzen photoprotektive Mechanismen entwickelt, um die übermäßige ROS-Produktion zu unterbinden. Nicht-photochemisches Quenchen (NPQ) ist als einer der photoprotektiven Mechanismen bekannt, bei der überschüssige absorbierte Energie als Wärme abgeleitet wird.

Es gibt zahlreiche Studien mit verschiedenen Pflanzen, die den Einfluss von Mg-Mangel auf die TM-Bildung, das Spross- und Wurzelwachstum, die ROS-Konzentration und die Aktivität von ROS-Fängern untersuchen. Eine kritische Mg-Konzentration, die jeden der genannten Parameter beeinflusst, wurde jedoch nicht eingeführt.

In dieser Dissertation wird der Einfluss von Mg-Mangel auf photosynthetische Parameter, Photoprotektion und Pflanzenphysiologie am Beispiel von Weizen, Sonnenblume, Gerste und Spinat untersucht. Die Pflanzen wurden hydroponisch kultiviert. Im zweiten Kapitel wurde in sieben Stufen ein schmaler Bereich von Mg-Mangel induziert; in den folgenden Kapiteln wurden diese nochmals auf drei Behandlungen verringert.

Der Mg-Mangel verringerte die CO_2-Assimilation signifikant in allen genannten Kulturpflanzen außer Weizen. Eine verringerte Assimilationsrate ist häufig mit verringerten Aktivitäten von Enzymen, die an der CO_2-Fixierung beteiligt sind, wie Rubisco und Rubisco-Aktivase, verbunden. Darüber hinaus hat die verringerte Translokation von Assimilaten von den Quellorganen zu den Senken und die Akkumulation von Assimilaten in den Quellorganen einen negativen Einfluss auf die Rubisco-Aktivität. In dieser Hinsicht wies die erfasste Spross- und Wurzel-TM in Spinatpflanzen auf verringerte Translokation von Assimilaten hin, die eine Reduktion des Wurzelwachstums unter Mg-Mangel einschloss.

Die Chlorophyll-Fluoreszenzmessungen liefern eine relativ gute Information über die photosynthetische Effizienz. Einige der Parameter, die mit dieser Methode gemessen wurden, sind die maximale Quanteneffizienz (F_v/F_m), das photochemische Quenching (qP), NPQ und die Elektronentransportrate (ETR), um nur einige zu nennen. qP war in Sonnenblume, Weizen und Spinat unter Mg-Mangel unbeeinflusst, während es in Gerste signifikant verringert war. qP ist ein Indikator für den Anteil der offenen Reaktionszentren. Daher spiegelt die Verringerung seines Wertes die erhöhte Anzahl geschlossener Reaktionszentren wider, und darüber hinaus eine Verringerung der Kapazität des Elektronentransports und folglich eine Verringerung der ETR. ETR wurde in Sonnenblume und Gerste erheblich reduziert, während es in Weizen und Spinat unbeeinflusst blieb. Die Abnahme von ETR schädigt die gesamte Elektronentransportkette vom Photosystem II (PSII) zum Photosystem I und reduziert somit die Assimilationsrate. Mg-Mangel führte zu Reduzierungen von F_v/F_m in Sonnenblume, Gerste und Weizen. Reduzierungen von F_v/F_m können ein Indikator für eine Schädigung des photosynthetischen Apparates sein. Außerdem kann ein Rückgang von F_v/F_m auf eine Photoinhibition von PSII aufgrund von photooxidativen Schäden hinweisen.

Photooxidative Schäden sind eine Folge von übermäßiger ROS-Bildung. Um die Photoprotektionseffizienz und das Niveau der photooxidativen Schäden zu untersuchen, wurde die Genexpression von ROS-fangenden Enzymen in Gerste und Spinat analysiert. In Gerste waren die Expressionsniveaus von Katalase, Glutathion-Reduktase und Superoxid-Dismutase erhöht, während kein Anstieg der Ascorbat-Peroxidase-Expression beobachtet wurde. In Spinat wurde keine Erhöhte Expression der genannten Gene beobachtet. Die ROS-Bildungsrate unter Mg-Mangel ist zudem abhängig von der Lichtintensität, die unter den Wachstumsbedingungen zur Verfügung steht. Höhere Lichtintensitäten verstärken die ROS-Bildung unter Mg-Mangel.

Der Violaxanthin (Vx)-Zyklus ist als einer der wichtigsten photoprotektiven Mechanismen in hochlichtakklimatisierten Pflanzen bekannt und trägt zur Verhinderung einer übermäßigen ROS-Bildung durch NPQ bei. Die am Violaxanthin (Vx)-Zyklus beteiligten Pigmente Antheraxanthin (Ax) und Zeaxanthin (Zx) wurden quantifiziert, um die Auswirkungen von Mg-Mangel auf die Photoprotektion in Gerste und Spinat zu untersuchen. Die Zunahme der VAZ (Vx+ Ax+ Zx) - Poolgröße unter Mg-Mangel in Gerste deutete auf eine Notwendigkeit für den Photoprotektion gegen oxidative Schäden hin. Die Zunahme der VAZ-Poolgröße wird jedoch als NPQ-unabhängige Funktion gedeutet. In Übereinstimmung damit wurde keine Erhöhung des NPQ-Wertes durch Chlorophyll-Fluoreszenz-Messungen beobachtet. Der Mg-Mangel induzierte keinen Anstieg des NPQ-Wertes oder der VAZ-Pool-Größe im Spinat. Daher konnten wir keinen photooxidativen Stress in Spinatpflanzen unter Mg-Mangel bestätigen.

Insgesamt trägt diese Studie zu einem besseren Verständnis der Einflüsse von Mg-Mangel auf photosynthetische und photoprotektive Mechanismen bei. Sie hilft, die Wissenslücken zu

schließen, die hinsichtlich der kritischen Mg-Versorgung für bestimmte physiologische und biochemische Mechanismen bestehen. Darüber hinaus verbessert es die Umsetzung von Düngungsstrategien.

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Declarations

1. I, hereby, declare that this Ph.D. dissertation has not been presented to any other examining body either in its present or a similar form.

   Furthermore, I also affirm that I have not applied for a Ph.D. at any other higher school of education.

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2. I, hereby, solemnly declare that this dissertation was undertaken independently and without any unauthorized aid.

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