# Magnesium deficiency in maize and effectiveness of nutrient supply through MgSO<sub>4</sub> leaf-application

#### **Dissertation**

submitted for the Doctoral Degree
awarded by the Faculty of Agricultural and Nutritional Sciences
of the Christian-Albrechts-Universität Kiel

submitted by

M.Sc. Mareike Jezek

Kiel, 2016

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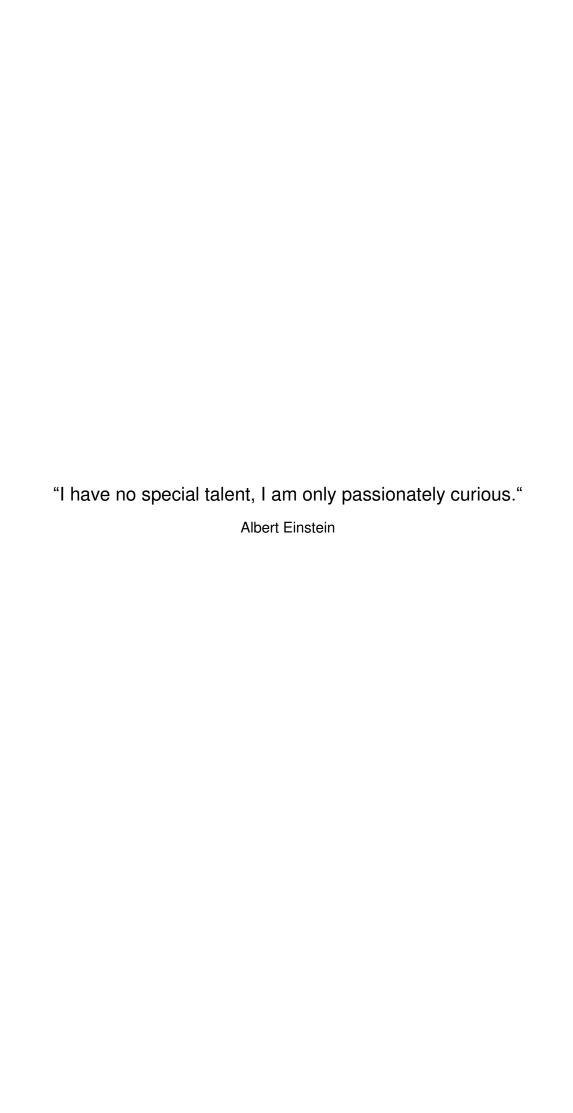
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Global agriculture faces the major challenge to provide nutritious and healthy food for an increasing world population in times of climate change, ongoing soil degradation and increasing soil sealing. According to projections from the United Nations Organization, the current world population of 7.4 billion people will increase by 1.1 billion until 2030 and reach 9.7 billion in 2050 (United Nations Organization, 2015b). Centuries of overuse and unsustainable land management have left soils throughout the world compacted, eroded and depleted of nutrients. Approximately 52% of the land used for agriculture worldwide is moderately or severely affected by soil degradation, and increasing arable land is lost due to desertification (United Nations Organization, 2015a). Consequently, global agriculture is confronted with the problem to achieve higher yields on a decreasing land area, in order to appease the population's increasing hunger not only for food – especially meat because of a changing diet in many societies (World Health Organization, 2003) – but also for energy (International Energy Agency, 2015). Nowadays, an increasing part of the farmland is converted to cultivate crops for biofuel production or electricity generation.

Both high crop yield and high food quality are needed to fight hunger and malnutrition. In order to achieve maximal production levels, crops have to be supplied with essential nutrients. Agricultural research focused mainly on the 'primary' plant nutrients nitrogen (N), phosphorous (P), and potassium (K), because they are most frequently required in crop fertilization programs and they have the highest impact on yield formation. However, deficiency of 'secondary' nutrients also adversely impacts plant growth and consequently decreases crop yield and food nutritional value. The essential plant nutrient magnesium (Mg) is declared as the 'forgotten nutrient' in plant nutrition research (McHoul, 2006). Magnesium may be secondary in terms of quantitative requirement, but it certainly is not secondary in terms of importance for plant development, growth, and reproduction. However, Mg nutrition of plants is frequently overlooked and Mg deficiency is increasingly becoming an important limiting factor in both intensive crop production systems (Cakmak and Yazici, 2010) and subsistence agriculture (Krishna, 2014). Magnesium is most prominent for its role as the central atom of the photosynthesis pigment chlorophyll but its impact on a range of other metabolic processes apart from photosynthesis remains obscure and increased knowledge about the physiological responses to Mg deficiency is needed. Magnesium shortage in the field can be ameliorated by soil fertilization. Additionally, leaf application of fertilizer has become a common standard practice on some crops (Fernández and Eichert, 2009; Fernández et al., 2013) and can display an environmentally and economically beneficial means of correcting nutrient deficiency.

This chapter will give an overview on the physiological functions of Mg in plant metabolism and the current knowledge of the effects of Mg deficiency. Furthermore, several parameters affecting Mg availability and Mg uptake will be addressed. Moreover, information about the cultivation of the test plant maize (*Zea mays* L.) will be given and the objectives of this Ph.D. work will be outlined.

#### 1.1 Physiological Functions of Magnesium in Plants

Magnesium (Mg) is an essential plant macronutrient with diverse physiological functions and it is vital for plant growth, development, and reproduction. About 250 enzymes in plant metabolism are Mg-dependent (Barker and Pilbeam, 2007). Magnesium either binds directly to the enzyme, changes its structure and leads to its activation, or it acts as a cofactor by binding to ATP or pyrophosphate to yield Mg-ATP and Mg-P<sub>2</sub>O<sub>7</sub>, respectively, which are the biologically active forms of these molecules (Cole and Schimmel, 1970). Magnesium neutralizes the electrostatic repulsion between the enzyme and ATP or PP<sub>i</sub>, respectively, and hence enables their binding to the active site. In fact, due to the negative charge of phosphate groups in general, all enzymatic processes that involve a phosphoryl-transfer and hence all phosphorylation or de-phosphorylation reactions, including the hydrolysis or synthesis of ATP, require Mg. Accordingly, Mg is also relevant for the activity of the plasma membrane H<sup>+</sup>-ATPase, which is involved in important physiological processes such as stomata regulation, cell expansion, phloem loading, intracellular pH regulation, and nutrient uptake and transport. The most prominent role of Mg is its function in photosynthesis. Magnesium is the central atom of the light-capturing pigment chlorophyll and it is vital for its function to harvest photons. Furthermore, Mg regulates the activity of several key enzymes of the Calvin-Benson cycle. During the light reaction of photosynthesis, H<sup>+</sup> are transported into the thylakoid lumen, creating a pH gradient across the thylakoid membrane. The accompanying electrical gradient is compensated by concomitant fluxes of Mg into the chloroplast stroma (Shaul, 2002). These light-induced increases in stromal Mg concentration and stromal pH activate fructose-1,6bisphospatase and sedoheptulose-1,7-bisphosphatase that function in the synthesis of ribulose-1,5-bisphospate, the substrate for the CO<sub>2</sub> assimilating enzyme Ribulose-1,5 bisphosphate Carboxylase/Oxygenase (RubisCO). The activity of RubisCO itself also requires a high stromal pH and is enhanced in the presence of Mg, which stabilizes its active carbamylated form (Andersson, 2008).

About 75% of the intracellular Mg is bound to ATP and the remaining portion is attached to numerous, negatively charged organic ligands within the cell, mostly phosphates and carboxylates. Magnesium, as the major free divalent cation in the cytosol, neutralises and stabilises cell membranes, and assists in folding and maintaining the structure of proteins and nucleic acids (Barker and Pilbeam, 2007; Brion and Westhof, 1997; Shaul, 2002). Therefore, DNA replication, gene transcription, and translation crucially depend on adequate levels of Mg (Maathuis, 2009). Furthermore, Mg is critical for the stability of ribosomes, which catalyse the synthesis of proteins during translation, by mediating an interaction between ribosomal proteins and ribosomal RNA (Petrov *et al.*, 2012). In order to be incorporated into the nascent polypeptide chain inside the ribosome, amino acids have to be bound to their corresponding tRNA. This amino acid activation is mediated by Mg, as well (Cole and Schimmel, 1970).

#### 1.2 Magnesium Uptake, Transport, and Homeostasis

Magnesium transporters were first identified and functionally analysed in bacteria (Hmiel *et al.*, 1986). Functional homologues of these CorA type membrane proteins have also been characterized in eukaryotic cells and they all share the universally conserved Gly-Met-Asn (GMN) motif at the end of the first of the two conserved transmembrane domains near the C-terminus (Knoop *et al.*, 2005). In *Arabidopsis thaliana* ten homologues of the bacterial CorA Mg transporter have been identified by two research groups independently. Schock *et al.* (2000) named this transporter family *AtMRS2*, because one of its members complemented the yeast deletion mutant *mrs2* (*Mitochondrial RNA Splicing2*), whereas Li *et al.* (2001) named the

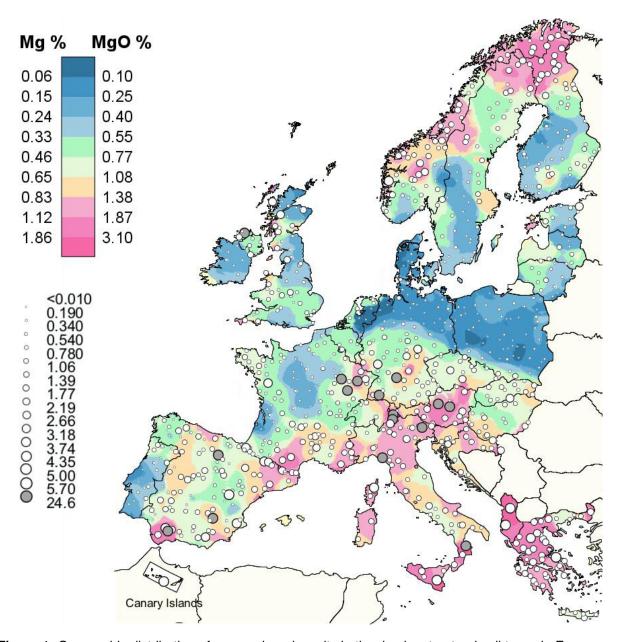
gene family AtMGT (Magnesium transport). Members of the MGT/MRS2 family are expressed in a broad range of plant tissue, and the transporters are located in the plasma membrane or in intracellular organelles such as mitochondria, plastid, and tonoplast (Mao et al., 2014). They are expressed differently depending on the developmental stage and the internal and external Mg status. Various Mg transporters seem to mediate Mg uptake under low or high Mg conditions in the growing medium, which suggests a metabolic adaptation of plants to the root environment. For example, six MGT/MRS2 genes are active in different root zones under high Mg nutrition (Gebert et al., 2009; Oda et al., 2016) and MRS2-7, which is located in the endoplasmatic reticulum (ER) of root cells, represents a Mg transporter that is important for plant growth under Mg limitation (Gebert et al., 2009). Furthermore, MGT6/MRS2-4 is expressed mainly in plant aerial tissues under high Mg nutrition and its expression is highly induced in the roots under Mg deficiency (Mao et al., 2014). Next to these Mg-specific transporters, Mg can also be taken up and translocated via nonselective cation channels (NSCCs), which represent a diverse group of ion channels found in the plasma membrane, the tonoplast, and other endomembranes (Demidchik et al., 2002). They are characterized by their low discrimination between cations, and their activation and gating mechanisms depend on several factors such as membrane polarization and Ca<sup>2+</sup> concentration. Magnesium competes with other cellular cations for transport through NSCCs. Due to its small atomic radius, Mg ions have a large charge density compared to other basic cations. Consequently, they are surrounded by a large hydration water shell. However, channel transport occurs in the dehydrated form and a high dehydration energy is needed to facilitate channel-mediated Mg transport. Therefore, Mg has got a lower transportation rate compared to other basic ions.

Magnesium is taken up passively into the root and it is transported into the shoot via the xylem. Furthermore, Mg is a phloem-mobile element and is readily translocated to fruit, seed and tubers (White and Broadley, 2009), and basipetal transport of leaf-applied Mg inside the phloem was demonstrated with <sup>28</sup>Mg isotopes (Steucek and Koontz, 1970). The highest Mg concentration is usually found in shoots, given its prominent role in chloroplasts (Maathuis, 2009). Plants supplied with adequate Mg have an average Mg concentration of 1 – 10 mg g<sup>-1</sup> dry matter in the shoot (White and Broadley, 2009). Magnesium can be bound to negatively charged sites in the apoplast and the apoplast supposedly contributes to Mg storage and permits its mobilization upon acidification (Grignon and Sentenac, 1991). Most of the symplastic Mg is stored in the vacuole, where it contributes to turgor generation and charge balancing of anions. The metabolically active Mg pool is distributed between the cytosol, the chloroplast, and the mitochondria. Magnesium homeostasis is regulated mainly by ion transport across the vacuolar membrane (tonoplast). Next to Mg-channels from the MGT/MRS-2 family (Conn et al., 2011), a tonoplast-located Mg/H+ antiporter (MHX) contributes to the uptake of Mg into the vacuole and regulates Mg homeostasis. MHX is mainly found in close association with the xylem tracheary elements and it is suggested that it functions in the partitioning of Mg between various plant organs (Shaul et al., 1999). Furthermore, voltagedependent slow vacuolar (SV) channels in the tonoplast can mediate passive exchange of Mg between the vacuole and the cytosol (Pottosin and Schonknecht, 2007). Theoretically, the SV channel could mediate uptake of cations into the vacuole, but the direction of the electrochemical potential gradient only allows passive release from the vacuole in vivo. Therefore, once gated open, SV channels will mediate the passive efflux of vacuolar Mg into the cytosol.

#### 1.3 Magnesium in the Soil and Magnesium Deficiency in Agriculture

The Mg concentration in uncultivated soil varies largely across Europe and depends on the source rock material containing various forms of silicates such as magnesite (MgCO<sub>3</sub>), dolomite (MgCO<sub>3</sub> x CaCO<sub>3</sub>) and serpentine (Mg<sub>3</sub>Si<sub>2</sub>O<sub>5</sub>(OH)<sub>4</sub>). Lowest values are found in Northern Germany, Poland, Portugal, Ireland, and large parts of France and Sweden (Fig. 1). By far the largest fraction of soil Mg is in the non-exchangeable form of primary minerals or secondary clay minerals (Bolan *et al.*, 2006; Kirkby and Mengel, 1976), and hence, cannot be taken up readily by plant roots. The plant-available Mg pool is made up of water-soluble Mg and exchangeable Mg (Grzebisz, 2013). The amount of soil Mg incorporated into minerals accounts for about 90 – 95% (Senbayram *et al.*, 2015). Consequently, soils can be classified as medium in the total Mg contents but be rated as poor in available Mg. Soils in Northern Europe are typically low in total Mg (0.01% - 0.2%) and the plant-available Mg fraction is even less (Fig. 1). Therefore, these soils are rated as too low in available Mg to sustain intensive cropping without the use of Mg fertilizers (Metson, 1974). The Mg concentration in the soil solution extracted under field capacity ranges between 125  $\mu$ M and 8.5 mM (Conn *et al.*, 2011).

Magnesium is transported to the roots mainly by mass flow. Therefore, the Mg availability is highly dependent on the soil moisture and the rate of transpiration-driven water flow (Conn *et al.*, 2011). Magnesium depletion in the rhizosphere is rare, but in these cases also diffusion contributes to the transport of Mg towards the root (Kirkby and Mengel, 1976).



**Figure 1:** Geographic distribution of magnesium deposits in the dominant natural soil types in Europe. Colours show the Mg/MgO concentration in the topsoil (0 - 25 cm) (European Soil Bureau Network 2005; modified).

Two forms of Mg deficiency can be distinguished. Too low concentrations of soil Mg in plant-available forms leads to 'absolute deficiency' (Metson and Brooks, 1975). Since Mg has got a small atomic radius and hence a high-charge density, it is present in a highly hydrated form in the soil solution (Maguire and Cowan, 2002). Therefore, Mg ions are not attached firmly to cation exchange sites on soil particles and consequently, the risk for 'absolute Mg deficiency' is highest in light-textured, freely drained soils with high Mg leaching losses. On sandy soils, Mg loss by leaching frequently dominates the release of Mg by weathering (Kirkby and Mengel, 1976). This problem is exacerbated in humid climates with heavy rainfalls.

'Induced Mg deficiency' occurs on soils with sufficient Mg content and is caused by factors that disturb the Mg uptake at the root (Metson and Brooks, 1975). 'Induced Mg deficiency' is mainly caused by high amounts of other cations in the soil, which compete with Mg for binding at the negatively charged soil particles and at cation exchange sites in the root cortex. Due to their

relatively large hydration shell, Mg ions bind less firmly to soil particles than other cations and consequently, Mg can easily be replaced at cation exchange sites in the soil and at the root. Nutrient antagonism occurs in soil rich in calcium (Ca<sup>2+</sup>), acidic soil with excess of protons (H<sup>+</sup>), aluminium (Al3+) and manganese (Mn2+), and saline soils with high sodium (Na+) concentrations (Gransee and Führs, 2013; Mengel and Kirkby, 2001; Shaul, 2002), leading to elevated risk of induced Mg deficiency and Mg leaching in these soils. Magnesium is released from clay particles into the soil solution upon soil acidification. However, H<sup>+</sup> and Al<sup>3+</sup> ions hamper the Mg uptake into the root on acid soils and hence, higher soluble Mg concentrations in the soil solution do not necessarily lead to higher uptake (Metson and Brooks, 1975). Liming of acid soils can also reduce the Mg availability because the added Ca<sup>2+</sup> displaces Mg and Al<sup>3+</sup> at soil particles leading to an occlusion or co-precipitation of Mg with Al3+ (Mayland and Wilkinson, 1989). In most soils, exchangeable Mg accounts for only 4 - 20% of the cation exchange capacity, which is considerably lower than Ca that makes up about 80% of the soil CEC (Kirkby and Mengel, 1976). Furthermore, strong antagonistic interactions regarding uptake exist among potassium (K+) and ammonium (NH<sub>4</sub>+) and Mg (Cai et al., 2012; Craighead, 2001; Guiet-Bara et al., 2007). The application of nitrate fertilizers promotes the downward mobility of Mg and the risk of leaching losses. Hence high-production systems, which are characterized by growing of high-yielding crop varieties and abundant input of synthetic nitrogen-phosphate-potassium (NPK) fertilizer, harbour the risk of Mg deficiency, because the unbalanced application of those primary nutrients leads to a lack of secondary elements such as Mg, which impacts production and nutritional quality of harvests (Hermans et al., 2013). The risk of Mg deficiency is increased by further external factors. For example, high rainfalls on acid, sandy soils are thought to be the driver for Mg deficiency in potatoes in the northern coastal area of New South Wales, Australia (Craighead, 2001). In fact, Mg deficiency is most likely to occur on acid, sandy soils in humid regions (Mayland and Wilkinson, 1989). Furthermore, the general degradation of arable soils is a massive problem also for the Mg nutrition of crops. Poor soil structure and increased soil compaction limit the amount of nutrient binding in the soil and nutrient uptake into the root. Also, the loss of soil organic matter, which increases the soil cation exchange capacity and improves water holding capacity, thereby reducing the risk of Mg leaching, is problematic for an adequate Mg nutrition.

#### 1.4 Physiological Responses and Symptoms of Magnesium Deficiency

Magnesium deficiency in plants occurs worldwide (White and Broadley, 2009) due to the multitude of external factors that limit the Mg phyto-availability, as outlined above. The typical visible symptom of acute Mg deficiency is leaf chlorosis, followed by necrosis. In dicotyledons, chlorosis starts between the leaf veins of fully expanded, older leaves (Fig. 2A), because Mg - as a phloem-mobile nutrient - is readily transported to younger, expanding plant parts (Senbayram et al., 2015). Monocotyledonous leaves show chlorosis in the form of stripes and in some crops, such as oat, green spots are arranged in string-like pearls along the leaf veins (Fig. 2B). In some species, which are naturally high in anthocyanins, such as grape, black currant bushes, and fruiting trees, Mg deficiency is accompanied by leaf purpling (Jacob, 1955). This symptom can also be observed in other crops, for example maize (Fig. 5C) and rice, under experimental conditions (Kobayashi et al., 2013). Anthocyanin accumulation is often explained as the direct consequence of sugar accumulation, but the ecophysiological function of this phenomenon is controversially discussed (Chalker-Scott, 1999, 2002; Gould, 2004; Gould et al., 2002; Landi et al., 2015; Lev-Yadun and Gould, 2009), and the underlying biochemical mechanisms of Mg deficiency-induced anthocyanin synthesis remain unclear (Kobayashi et al., 2013).



**Figure 2:** Magnesium deficiency symptoms. (A) Leaves of fava bean (*Vicia faba* L.) grown under sufficient (left) and deficient (right) Mg supply. Leaves were taken from an entire plant (oldest leaves at the bottom) (Neuhaus, 2013). (B) Oat (*Avena sativa* L.) leaves with typical Mg deficiency symptoms (see text) (Bergmann, 1993).

In some crops such as bean and wheat, the root growth is more inhibited by Mg deficiency than the shoot growth, leading to increased shoot-to-root ratio (Andrews *et al.*, 1999; Cakmak *et al.*, 1994b). Inhibited root growth can easily be overlooked in the field and leads to yield reductions due to the high importance of the root system in nutrient acquisition and water uptake. Therefore, special attention should be given to the Mg nutritional status of plants before the development of any visible deficiency symptoms (Cakmak and Yazici, 2010). Reduced root growth is thought to be the consequence of impaired phloem loading with photosynthates in source leaves (Cakmak *et al.*, 1994a). Accumulation of reduced sugars, amino acids and starch have been found in several species under Mg deficiency such as spinach, field bean, and sugar beet (Cakmak *et al.*, 1994a; Fischer *et al.*, 1998; Hermans *et al.*, 2005).

In fact, the onset of Mg deficiency and latent deficiency is often not visible and hard to diagnose but negatively affects crop yields (Gransee and Führs, 2013). Several metabolic pathways are impaired or down-regulated before the first visible symptoms can be detected. The accumulation of reduced sugars and starch is supposed to be the primary metabolic consequence in the hierarchy of physiological responses induced by Mg deficiency (Cakmak *et al.*, 1994a; Hermans and Verbruggen, 2005). The reason for this is not consensually resolved. Cakmak *et al.* (1994a) suppose that the activity of the plasma membrane (PM) H<sup>+</sup>-ATPase is impaired, caused by a decrease in the cytosolic concentration of the enzyme's substrate Mg-ATP. This ultimately leads to sugar and amino acid accumulation in source leaves, because the proton motive force in the sieve element-companion cell complex

generated by the PM H+-ATPase enables the symport of sugars or amino acids and H+ into the phloem (Lalonde et al., 2003). This is in line with studies by Hermans et al. (2005), who propose that phloem loading of sucrose in source leaves is a primary target of Mg deficiency in sugar beet. However, the authors state that the observed effect of Mg deficiency on carbon export may also emerge from a direct effect on the sucrose symporter activity. Fischer et al. (1998) found increased starch and amino acid concentrations in source leaves and elevated levels of sucrose, hexoses, starch, and amino acids in sink leaves of Mg-deficient spinach. By shading sink leaves and hence interrupting the photosynthetic CO<sub>2</sub>-assimilation they proved that the accumulated carbohydrates were imported from source leaves. The authors conclude that the growth of sink leaves and roots in Mg-deficient spinach plants is not limited by carbohydrate or amino acid supply from source leaves, which is corroborated by the finding that the sucrose and amino acid concentrations in the phloem sap was not decreased under Mg deficiency compared to well-nourished control plants. Hence, they propose that the accumulation of assimilates in the source leaves of Mg-deficient plants results from a lack of utilization of these metabolites in the sink leaves. Interestingly, starch accumulation in needles of Mg-deficient conifers is ascribed to a collapse of sieve cells in the phloem (Fink, 1991; Puech and Mehne-Jakobs, 1997), but this phenomenon has not been observed in Mg-deficient angiosperms so far. The difference may result from the specific symplastic phloem loading mechanism in conifer needles (Fink, 1991).

As a result of increased sugar concentrations the photosynthetic CO2-fixation is impaired because of a feedback regulation by carbon metabolites and a down-regulation of photosynthesis gene expression (Oswald et al., 2001). This genetic adaptation occurs several days before the appearance of any visible symptoms of Mg deficiency, such as decreased chlorophyll concentrations. Cab2 (chlorophyll a/b binding protein gene) is one of the first downregulated genes at the onset of Mg deficiency in Arabidopsis thaliana. This might be the reason for the chlorophyll breakdown upon Mg deficiency, rather than a lack of Mg atoms for chelating chlorophyll molecules (Hermans and Verbruggen, 2005). Chlorophyll catabolism may be a strategy to dechelate Mg from pigment molecules and to recycle the Mg in favour of young tissue growth (Verbruggen and Hermans, 2013). Another adaptation to low Mg availability is the decline of PSII photochemistry due to a loss of light-harvesting complexes (LHC II) and down-regulation of PSI primarily through a loss of reaction centres, thereby reducing the linear e transport between the photosystems to prevent over-reduction of the e transport chain during conditions of restricted sugar export (Hermans et al., 2004). The sugar-induced feedback regulation also alters the RubisCO activity in favour of the oxygenase reaction (Marschner and Marschner, 2012), which is followed by superoxide radical ( $O_2^{-1}$ ) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) formation in peroxisomes. Over-reduction of the e<sup>-</sup> transport chain also leads to the evolution of cell-toxic reactive oxygen species (ROS) and consequently, various antioxidant defence mechanisms such as superoxide dismutase (SOD), and the anti-oxidant glutathione-ascorbate cycle are induced (Cakmak and Marschner, 1992; Candan and Tarhan, 2003a, b; Tewari et al., 2006; Tewari et al., 2004). The transcriptomic changes induced by Mg deficiency were analysed in detail in Arabidopsis thaliana (Hermans et al., 2010a; Hermans et al., 2010b). Next to genes of the photosynthetic apparatus, genes associated with the circadian clock, abscisic acid (ABA) signalling, ethylene biosynthesis, and the detoxification of reactive oxygen species (ROS) were expressed differently under Mg deficiency. Hermans et al. (2010a) suppose that ABA signalling could play a major role in signalling of Mg shortage.

Next to the detrimental effects of reduced photosynthetic CO<sub>2</sub>-fixation, restricted protein biosynthesis has been suggested as a reason for reduced shoot and root growth under Mg deficiency. It has even been proposed that a disturbed polypeptide synthesis in the ribosomes

is the primary effect of Mg undersupply, leading to restricted protein production and growth inhibition (Mengel and Kirkby, 2001). Impaired polypeptide synthesis seems likely, considering that the largest pool of Mg is thought to be devoted to protein synthesis by bridging ribosome subunits (Waters, 2011). However, the effects of Mg deficiency on protein metabolism are poorly investigated.

#### 1.5 Magnesium Fertilization Practice

#### 1.5.1 Soil Fertilization

As outlined above, the major part of soil Mg is in the non-exchangeable form of primary or secondary minerals. Hence, the plant-available fraction in arable soils is most often too low to sustain high crop yield and quality. Therefore, the application of Mg fertilizer is crucial for optimal crop production, especially on soils that bear the risk of nutrient antagonism and Mg leaching. In traditional agriculture, Mg supply to the soil used to be a side benefit of applying fertilizers such as superphosphate or the potassium fertilizer kainite (MgSO<sub>4</sub>⋅KCl⋅3H<sub>2</sub>0) that contain Mg impurities high enough to ameliorate the Mg levels in many potentially deficient soils of Northern Europe (Metson, 1974). However, the use of Mg-containing serpentine superphosphate is declining nowadays (Bolan et al., 2006) and the application of purified fertilizers becomes increasingly common (Metson, 1974). This raises the requirement of applying specific Mg fertilizer in order to meet the nutrient demand of crops especially in intensive, industrial farming systems, which are characterized by the high input of NPKfertilizers and growing of high-yield crop varieties with consequently increasing Mg removal at crop harvest. High Mg fertilizer rates may also be required on Mg-deficient, alkaline soils because of the large amounts of the antagonistic ions Ca, Na, and K present (Mayland and Wilkinson, 1989). Magnesium soil fertilizers can be divided into two groups. Solid minerals such as ground dolomite limestone with a slow Mg release and soluble, fast-release Mg fertilizers such as Epsom salt (MgSO<sub>4</sub>·7H<sub>2</sub>O) and kieserite (MgSO<sub>4</sub>·H<sub>2</sub>O). The effectiveness of these different forms depends on the Mg content, the material coarseness, soil properties such as pH, and the prevailing weather conditions.

#### 1.5.2 Foliar Fertilization

Senbayram et al. (2015) suggested that Mg fertilizer supply in many agricultural systems is often inadequate and the authors call for the development of precise site- and crop-specific Mg fertilizer management practices. As explained above, the soil capacity for storing Mg in plant-available depths, the Mg binding within the root cortex and the Mg uptake into the root symplast can be constrained by a range of soil characteristics. Also, the weather conditions can preclude efficient Mg uptake from the soil. Therefore, it may be of advantage to supply Mg to the plants by foliar fertilization instead of soil application. This strategy can be environmentally and economically beneficial because the risk of losing Mg fertilizer by leaching into plant-unavailable soil depths is reduced. This is consistent with the '4R Principles of Nutrient Stewardship', proclaimed by The International Plant Nutrition Institute. The '4R Nutrient Stewardship' concept comprises the use of the right fertilizer source at the right rate. the right time and on the right place, in order to achieve increased crop production, increased farmer profitability, and improved environmental sustainability (The Fertilizer Institute, 2016). Applied nutrient solution can be absorbed by the foliage via stomata, the cuticle, and modified epidermal cells such as trichomes. The addition of adjuvants to the foliar fertilizer allows proper wetting, spreading and penetration of the solution (Fernández and Eichert, 2009).

Magnesium leaf fertilization can enable a better management of nutrient application in order to meet the plant's changing internal nutrient demand during different developmental changes. For example, studies in soybean suggest that adequate Mg nutrition at later vegetative growing stages just before flowering is of particular importance for protein and oil accumulation in the seeds (Gerendas and Fuehrs, 2013). However, the release of Mg from slowly degrading soil Mg fertilizers is hard to control and varies with soil and weather conditions, and additionally, Mg uptake via the roots is decreasing at later growth stages (Gerendas and Fuehrs, 2013). Therefore, leaf application of nutrient solution can be more effective than soil fertilization in increasing the transport of C- and N-containing compounds to the seeds and hence, to raise soybean seed quality (Gerendas and Fuehrs, 2013). Furthermore, the nutrient is directly delivered to the plant organs with the highest Mg demand when applied onto the leaves and it can immediately become biologically active. Therefore, foliar fertilization displays an optimal method for instantly ameliorating an observed nutrient deficiency in the field in a timely manner. Foliar fertilization has become a widely adopted standard practice (Fernández and Brown, 2013; Fernández et al., 2013) and foliar applications of MgSO<sub>4</sub> are common in some crops (White and Broadley, 2009). For example, foliar application of MgSO<sub>4</sub> solution between ear emergence and end of flowering has been commonly used in cereals even on soils with high Mg status, in order to increase the grain weight and hence the yield. This late Mg foliar application is thought to delay the senescence-induced decrease in photosynthesis (Chalmers et al., 1999). Particularly in larger plants, foliar sprays can be much more effective for correcting visible Mg deficiencies in the short term than soil fertilization (Metson, 1974) and can be a costeffective fertilization method in perennial plants (Dordas, 2009). For example, a single application of 30% Mg solution could correct Mg deficiency and increase growth rates in tea plants (Obatolu, 1999). Positive effects of Mg foliar fertilization on plant performance and crop yield and quality have been shown in several cultivated plants such as maize, fava bean, soybean, potato and spinach (Borowski and Michalek, 2010; Ciećko et al., 2010; Geilfus et al., 2016; Jezek et al., 2015a; Jezek et al., 2015b; Neuhaus et al., 2014; Neuhaus et al., 2013; Vrataric et al., 2006). Increased dry matter and oil yield was also recorded for oregano treated with foliar Mg application (Dordas, 2009) and foliar Mg supply was shown to raise quality traits of sugar beet, such as the total and extractable sugar concentrations (Gerendas and Fuehrs, 2013).

However, the physicochemical properties of the fertilizer solution, anatomical leaf characteristics, the physiological plant status and environmental factors determine the efficacy of fertilizer uptake (Fernández *et al.*, 2013). Incorrect application of foliar fertilizer can even have detrimental effects such as leaf burns. Therefore, the effectiveness and the efficiency of Mg leaf-application needs to be carefully tested for different crop species and under several growing conditions.

#### 1.6 Maize (Zea mays L.)

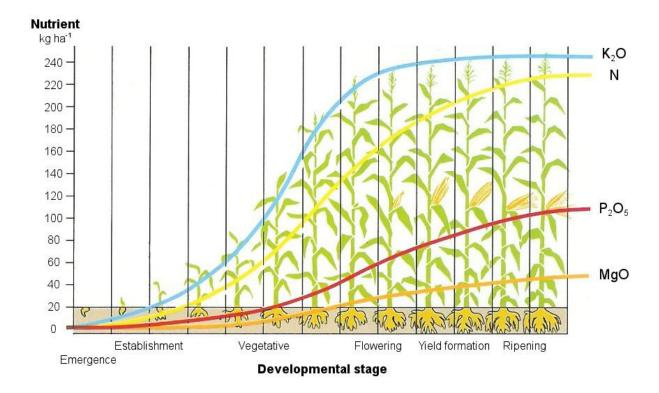
#### 1.6.1 Utilization and agronomic Cultivation

Maize is grown worldwide and it is the second most grown crop after wheat in terms of cultivation area. The global maize production has more than doubled in the last 25 years, reaching over 1 billion tons in 2014. Simultaneously, the average global yield increased from 3.7 t ha<sup>-1</sup> to 5.6 t ha<sup>-1</sup>. This indicates the cultivation of new high-yield varieties and the intensification of farming, which is characterized by the high input of fertilizer, plant growth regulators, pesticides and mechanization. The top producing countries are the United States, China, Brazil and Argentina (FAOSTAT, 2014). The same positive trend in maize production is seen in Germany, where the increase in intensive livestock farms concomitantly raised the cultivation area of maize. Furthermore, maize is widely used as a feedstock for biogas plants as a regenerative energy source to generate electricity in this country. In 2015, maize was grown on 2,555,900 ha in Germany (Deutsches Maiskomitee, 2016b). In the United States maize is mostly grown to feed livestock and to produce ethanol as a biofuel additive for gasoline (Ranum et al., 2014). Furthermore, corn starch serves as the substrate for corn syrup, also called glucose-fructose syrup, which is used as a sweetener for example in soft drinks in the US. Maize can also serve as a substrate for corn oil, whiskey, and other alcoholic beverages, and for the industrial production of plastics and fabrics.

Maize is Africa's most important staple food and increasing maize production offers hope for ameliorating Africa's long-term food crisis (Byerlee and Eicher, 1997). However, the major problem for many African production systems is declining soil fertility, and fertilizer recommendations must be developed in order to improve the efficiency of fertilizer use (Byerlee and Eicher, 1997).

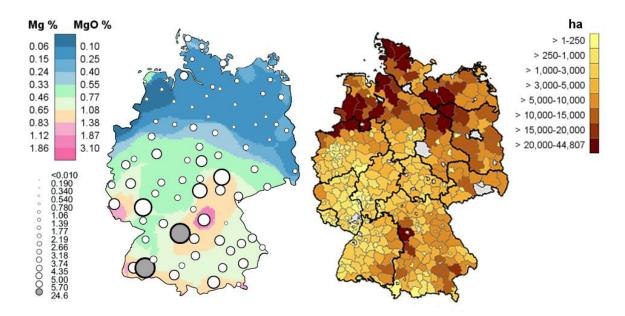
#### 1.6.2 Magnesium Requirement and Magnesium Deficiency in Maize

The average Mg demand is 30 kg ha<sup>-1</sup> MgO for grain maize and 70 kg ha<sup>-1</sup> MgO for silage maize (Deutsches Maiskomitee, 2016c), but the amount varies with soil type, weather conditions, maize cultivar, farming system and yield expectations. Maize has a period of especially intensive growth after a slow initial development (Finke *et al.*, 1999); therefore, the Mg demand is variable during the course of vegetation and depends on the growth rate (Fig. 3).



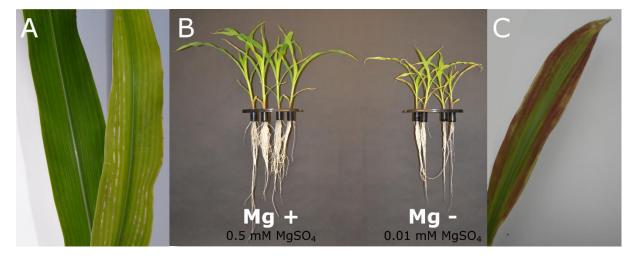
**Figure 3**: Nutrient uptake of maize in the course of plant development (Buchner and Sturm, 1980; modified).

Magnesium deficiency in maize is a widespread phenomenon on sandy soils of Western and Southern Africa (Abunyewa and Mercer-Quarshie, 2004; Krishna, 2014). A temporary water deficit can exacerbate Mg shortage in these semi-arid regions because Mg is transported to the roots by mass flow and hence is driven by the transpiration-driven water flow. Magnesium deficiency in maize can also occur in intensive high-yield farming systems. Leaf analysis in the year 2013 revealed that about 20% of the cultivated maize in Germany showed a slight Mg deficit and more than 10% was clearly deficient in Mg (YARA, 2014). As outlined in detail above, Mg deficiency is most likely to occur on sandy soils in humid regions, which coincides with the major maize production areas in Northern Germany (Fig. 4). Excess soil availability of N and K can typically be found in arable land used for maize cultivation and contributes to the risk of induced Mg deficiency. High N fertilization is often the case in intensive maize farming systems, to raise crop yield and protein levels. Maize tolerates excess levels of N without yield loss (Finke et al., 1999), but it can 'dilute' the Mg concentration in plant tissue due to rapid growth (Craighead, 2001). High K fertilization is also common in maize, because of its high K requirement (Finke et al., 1999). Additionally, new maize varieties bred for high growing and yield become more easily depleted in Mg.



**Figure 4:** Geographical distribution of Mg deposits in Germany (left) and cultivation area of silage maize in German administrative districts in the year 2010 (right) (Deutsches Maiskomitee, 2016a; European Soil Bureau Network 2005; modified).

Visual symptoms of Mg deficiency in maize usually start in older leaves because of the high mobility of Mg within the plant. Leaves show interveinal chlorosis (Fig. 5A), the growth of shoots and roots is depressed (Fig. 5B), and in some cases, anthocyanins are synthesized in lower leaf levels (Fig. 5C). Even latent Mg deficiency without visual symptoms can impair maize growth. Correcting slight Mg deficits in order to reach the full growing potential is nowadays more important than ever, because yields of current maize varieties and hybrids approach the yield ceiling and therefore the margin of error between optimal nutrition and nutrient deficiency decreases markedly (Dobermann and Cassman, 2002).



**Figure 5:** Visual symptoms of magnesium deficiency in maize. (A) Leaves of maize grown under optimal (0.5 mM MgSO<sub>4</sub>; left) and Mg-deficient (0.01 mM MgSO<sub>4</sub>; right) conditions. Deficient leaves show interveinal chlorosis and necrosis. (B) 18-days-old maize plants grown under optimal (0.5 mM MgSO<sub>4</sub>; left) and Mg-deficient (0.01 mM MgSO<sub>4</sub>; right) conditions. Deficient plants show severe growth depression of shoot and root. (C) Maize leaf showing anthocyanin synthesis in response to Mg deficiency.

Although maize production has expanded greatly in the last decades, its response to Mg fertilization is less recognized than for other cereals (Grzebisz, 2013). Therefore, a greater knowledge about the physiological effects of maize under Mg shortage and the response to Mg application is required to manage maize production at high yield levels. On the one hand, fertilization must provide adequate N and K levels for desired crop yield, but without diminishing Mg uptake on the other hand. Therefore, Mg fertilization by leaf application might be of advantage to correct temporal Mg deficiencies, because the nutrient is applied directly to the plant organ with the highest Mg demand and soil-born factors that impair the plant Mg status are circumvented. This fertilization method furthermore allows to meet the Mg demand of maize at the right developmental stage and consequently, to achieve the highest growing potential.

The purpose of Mg fertilization in maize is not only reaching a sufficiently high Mg level for maximal plant production, but the Mg concentration is also an important determinant of the nutritional value of the produced crop. Silage maize has become one of the major forage components in the diet of dairy cows (Khan et al., 2015). Ruminants deficient in Mg suffer from the metabolic disorder hypomagnesaemic tetany, which is characterized by muscle twitching and staggering because of the pivotal functions of Mg in muscle contraction and the nervous system. Hypomagnesaemia can be a major cause of lower milk production (Senbayram et al., 2015) and therefore optimal Mg supply is of special importance for dairy cattle particularly at the end of pregnancy and in early lactation periods (Bolan et al., 2006). Magnesium deficiency has also become a worldwide clinical problem for humans over the last 40 years, resulting from insufficient Mg intake in the diet (Shaul, 2002). Human hypomagnesemia results in neuromuscular, cardiac or nervous disorders (Jahnen-Dechent and Ketteler, 2012). Maize grains are an excellent source of Mg, containing 127 mg Mg per 100 g (US Department of Agriculture, 2016). Regarding the fact that maize displays Africa's most important staple food, maintaining a high nutritional quality of maize in terms of Mg concentration is of massive importance. Magnesium fertilization can even be used for agronomic biofortification to increase the concentration of Mg in edible tissues (White and Broadley, 2009).

#### 1.7 Objectives

evaluated in maize seedlings.

Magnesium deficiency is an increasing agricultural problem worldwide. However, Mg is known as the 'forgotten nutrient' in plant nutrition research (McHoul, 2006) and therefore the impact of Mg shortage on basic physiological processes and crop growth are largely unknown. Certain aspects of the metabolic adaptation of maize to Mg deficiency are not conclusively clarified so far and were addressed in this Ph.D. work. The leitmotiv of this work was to examine the effects of Mg deficiency on specific physiological processes in maize and to test the effectiveness and efficiency of Mg leaf-application as an alternative means to correct nutrient deprivation. Furthermore, the suitability of non-destructive chlorophyll fluorescence measurement for early-stage diagnosis of Mg deficiency in the field and in plant research was

### 1.7.1 Glutamine synthetase activity in leaves of *Zea mays* L. as influenced by magnesium status (Chapter 2)

The relationship between nitrogen (N) metabolism and Mg nutrition is poorly understood. It has previously been reported that Mg deficiency may impair N assimilation in rice and spinach because of decreased activity of the enzyme glutamine synthetase (GS) (Ding *et al.*, 2006; Yin *et al.*, 2009). In fact, Mg is an integral part of GS and is furthermore involved in GS activation and post-translational regulation (Eisenberg *et al.*, 2000; Finnemann and Schjoerring, 2000); therefore, impaired GS activity is likely to occur in response to Mg deficiency. Glutamine synthetase catalyses the ligation of inorganic ammonium (NH<sub>4</sub>+) and the amino glutamic acid (Glu) to form glutamine (Gln). Since the GS-catalysed Gln synthesis is the most important reaction allowing the assimilation of inorganic N into organic molecules, it is of paramount importance for plant growth and crop yield. Therefore, the effects of severe Mg shortage on the total GS activity was tested in maize leaves. In this context, foliar amino acid and protein concentrations were examined. Additionally, these parameters were analysed in Mg-deficient maize after repeated Mg leaf-application, in order to evaluate the effectiveness of Mg foliar fertilization for ameliorating Mg deficiency.

### 1.7.2 Photosynthetic capacity, nutrient status, and growth of maize (*Zea mays* L.) upon MgSO4 leaf-application (Chapter 3)

Leaf fertilization is an alternative means of Mg supply, which might be of advantage in case of restricted Mg root uptake. The potential for ameliorating Mg deficiency symptoms by MgSO<sub>4</sub> leaf-application was tested in hydroponically grown maize. The effectiveness and efficiency of foliar fertilization were evaluated based on the parameters biomass accumulation, photosynthetic capacity, transpiration rate and homeostasis of certain cations.

# **1.7.3 Early-Stage Diagnosis of Nutrient Deficiency by Chlorophyll Fluorescence in Maize Leaves** (Chapter 4)

In order to counteract Mg deficiency before it significantly impairs crop growth, early-stage diagnosis is important. In this work, the suitability of a hand-held leaf-clip device for rapid, non-destructive assessment of epidermal flavonoids for diagnosis of nutrient deficiency was tested. The technique is based on chlorophyll fluorescence measurements at different wavelengths to calculate leaf chlorophyll and epidermal flavonol and anthocyanin levels (Force-A, 2015). This method might be useful for agricultural and scientific purposes in relation to Mg deficiency

because chlorophyll degradation and foliar anthocyanin synthesis are typical symptoms of Mg shortage in certain crops.

### **1.7.4** The Relationship between Plant Nutrition and Anthocyanins and their Significance for Agriculture (Chapter 5)

Transient foliar anthocyanin accumulation can serve as a stress indicator for nutrient demandoriented fertilization management. However, leaf purpling is not a Mg deficiency-specific symptom but occurs in response to different abiotic stresses, including a large variety of nutritional imbalances. Furthermore, anthocyanins in fruits are of major interest in agriculture, because they highly impact the quality of agricultural goods and determine their market value. In this review, the current knowledge of the influence of plant nutritional aspects – including Mg deficiency – on anthocyanin synthesis and the concomitant physiological changes that alter crop quality and stress resistance are summarized and discussed.

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2. Glutamine synthetase as influenced by magnesium status

### Chapter 2

# Glutamine synthetase activity in leaves of *Zea mays* L. as influenced by magnesium status

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#### ORIGINAL ARTICLE

# Glutamine synthetase activity in leaves of Zea mays L. as influenced by magnesium status

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#### **Abstract**

Main conclusion The total capacity of the GS-mediated ligation of free ammonium and glutamate to form glutamine in the leaves of maize plants is not impaired upon severe magnesium starvation. Magnesium deficiency does not obligatorily lead to the decreased total protein concentrations in the leaves.

Abstract Magnesium (Mg) is an integral component of the enzyme glutamine synthetase (GS), having both a structural and a catalytic role. Moreover, Mg is relevant for the post-translational regulation of the GS. Glutamine synthetase is one of the key enzymes in nitrogen assimilation, ligating-free ammonium (NH<sub>4</sub><sup>+</sup>) to glutamate to form glutamine and it is therefore crucial for plant growth and productivity. This study was conducted in order to test whether a severe Mg-deficiency impairs the total capacity of the GS-catalyzed synthesis of glutamine in maize leaves. Maize was grown hydroponically and the GS activity was analyzed dependent on different leaf developmental stages. Glutamine synthetase activity in vitro assays in combination with immune-dot blot analysis revealed that both the total activity and the abundance of glutamine synthetase was not impaired in the leaves of maize plants upon 54 days of severe Mg starvation. Additionally, it was shown that Mg deficiency does not obligatorily lead to decreased total protein concentrations in the leaves, as assayed by Bradford protein quantification. Moreover, Mg

**Keywords** Magnesium deficiency · Maize · Glutamine synthetase · Protein concentration · Amino acid pattern · Nitrogen assimilation · Foliar fertilization

#### **Abbreviations**

Asn Asparagine FW Fresh weight

GHA γ-glutamyl hydroxamate

Gln Glutamine Glu Glutamate

GS Glutamine synthetase NS Nutrient solution

#### Introduction

Magnesium (Mg) is an indispensable plant mineral nutrient, having physiological functions far beyond its well-known role as central atom of chlorophyll molecules. Magnesium is involved in multiple metabolic processes but, however, the effects of Mg deficiency on growth and development of plants are not entirely understood. Despite the fact that Mg deficiency is becoming an increasing problem in a range of different soils used for crop and pasture plant production

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resupply to the roots or the leaves of Mg-deficient plants reversed the Mg-deficiency-induced accumulation of free amino acids in older leaves, which indicates impaired phloem loading. The results of our study reveal that the total GS-mediated primary or secondary assimilation of free NH<sub>4</sub><sup>+</sup> is not a limiting enzymatic reaction under Mg deficiency and thus cannot be accountable for the observed restriction of plant growth and productivity in Mg-deficient maize.

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(Gransee and Führs 2013), the effects of varying Mg supply on processes related to leaf nitrogen assimilation have rarely been investigated (Yin et al. 2009). Glutamine synthetase (GS, EC 6.3.1.2) is a key enzyme of nitrogen assimilation, because it is the only enzyme that corporates free ammonium (NH<sub>4</sub><sup>+</sup>) into glutamine (Hirel et al. 2005b) and is therefore crucial for plant growth and biomass formation (Seabra et al. 2013). Adequate plant Mg status seems to be indispensable for optimal GS activity for several reasons. First, Mg is an integral component of the GS, having both a structural and a catalytic role (Eisenberg et al. 2000). Second, Mg is relevant for the enzyme's post-translational regulation. In fact, the cytosolic isoform GS1 is protected against oxidative inactivation by phosphorylation. This process requires the action of protein kinases which are only active in the presence of both Mg and ATP (Finnemann and Schjoerring 2000). It has further been revealed that phosphorylation and subsequent binding of a 14-3-3 protein is pivotal for the activity of the chloroplastic isoform GS2 (Riedel et al. 2001; Lima et al. 2006). Both processes are known to be Mg-dependent reactions (Kaiser et al. 1999). Additionally, the GS activity appears to be controlled by end-product feedback inhibition, particularly by high levels of free glutamine (Rhodes et al. 1975) and glutamine-dependent amino acids such as asparagine (Eisenberg et al. 2000). An accumulation of free amino acids in leaves is known to be a typical symptom of Mg deficiency (Cakmak et al. 1994; Fischer et al. 1998). Considering all these impacts of Mg on the functionality of the GS, decreased GS activity is expected to be a likely response to inadequate Mg availability. Despite this relevance of the GS as a major bottleneck during the ligation of free NH<sub>4</sub><sup>+</sup> into organic carbon skeletons and the resulting crucial role in plant growth, biomass accumulation, and grain production (Gallais and Hirel 2004; Martin et al. 2006; Bernard and Habash 2009; Thomsen et al. 2014), the effects of a Mg deficiency on the GS activity still await clarification.

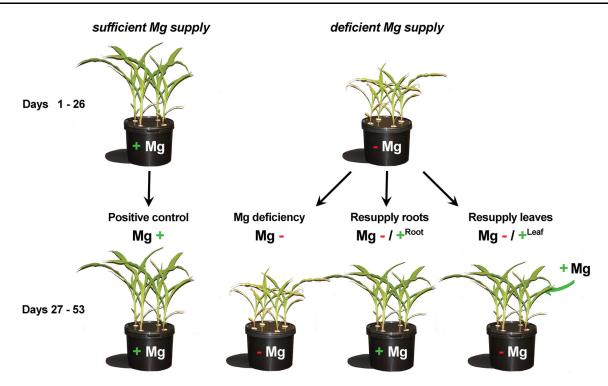
This study was conducted in order to test whether a severe Mg-deficiency impairs the total GS activity in maize leaves. Maize was selected since it shows constant GS activity in different leaf stages throughout aging and development (Hirel et al. 2005b), whereas in many other crop species senescence is known to induce strong reductions in total leaf GS activity (Kamachi et al. 1991; Masclaux et al. 2000; Kichey et al. 2005). Despite the great relevance of Mg for the GS activity, this study surprisingly revealed that the GS activity is not impaired in the leaves of maize plants upon 53 days of severe magnesium starvation.

#### Materials and methods

#### Plant cultivation and harvest

Maize (Zea mays L. cv. Susann, Nordsaat Saatzucht, Langenstein, Germany) was grown hydroponically in the greenhouse at 20–25 °C with 70 % relative humidity. Plants were cultivated under lamps (lamp: Professional Lighting, SON-K 400, Philips Deutschland GmbH, Hamburg, Germany; bulb: Philips SON-T Agro 400 watt, Philips Deutschland GmbH; light intensity:  $\sim 350 \, \mu \text{mol m}^{-2} \, \text{s}^{-1}$ , as measured with a Li-Cor Light Meter LI-189, Lincoln, NE, USA) for three weeks and under natural light regime thereafter. Seeds were soaked in 2-mM-aerated CaSO<sub>4</sub> solution for 2 days and germinated in sterile quartz sand, which was kept moistened with 2 mM CaSO<sub>4</sub>. Six-day-old seedlings were transferred into 9 L containers (four plants per vessel) with 25 % strength nutrient solution, which was raised stepwise to 100 % strength within four days. The fullstrength nutrient solution (NS) had the following composition: 1.3 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.7 mM NH<sub>4</sub>NO<sub>3</sub>, 2.0 mM CaCl<sub>2</sub>, 1.0 mM K<sub>2</sub>SO<sub>4</sub>, 0.2 mM KH<sub>2</sub>PO<sub>4</sub>, 200 μM Fe-EDTA,  $5 \mu M H_3 BO_3$ ,  $2 \mu M Mn SO_4$ ,  $0.5 \mu M Zn SO_4$ ,  $0.3 \mu M$ CuSO<sub>4</sub>, and 0.01 µM (NH<sub>4</sub>)<sub>2</sub>Mo<sub>7</sub>O<sub>24</sub>, and it was exchanged weekly. Four experimental groups differently supplied with Mg were cultivated (Fig. 1), and biologically replicated four times and vessels were arranged fully randomized. Plants of the positive control group (Mg+) were sufficiently supplied with Mg, receiving 0.5 mM MgSO<sub>4</sub> via the NS. The remaining plants grew under Mg-deficient conditions (Mg-) and received only 0.02 mM MgSO<sub>4</sub> for a period of 6 days to ensure proper seedling development. In order to induce clear Mg-deficiency symptoms, [Mg] was reduced to 0.01 mM MgSO<sub>4</sub> thereafter. After a growing period of 27 days, the Mg-deficient plants were subdivided into three experimental groups: the [Mg] in the NS of the first sub-group remained low at 0.01 mM MgSO<sub>4</sub> (Mg-), in the second sub-group the [Mg] in the NS was raised to sufficient 0.5 mM MgSO<sub>4</sub> (Mg-/+Root), and in the third sub-group Mg was exogenously supplied over the leaves while the plants were still growing in 0.01 mM MgSO<sub>4</sub> NS (Mg-/+<sup>Leaf</sup>). Leaf-application solution containing 200 mM MgSO<sub>4</sub> was brushed onto the ad- and abaxial faces of the leaves number four to seven. The application solution contained 0.1 % Silwet as wetting agent and treatments took place in the morning to avoid leaf burnings by high irradiation. Leaves number four to six received four treatments within 10 days and leaf number seven was treated two times due to its later emergence. Plants were harvested after 53 days and fresh weights (FW) of the total shoot, single leaves and the roots were determined. Subsequently, leaves were frozen in liquid N<sub>2</sub> for further analysis.





**Fig. 1** Schematic overview of the experimental setup showing four distinct treatments of hydroponically grown maize plants. For each experimental group, four pots with four maize plants each were installed. Mg+ 0.5 mM MgSO<sub>4</sub> in nutrient solution (NS); Mg-0.01 mM MgSO<sub>4</sub> in NS; Mg-/+<sup>Root</sup> 0.01 mM MgSO<sub>4</sub> in NS for

26 days and 0.5 mM MgSO $_4$  in NS for the following 27 days; Mg $_-$ /+  $_-$ Leaf 0.01 mM MgSO $_4$  in NS and leaf-application of 200 mM MgSO $_4$  onto the leaves number four to seven (described in detail in the "Materials and methods" paragraph)

#### Protein extraction and quantification

Frozen leaf samples were ground to a homogenous powder with liquid  $N_2$ , and proteins were extracted with buffer containing 50 mM TrisHCl, 1 mM EDTA, and 1 mM DTT at a pH 7.8. Protein concentration of tissue extracts was determined according to the method of Bradford (1976) using BSA as a protein standard.

#### Measurement of GS activity

Glutamine synthetase activity was measured for the synthetase reaction according to a modified method of O'Neal and Joy (1973). All operations were performed at 4 °C. 200  $\mu$ L of protein extract were incubated with 200  $\mu$ L of reaction buffer (50 mM Tris-Base, 20 mM MgSO<sub>4</sub>, 80 mM L-glutamate, 30 mM NH<sub>2</sub>OH, 24 mM ATP; pH 7.8) at 37 °C. Reaction was stopped with stopping solution (370 mM FeCl<sub>3</sub> and 670 mM HCl) after 25 min. Samples were centrifuged for 5 min at 10,000g. The amount of  $\gamma$ -glutamyl hydroxamate in the supernatant was determined photometrically (540 nm) against an immediately stopped parallel sample. A standard curve was made using authentic  $\gamma$ -glutamyl hydroxamate (GHA). Specific enzyme activity was determined as mmol of GHA

produced per  $\mu g$  of protein per h. This assay estimates the total activity of all GS isoforms together, which is straightforward for this study because the total GS-mediated capacity to ligate-free  $NH_4^+$  to glutamate was of interest, making isoform-specific analysis obsolete.

### Immunodetection of glutamine synthetase protein abundance

For dot blot analysis, 5 µL of protein extract containing 0.5 µg of protein were blotted onto wet PVDF membrane. Protein samples were diluted with ultrapure H<sub>2</sub>O to achieve equal protein concentrations. For activation, PVDF membrane was immersed in 100 % methanol for 5 s and was subsequently transferred to TBS buffer (10 mM Tris-Base, 150 mM NaCl, and pH 7.6). PVDF membrane was allowed to dry at room temperature for 60 min, and dry membrane was incubated in blocking solution (TBS buffer, 2.5 % skimmed milk powder) overnight. GS polypeptides were detected using a polyclonal antiserum raised against both the cytosolic and the chloroplastic enzyme forms GS1 and GS2 (GS1 GS2 glutamine synthetase global antibody, Agrisera AB, Vännas, Sweden). This composite detection was selected because the study aimed to analyze the total GS-mediated capacity to assimilate-free NH<sub>4</sub><sup>+</sup> and did not



target to investigate isoform-specific effects. Horseradish peroxidase conjugate was used as secondary antibody (Anti-Rabbit IgG (whole molecule)-Peroxidase, Sigma-Aldrich, St. Louis, MO, USA). The PVDF membrane was incubated in primary and subsequently in secondary antibody solution (TBS buffer, 0.1 % Tween 20, 2.5 % skimmed milk powder) for 1 h each with three washing steps in TBS-T solution (TBS buffer, 0.1 % Tween 20) in between and two washing steps in TBS-T solution plus two washing steps in TBS buffer afterward, respectively. Each washing step lasted for 5 min and the washing was performed with gentle agitation. Blots were developed with ECL reagent with subsequent light detection with Kodak BioMax light film (Sigma-Aldrich). The exposure time of the light film to the membrane amounted to approximately 40 s. Mean gray values of the dots were analyzed using ImageJ (US National Institutes of Health, Bethesda, MD, USA).

#### Extraction and analysis of free amino acids

Amino acids were extracted with chloroform/methanol (30/70; v/v) from freeze-dried leaf tissue powder with subsequent reverse-phase cartridge purification (Strata C18-E, Phenomex, Torrance, CA, USA). Free amino acids were measured by cation-exchange chromatography followed by post-column derivatization with ninhydrin and photometrical detection at 570 nm wavelength, using the amino acid analyzer Biochrom 30 (Laborservice Onken, Gründau, Germany).

#### Analysis of Mg concentration

For mineral analysis, freeze-dried leaf tissue powder was digested in 69 % HNO<sub>3</sub> using a 1800 W microwave oven (MARS 6 Xpress; CEM Corporation, Matthews, NC, USA) under the following procedure: 2 min at 100 °C, 1 min at 120 °C, 20 min at 180 °C, and 20 min cooling time.

Samples were diluted with distilled water to 50 mL and Mg concentrations were quantified by atomic absorption spectrometry with the S Series AA spectrometer (Thermo Fisher Scientific, Waltham, MA, USA).

#### **Statistics**

Data were statistically analyzed using R statistical package software version 3.0.1 (The R foundation for statistical computing, Vienna, Austria, 2013). Effects of treatments were tested by comparisons with the positive control group (Mg+) using Student's t test. In tables and figures, asterisks indicate significant differences between treatments and positive control (Mg+) at  $P \leq 0.05$  (\*),  $P \leq 0.01$  (\*\*) and  $P \leq 0.001$  (\*\*\*), respectively, and n.s. indicates nonsignificance.

#### **Results**

#### Plant growth

A low supply of Mg (Mg-, 0.01 mM MgSO<sub>4</sub> in nutrient solution (NS)) significantly reduced fresh weights (FW) of shoots and roots by about 85 and 84 %, respectively, compared to plants that were sufficiently supplied with Mg (Mg+, 0.5 mM MgSO<sub>4</sub> in NS) (Table 1). This FW reduction was also apparent in the individually analyzed leaves number six, seven, and eight (Table 1). Compared to the positive control, fresh weights of shoots and roots of plants that were resupplied with Mg after 26 days of Mg deficiency were reduced by 70 and 73 %, respectively, if [Mg] of the NS was raised (Mg-/+<sup>Root</sup>), and by 75 and 80 %, respectively, if Mg was applied to the leaves (Mg-/+<sup>Leaf</sup>). Single leaf FWs of these groups were also significantly reduced in comparison to the positive control.

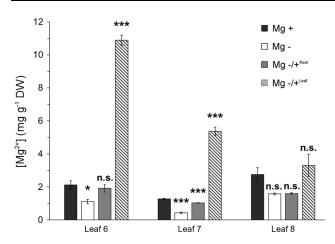
Table 1 Fresh weights (FW) of total shoots and roots as well as single leaves in response to differing Mg supply

	Positive control Mg+ Mean (g) ± SE	Mg-deficiency Mg- Mean (g) ± SE	Root resupply $Mg-/+^{Root}$ Mean $(g) \pm SE$	Leaf resupply $Mg-/+^{Leaf}$ Mean (g) $\pm$ SE
Shoot	$142.63 \pm 2.57$	21.65 ± 2.04***	42.74 ± 1.43***	35.29 ± 2.22***
Root	$20.84 \pm 0.57$	$3.40 \pm 0.36***$	$5.64 \pm 0.34***$	$4.19 \pm 0.33***$
Leaf 6	$8.38 \pm 0.55$	$2.16 \pm 0.10***$	$3.08 \pm 0.06***$	$3.08 \pm 0.07***$
Leaf 7	$8.88 \pm 0.27$	$2.79 \pm 0.25***$	$3.83 \pm 0.08***$	$3.05 \pm 0.11***$
Leaf 8	$8.63 \pm 0.13$	$2.73 \pm 0.22***$	$3.93 \pm 0.03***$	$3.32 \pm 0.15***$

The leaves four to six were treated four times and leaf seven was treated two times with Mg solution due to its later emergence. Values are means of 4 biological replicates  $\pm SE$ 

Mg+0.5 mM  $MgSO_4$  in nutrient solution (NS), Mg-0.01 mM  $MgSO_4$  in NS,  $Mg-/+^{Root}$  0.01 mM  $MgSO_4$  in NS for 26 days, and 0.5 mM  $MgSO_4$  in NS for 27 days,  $Mg-/+^{Leaf}$  0.01 mM  $MgSO_4$  in NS and leaf-application of 200 mM  $MgSO_4$  onto the leaves number four to seven Asterisks indicate significant differences between treatments and positive control (Mg+) at  $P \leq 0.001$  (\*\*\*)





**Fig. 2** Magnesium concentration ([Mg<sup>2+</sup>]) in the leaves six to eight in response to differing Mg supply Mg+ 0.5 mM MgSO<sub>4</sub> in nutrient solution (NS); Mg- 0.01 mM MgSO<sub>4</sub> in NS; Mg-/+ $^{\text{Root}}$  0.01 mM MgSO<sub>4</sub> in NS for 26 days and 0.5 mM MgSO<sub>4</sub> in NS for 27 days; Mg-/+ $^{\text{Leaf}}$  0.01 mM MgSO<sub>4</sub> in NS and leaf-application of 200 mM MgSO<sub>4</sub> onto the leaves number four to seven, whereby the leaves four to six were treated four times and leaf seven was treated two times with Mg solution due to its later emergence. Values are means of 4 biological replicates  $\pm$  SE. *Asterisks* indicate significant differences between treatments and positive control (Mg+) at  $P \leq 0.05$  (\*) and  $P \leq 0.001$  (\*\*\*); n.s., not significant

#### **Magnesium concentration**

Mg deficiency resulted in significantly reduced Mg concentrations in leaves number six and seven, containing  $1.11 \pm 0.14$  and  $0.44 \pm 0.04$  mg Mg<sup>2+</sup> g<sup>-1</sup> DW, respectively, compared to sufficiently Mg-supplied plants  $(2.13 \pm 0.25 \text{ and } 1.28 \pm 0.03 \text{ mg Mg}^{2+} \text{ g}^{-1} \text{ DW})$  (Fig. 2). The Mg concentration in leaf number 8 was with  $1.58 \pm 0.05 \text{ mg Mg}^{2+} \text{g}^{-1} \text{DW}$  not significantly lower than in the positive control (2.76  $\pm$  0.41 mg Mg<sup>2+</sup> g<sup>-1</sup> DW). Magnesium resupply via the roots raised the Mg concentrations in leaf number six (1.92  $\pm$  0.22 mg Mg<sup>2+</sup> g<sup>-1</sup> DW) and in leaf number eight (1.60  $\pm$  0.06 mg Mg<sup>2+</sup> g<sup>-1</sup> DW) to the level of the positive control plants, whereas the Mg concentration in leaf number seven (1.03  $\pm$  0.01 mg Mg<sup>2+</sup> g<sup>-1</sup> DW) was significantly reduced. Exogenous application of Mg onto the leaves resulted in significantly increased Mg concentrations in the directly treated leaves number six  $g^{-1}$  $(10.89 \pm 0.31 \text{ mg Mg}^{2+})$ DW) seven  $(5.36 \pm 0.25 \text{ mg Mg}^{2+} \text{ g}^{-1} \text{ DW})$ . The Mg concentration in leaf number eight, which was not brushed with MgSO<sub>4</sub> solution due to its later emergence, was with  $3.31 \pm 0.67$  mg Mg<sup>2+</sup> g<sup>-1</sup> DW not significantly different from the positive control.

#### Glutamine synthetase activity and abundance

Plants that were well supplied with Mg had a total GS activity of 14.6  $\pm$  1.5, 5.6  $\pm$  0.3 and 8.1  $\pm$  0.7  $\mu mol$ 

GHA  $\mu g^{-1}$  protein  $h^{-1}$  in the leaves number six, seven, and eight, respectively. In comparison to this positive control, neither the permanent Mg deficiency (16.1  $\pm$  1.3  $\mu$ mol,  $4.9 \pm 0.3, 6.1 \pm 0.6 \; \mu \text{mol GHA } \mu \text{g}^{-1} \; \text{protein h}^{-1}) \; \text{nor the}$ Mg resupply to the NS after Mg starvation (14.4  $\pm$  0.8,  $6.4 \pm 0.5, 8.4 \pm 0.9 \ \mu mol \ GHA \ \mu g^{-1} \ protein \ h^{-1}) \ had \ a$ significant effect on the total GS activities in these leaves (Fig. 3). Same was true for the total GS abundance in leaf number six. Immuno-dot blot analysis revealed mean gray values of 111.4  $\pm$  5.2, 97.3  $\pm$  10.6, and 121.5  $\pm$  7.8 for Mg+, Mg-, and Mg-/+Root, respectively (Fig. 4). In contrast, the Mg application onto the ab- and adaxial leaf surfaces after 26 days of Mg starvation significantly increased the total GS activity in the leaves number six and seven up to  $21.4 \pm 1.1$  and  $8.0 \pm 0.4$  µmol GHA µg<sup>-1</sup> protein h<sup>-1</sup>. In accordance, the mean gray value of the immuno-dot blot analysis of this treatment was  $147.7 \pm 3.9$ , which indicated a significantly increased total GS abundance in leaf number six compared to the positive control.

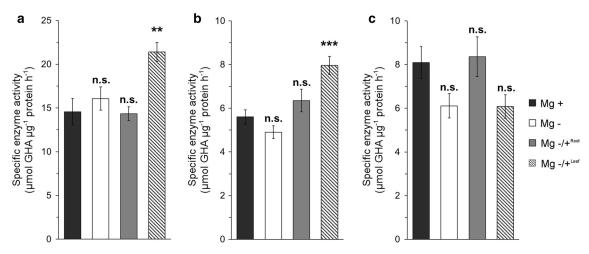
#### **Protein concentration**

Permanent Mg deficiency resulted in a significantly decreased protein concentration in leaf number six, containing 15.4  $\pm$  0.4  $\mu g$  protein mg<sup>-1</sup> DW, compared to the positive control containing  $31.9 \pm 3.7 \,\mu g$  protein mg<sup>-1</sup> DW (Fig. 5). The protein concentrations in leaf number seven and eight amounted to 35.8  $\pm$  2.1 and 50.5  $\pm$  3.2  $\mu g$ protein mg<sup>-1</sup> DW, respectively, which did not differ from well-nourished plants, containing  $42.5 \pm 3.4$  and  $40.0 \pm 3.6 \,\mu g$  protein mg<sup>-1</sup> DW, respectively, in these leaves. Magnesium resupply via the roots or the leaves increased the protein concentrations in all analyzed leaves to the level of the positive control. Root resupply resulted in protein concentrations of  $29.5 \pm 1.4$ ,  $39.4 \pm 3.6 \,\mu g$ protein mg<sup>-1</sup> DW and  $38.8 \pm 3.0 \,\mu g$  protein mg<sup>-1</sup> DW, respectively, in the leaves number six, seven, and eight. Application of Mg to leaves increased the protein concentrations to  $22.2 \pm 1.9$ ,  $33.5 \pm 3.4 \,\mu g$  protein mg<sup>-1</sup> DW, and  $52.5 \pm 4.5 \,\mu g$  protein mg<sup>-1</sup> DW, respectively.

#### Concentration of free amino acids

The total concentration of free amino acids significantly increased in the leaves number six and seven upon Mg deficiency to 207 and 169 %, respectively, to that of the positive control (Table 2). In leaf number six, this increment was mainly caused by asparagine (Asn), whose concentration was 29-fold higher as under adequate Mg nutrition. Similarly, in leaf number seven the Asn concentration rose about 745 % and, furthermore, the glutamine (Gln) concentration increased 569 % as compared





**Fig. 3** Effects of differing Mg supply on the specific GS activity (composite measurement of all isoforms) in the leaves number six (a), seven (b), and eight (c). Mg+ 0.5 mM MgSO<sub>4</sub> in nutrient solution (NS); Mg- 0.01 mM MgSO<sub>4</sub> in NS; Mg-/+ $^{Root}$  0.01 mM MgSO<sub>4</sub> in NS for 26 days and 0.5 mM MgSO<sub>4</sub> in NS for 27 days; Mg-/+ $^{Leaf}$  0.01 mM MgSO<sub>4</sub> in NS and leaf-application of 200 mM MgSO<sub>4</sub> onto

the leaves number four to seven, whereby the leaves four to six were treated four times and leaf seven was treated two times with Mg solution due to its later emergence. Values are means of 4 biological replicates and twofold technical replication  $\pm$  SE. *Asterisks* indicate significant differences between treatments and positive control (Mg+) at  $P \leq 0.01$  (\*\*) and  $P \leq 0.001$  (\*\*\*); n.s., not significant

■ Mg +

□ Mg -

■ Mg -/+<sup>Roo</sup>

Mg -/+
 Leaf
 Mg -/+
 Leaf
 Mg -/+
 Leaf
 Mg -/+
 Leaf
 Mg -/+
 Mg -/ Mg -

n.s

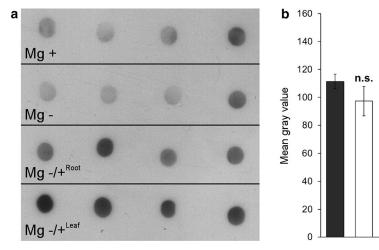


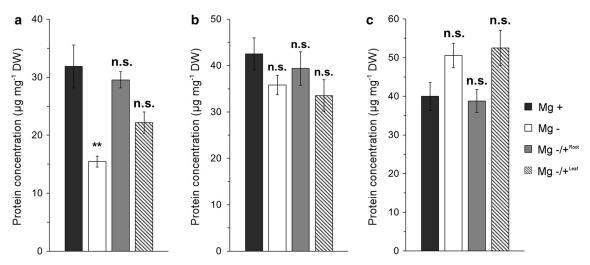
Fig. 4 Effects of differing Mg supply on the relative GS abundance (composite measurement of all isoforms in leaf number six. a Immuno-dot blot analysis of the GS protein abundance. Equal amounts of protein (0.5  $\mu g$ ) in equal solution volumes (5  $\mu L$ ) were loaded onto an activated PVDF membrane, which was subsequently probed with antiserum raised in rabbits against the GS1 and GS2 isoforms. b Signal intensity analysis as detected by mean gray value determination of the dots. Values are means of four biological replicates, technically replicated four times  $\pm$  SE. Asterisks indicate

significant differences between treatments and positive control (Mg+) at  $P \leq 0.01$  (\*\*); n.s., not significant. Mg+ 0.5 mM MgSO<sub>4</sub> in nutrient solution (NS); Mg- 0.01 mM MgSO<sub>4</sub> in NS; Mg-/+ $^{\rm Root}$  0.01 mM MgSO<sub>4</sub> in NS for 26 days and 0.5 mM MgSO<sub>4</sub> in NS for 27 days; Mg-/+ $^{\rm Leaf}$  0.01 mM MgSO<sub>4</sub> in NS and leaf-application of 200 mM MgSO<sub>4</sub> in the leaves number four to seven, whereby the leaves four to six were treated four times and leaf seven was treated two times with Mg solution due to its later emergence

to positive control. Leaf number eight of the Mg-deficient plants contained, among others, significantly higher concentrations of Gln, but the total concentration of free amino acids was not increased in this leaf. Magnesium resupply to Mg-deficient plants after 27 days significantly decreased the total concentration of free amino acids in the leaves number seven and eight to 78 and 87 %, respectively, to those of the positive control for root resupply and in leaves

six, seven and eight to 65, 66, and 85 %, respectively, for leaf resupply. The concentrations of several major amino acids decreased in consequence of the Mg restoration in all leaves, whereas the summarized concentration of other minor amino acids increased in most leaves (see Table 2 for detailed information, where significant increases are highlighted in green and significant decreases are highlighted in red).





**Fig. 5** Effects of differing Mg supply on the protein concentration in the leaves six (a), seven (b), and eight (c). Mg+ 0.5 mM MgSO<sub>4</sub> in nutrient solution (NS); Mg- 0.01 mM MgSO<sub>4</sub> in NS; Mg-/+ $^{\rm Root}$  0.01 mM MgSO<sub>4</sub> in NS for 26 days and 0.5 mM MgSO<sub>4</sub> in NS for 27 days; Mg-/+ $^{\rm Leaf}$  0.01 mM MgSO<sub>4</sub> in NS and leaf-application of 200 mM MgSO<sub>4</sub> in the leaves number four to seven, whereby the

leaves four to six were treated four times and leaf seven was treated two times with Mg solution due to its later emergence. Values are means of four biological replicates, technically replicated two times  $\pm$  SE. Asterisks indicate significant differences between treatments and positive control (Mg +) at  $P \leq 0.001$  (\*\*\*); n.s., not significant

#### **Discussion**

Magnesium is essential for numerous cellular processes and is involved in diverse physiological reactions. Surprisingly little attention has been paid to its relevance for nitrogen metabolism. The Mg-containing enzyme glutamine synthetase (GS) is a key enzyme of nitrogen assimilation, ligating ammonium (NH<sub>4</sub><sup>+</sup>) to glutamate to form glutamine. The GS activity is not only crucial for the primary assimilation of inorganic nitrogen, derived from direct NH<sub>4</sub><sup>+</sup> uptake or reduction of nitrate. Furthermore, free NH<sub>4</sub><sup>+</sup> can also be produced endogenously in the plant and is reassimilated by the GS again. Ammonium ions can be released during processes like photorespiration, phenylpropanoid biosynthesis, or amino acid catabolism following protein degradation (Bernard and Habash 2009; Betti et al. 2012). The GS activity is known to structurally and catalytically rely on the availability of Mg (O'Neal and Joy 1974; Eisenberg et al. 2000) and Mg is furthermore involved in the activation and stabilization of the enzyme via phosphorylation reactions (Finnemann and Schjoerring 2000; Riedel et al. 2001). Proper Mg nutrition therefore seems to be pivotal for optimal GS activity, which is of particular interest for food and feed security and agricultural production systems since GS is likely to be a major bottleneck controlling plant growth and productivity (Martin et al. 2006; Seabra et al. 2013). For this reason, we investigated the impact of a varying Mg supply on the total GS activity in maize leaves at different developmental stages. Maize was studied because in this species the total GS activity operates in a very efficient manner throughout the whole plant-life cycle as well as in different leaf stages (Hirel et al. 2005b) and is therefore only marginally influenced by senescence, a process which is known to decrease GS activity in many other plants species (Masclaux et al. 2000; Kichey et al. 2005). Therefore, changes in the total GS activity of Mg-deficient maize plants are considered to mainly originate from the lack of Mg as a catalytic and activating component of the GS enzyme.

### Magnesium deficiency is not necessarily accompanied by decreased GS activity

In vitro measurements in different leaves of maize growing under Mg deficiency revealed that neither the activity nor the abundance of this enzyme is directly correlated to the concentration of Mg in the plant. This contrasts with previous studies conducted by Ding et al. (2006) and Yin et al. (2009) who reported about a negative impact of Mg deficiency on the GS activity in rice and spinach. Although the Mg concentrations in the mature leaves number six and seven were significantly reduced under Mg-deficient conditions (Fig. 2), no inhibition of total GS activity could be observed in comparison to the positive control plants (Fig. 3a, b). Likewise, the total GS abundance in leaf number six was not significantly lower under permanent Mg restriction (Fig. 4). These results are supported by the concentrations of the free amino acids in the leaves of Mgdeficient plants: neither glutamate (Glu), the substrate of the GS, was increased nor glutamine (Gln), the product of the GS, was decreased (Table 2), as shown under conditions of inhibited GS activity (Watanabe et al. 1997;



Table 2 Effects of differing Mg supply on the concentrations of free amino acids in the leaves six, seven, and eight

Leaf number	Amino acid	Positive control Mg+ Mean ( $\mu$ mol g <sup>-1</sup> DW) $\pm$ SE	Mg-deficiency Mg- Mean ( $\mu$ mol g <sup>-1</sup> DW) $\pm$ SE	Root resupply $Mg-/+^{Root}$ Mean ( $\mu$ mol $g^{-1}$ DW) $\pm$ SE	Leaf resupply $Mg-/+^{Leaf}$ Mean ( $\mu$ mol $g^{-1}$ DW) $\pm$ SE
6	Asp	$2.52 \pm 0.14$	$2.27 \pm 0.19$ n.s.	$2.43 \pm 0.30 \text{ n.s.}$	0.93 ± 0.06***
	Thr	$1.05\pm0.08$	$1.73 \pm 0.12**$	$0.62 \pm 0.06$ *	$0.51 \pm 0.03**$
	Ser	$2.24 \pm 0.20$	$4.19 \pm 0.32**$	$1.71 \pm 0.08$ n.s.	$1.80 \pm 0.24 \text{ n.s.}$
	Asn	$0.81 \pm 0.11$	<b>23.22</b> ± 2.41***	$1.45 \pm 0.44$ n.s.	$1.22\pm0.16$ n.s.
	Glu	$6.61 \pm 0.39$	$6.01 \pm 0.28 \text{ n.s.}$	$6.62 \pm 0.65$ n.s.	$4.37 \pm 0.21**$
	Gln	$2.52 \pm 0.13$	<b>6.22</b> ± 0.33***	$1.05 \pm 0.05***$	$0.93 \pm 0.18***$
	Gly	$2.21 \pm 0.15$	<b>4.78</b> ± 0.49**	$2.49 \pm 0.42 \text{ n.s.}$	$3.48 \pm 0.49$ n.s.
	Ala	$12.94 \pm 0.68$	$12.82 \pm 0.65$ n.s.	$10.01 \pm 0.32*$	$6.76 \pm 0.52***$
	Others	$3.02 \pm 0.18$	<b>9.00</b> ± 0.43***	$4.13 \pm 0.22*$	$5.22 \pm 0.55$ *
	Sum	$33.92 \pm 0.70$	<b>70.25</b> ± 4.09***	$30.51 \pm 1.53$ n.s.	$22.06 \pm 1.68**$
7	Asp	$2.55 \pm 0.10$	$1.58 \pm 0.05***$	$2.03 \pm 0.20 \text{ n.s.}$	$1.38 \pm 0.07***$
	Thr	$1.12 \pm 0.04$	$0.82 \pm 0.06**$	$0.54 \pm 0.05***$	$0.54 \pm 0.05***$
	Ser	$1.74 \pm 0.12$	$2.68 \pm 0.22*$	$1.52 \pm 0.07$ n.s.	$1.39 \pm 0.07$ n.s.
	Asn	$0.88 \pm 0.17$	$6.54 \pm 1.24*$	$0.28 \pm 0.03*$	$0.23 \pm 0.04*$
	Glu	$5.64 \pm 0.09$	$3.23 \pm 0.27***$	$3.74 \pm 0.34**$	$2.89 \pm 0.19***$
	Gln	$1.58 \pm 0.04$	<b>9.01</b> ± 1.35**	$1.57 \pm 0.14 \text{ n.s.}$	$1.35 \pm 0.03**$
	Gly	$1.74 \pm 0.19$	<b>6.26</b> ± 0.66**	$1.78 \pm 0.19$ n.s.	$2.22 \pm 0.30$ n.s.
	Ala	$12.47 \pm 1.14$	$7.36 \pm 0.21**$	$10.26 \pm 0.45$ n.s.	$7.40 \pm 0.23**$
	Others	$3.09 \pm 0.13$	<b>7.80</b> ± 0.94**	$2.23 \pm 0.15**$	$2.83 \pm 0.15 \text{ n.s.}$
	Sum	$30.80 \pm 1.43$	$52.10 \pm 7.08*$	$23.95 \pm 0.82*$	20.23 ± 0.86**
8	Asp	$3.39 \pm 0.04$	$2.68 \pm 0.14**$	$3.25 \pm 0.36$ n.s.	$2.60 \pm 0.19*$
	Thr	$0.91 \pm 0.07$	$0.86 \pm 0.08 \text{ n.s.}$	$0.55 \pm 0.06$ *	$0.76 \pm 0.02 \text{ n.s.}$
	Ser	$2.58 \pm 0.15$	$2.85 \pm 0.17$ n.s.	$2.07 \pm 0.02*$	$2.77 \pm 0.31$ n.s.
	Asn	$0.74 \pm 0.15$	$1.42 \pm 0.23$ n.s.	$0.29 \pm 0.02*$	$0.53 \pm 0.06$ n.s.
	Glu	$7.60 \pm 0.21$	$5.73 \pm 0.82 \text{ n.s.}$	$6.13 \pm 0.28$ *	$4.06 \pm 0.28***$
	Gln	$1.42 \pm 0.15$	$2.65 \pm 0.29*$	$1.09 \pm 0.10$ n.s.	$1.12 \pm 0.05$ n.s.
	Gly	$2.74 \pm 0.31$	<b>8.85</b> ± 1.06**	$2.63 \pm 0.31$ n.s.	<b>6.01</b> ± 1.01*
	Ala	$15.55 \pm 0.66$	8.92 ± 0.52***	$12.75 \pm 0.27*$	9.24 ± 0.58***
	Others	$4.97 \pm 0.17$	<b>9.66</b> ± 1.07**	$5.79 \pm 0.18*$	<b>6.99</b> ± 0.11***
	Sum	$39.91 \pm 0.31$	$43.83 \pm 2.69 \text{ n.s.}$	$34.55 \pm 0.45***$	$34.08 \pm 0.67***$

Significant increases are highlighted in bold, significant decreases are highlighted in italics

The leaves four to six were treated four times and leaf seven was treated two times with Mg solution due to its later emergence. Values are means of 4 biological replicates  $\pm$  SE

Mg+0.5 mM  $MgSO_4$  in nutrient solution (NS), Mg-0.01 mM  $MgSO_4$  in NS,  $Mg-/+^{Root}0.01$  mM  $MgSO_4$  in NS for 26 days, 0.5 mM  $MgSO_4$  in NS for 27 days,  $Mg-/+^{Leaf}0.01$  mM  $MgSO_4$  in NS, and leaf-application of 200 mM  $MgSO_4$  onto the leaves number four to seven Asterisks indicate significant differences between treatments and positive control (Mg+) at  $P \le 0.05$  (\*),  $P \le 0.01$  (\*\*) and  $P \le 0.001$  (\*\*\*), n.s. indicates non-significance

Caputo et al. 2009). The total GS activity was measured in vitro in the presence of Mg in order to assure high enzyme stability (O'Neal and Joy 1973). Therefore, the results of the GS activity measurements display the in vitro capacity of the enzyme to catalyze the formation of Gln. Nevertheless, the highly increased Gln concentrations in the leaves six and seven of Mg-deficient plants (Table 2) reveal considerable GS activity under physiological conditions in vivo, as well. The finding that a severe Mg

deficiency of 54 days, as induced by only 0.01 mM MgSO<sub>4</sub> in the NS, does not compromise both, the in vitro GS activity and its abundance in leaves, appears unexpected not only because Mg is an indispensable component for the structural integrity and activity of the enzyme. Moreover, former studies showed strong reductions in CO<sub>2</sub> fixation, chlorophyll concentrations, and overall growth of maize under such a severe Mg deficiency (Jezek et al. 2015). The results of the present study strongly indicate that the GS-



mediated ligation of NH<sub>4</sub><sup>+</sup> and glutamate is not a limiting enzymatic reaction under Mg deficiency in maize.

In this study, higher total GS activities were measured when plants were resupplied with Mg over the leaves after 27 days of Mg starvation (Fig. 4a, b). The quantification of total GS activity was based on total protein extracts but not pure GS enzyme protein. Therefore, differences in the total GS activity may be caused by variations in the relative amounts of the GS or variations in the enzymatic efficiency. The latter is determined by intrinsic attributes of the enzyme like its affinity to the substrates. Hence, the GS abundance was determined by dot blot analysis (Fig. 4). We have chosen an antibody that was raised against both the cytosolic and the chloroplastic enzyme forms GS1 and GS2 because it allowed quantifying its composite abundance in one identical blotting cycle. Thus, the antibody signal reflects the total abundance of both isoforms as affected by varying Mg supply. Immunoblotting revealed an increased GS abundance in leaf number six of plants with exogenous Mg resupply to the leaves. This prompts the conclusion that the reported increased GS activity, as measured in vitro by the production of  $\gamma$ -glutamyl hydroxamate per µg of protein per hour, relies on increased GS abundance. Nevertheless, changes in the enzyme catalytic efficiency induced by the treatment cannot completely be ruled out. Because both the GS activity as well as the abundance are subject to multilevel regulation including various C and N metabolites and since the mechanisms involved in sensing their levels and linking them to transcriptional regulation of GS are so far unknown (Thomsen et al. 2014), reasons for the increase in GS activity and abundance upon Mg leaf-application can only be discussed and further research in this area is anticipated. The increased protein concentrations in leaf number six (Fig. 5a) indicate enhanced de novo synthesis of proteins upon Mg resupply to Mg-deficient plants. Thus, the metabolic processes of nitrogen remobilization during Mg deficiency might have been replaced by nitrogen assimilation. Hirel et al. (2005a) found that GS activity in maize plants is high during nitrogen assimilatory phases. This would conceivably explain the increase in total GS activity and abundance upon Mg resupply to the leaves (Figs. 3a, b, 4).

The objective of this study was to investigate whether a pronounced Mg-deficiency impairs the ammonium assimilation in maize leaves by reducing the total GS activity. Based on the results of the GS activity in vitro assays, GS abundance and amino acid measurements, this assumption can be neglected. Since these findings are in contrast to observations in rice and spinach (Ding et al. 2006; Yin et al. 2009), it is necessary to generate more detailed knowledge on the molecular nature of the changes.

# Protein concentration in maize leaves is not related to Mg status

Magnesium is known to play an important role during protein biosynthesis. The largest pool of Mg is believed to be devoted to protein formation by bridging ribosome subunits (Mengel and Kirkby 2001; Waters 2011). Furthermore, Mg binds the transfer-RNA-amino acid complex to the ribosomes (Chatterjee et al. 1994). It has been postulated that a primary effect of Mg deficiency is a disturbed polypeptide synthesis thereby restricting protein production and growth (Mengel and Kirkby 2001). However, the protein concentrations in leaf number seven and eight were not decreased in Mg-deficient plants (Fig. 5b) although the Mg concentrations were significantly lower than in the positive control (Fig. 2). Hence, our results indicate no impairment of protein biosynthesis even though the Mg concentration in the leaves was markedly decreased under Mg deficiency. Therefore, it seems likely that the significantly lower-protein concentration in leaf number six originated rather from protein breakdown in consequence of Mg-deficiency-induced senescence than from disturbed protein synthesis. This assumption is strengthened by the immensely increased concentrations of asparagine (Asn) and glutamine (Gln) in this leaf, since both amino acids, by virtue of their high C:N ratio, are the major nitrogen transport forms for nitrogen retranslocation (Ireland and Lea 1999; Lea et al. 2007; Bernard and Habash 2009) into sink organs and are accumulated in consequence of senescence-induced protein breakdown (Balazadeh et al. 2014).

The results of our study show that an undersupply with Mg does not obligatorily lead to disturbed protein biosynthesis and thus cannot be the main reason for the observed growth reduction under Mg deficiency in maize (Table 1). Magnesium resupply via the roots or the leaves after 27 days of Mg starvation increased the protein concentration in leaf number six (Fig. 5a). Therefore, the Mg restoration by exogenous Mg resupply may have contributed to stopping the process of senescence-induced protein breakdown and/or increased protein biosynthesis.

# Magnesium deficiency increases free amino acid concentrations in older leaves

The concentration of free amino acids was considerably increased in the leaves number six and seven of Mg-deficient plants (Table 2). Higher concentrations of free amino acids, especially Gln and Asn, are a well-established indicator for nutrient deficiency-induced senescence and an associated protein breakdown (Martin et al. 2006; Lea et al. 2007; Bernard and Habash 2009). Usually, these free amino acids are translocated into sink organs



like developing leaves and roots via the phloem and xylem (Okumoto and Pilot 2011) in order to recycle the containing nitrogen. Interestingly, Cakmak et al. (1994) found decreased phloem loading caused by Mg deficiency in bean plants. The authors attributed this to the absolute requirement for Mg of the plasma membrane H<sup>+</sup>-ATPase (Balke and Hodges 1975; Hanstein et al. 2011). This enzyme generates the proton motive force across the plasma membrane that is necessary for phloem loading. Therefore, photosynthates like sucrose are accumulated in source leaves under Mg deficiency. These findings have been confirmed for sugar beet and spinach (Fischer et al. 1998; Hermans et al. 2005). Since the transport of amino acids from source to sink organs is also driven by the proton motive force generated by the H<sup>+</sup>-ATPase in dependency on available Mg-ATP (Lalonde et al. 2003), it seems likely that the increased concentrations of free amino acids in the leaves number six and seven resulted from Mg-deficiency-induced impairment of phloem loading. This assumption is in good agreement with the finding that Mg restoration by Mg addition to the NS or by leaf-application proved to successfully reverse this impairment leading to decreased concentrations of free amino acids (Table 2).

### Conclusion

The novelty of this study is that glutamine synthetase activity in vitro assays in combination with immune-dot blot analysis revealed that the total activity and abundance of glutamine synthetase was not impaired in the leaves of maize plants upon 54 days of severe Mg starvation. Therefore, the GS-mediated primary or secondary assimilation of free NH<sub>4</sub><sup>+</sup> is not a limiting enzymatic reaction under Mg deficiency in maize leaves and thus cannot be accountable for the observed restriction of plant growth and productivity in Mg-deficient maize. Notably, it was shown that Mg deficiency does not necessarily decreases total protein concentrations in the leaves as usually assumed. Moreover, Mg resupply to the roots or the leaves of Mg-deficient plants successfully reversed the Mg-deficiency-induced accumulation of free amino acids in older leaves, being indicative for impaired phloem loading.

Author contribution statement MJ and CMG conceived and designed the experiments. MJ conducted the experiment and analyzed the data. KHM contributed analytical tools and reagents. MJ and CMG wrote the manuscript. All authors read and approved the manuscript.

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3. Magnesium leaf-application in maize

# **Chapter 3**

# Photosynthetic capacity, nutrient status, and growth of maize (*Zea mays* L.) upon MgSO<sub>4</sub> leaf-application

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# Photosynthetic capacity, nutrient status, and growth of maize (*Zea mays* L.) upon MgSO<sub>4</sub> leaf-application

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The major plant nutrient magnesium (Mg) is involved in numerous physiological processes and its deficiency can severely reduce the yield and quality of crops. Since Mg availability in soil and uptake into the plant is often limited by unfavorable soil or climatic conditions, application of Mg onto leaves, the site with highest physiological Mg demand, might be a reasonable alternative fertilization strategy. This study aimed to investigate, if MgSO4 leaf-application in practically relevant amounts can efficiently alleviate the effects of Mg starvation in maize, namely reduced photosynthesis capacity, disturbed ion homeostasis and growth depression. Results clearly demonstrated that Mg deficiency could be mitigated by MgSO<sub>4</sub> leaf-application as efficiently as by resupply of MgSO<sub>4</sub> via the roots in vegetative maize plants. Significant increases in SPAD values and net rate of CO<sub>2</sub>-assimilation as well as enhanced shoot biomass have been achieved. Ion analysis furthermore revealed an improvement of the nutrient status of Mg-deficient plants with regard to [Mg], [K], and [Mn] in distinct organs, thereby reducing the risk of Mn-toxicity at the rootside, which often occurs together with Mg deficiency on acid soils. In conclusion, foliar fertilization with Mg proved to be an efficient strategy to adequately supply maize plants with Mg and might hence be of practical relevance to correct nutrient deficiencies during the growing season.

Keywords: foliar application, magnesium deficiency, photosynthesis, chlorophyll, cation interaction, Zea mays L.

### **INTRODUCTION**

The macronutrient magnesium (Mg) is involved in numerous physiological processes during plant growth and development, extending far beyond its well-known function as central atom of chlorophyll. Mg activates more than 300 enzymes, e.g., ribulose-1,5-bisphosphate-carboxylase/oxygenase (RubisCO), glutamine synthetase or glutathione synthase, and therefore participates in the assimilation of carbon, nitrogen, and sulfur, respectively (Marschner, 2012). Furthermore, Mg-binding to ATP is essential for the plasma membrane H<sup>+</sup>-ATPases activity, having Mg-ATP as substrate (Balke and Hodges, 1975). Consequently, phloem loading and partitioning of photoassimilates from source to sink organs depends on Mg availability (Cakmak et al., 1994a,b). Protein biosynthesis cannot take place without Mg, since it connects the subunits of ribosomes (Maathuis, 2009).

Being aware of its impact on plant metabolism, it seems astonishing that Mg has received little attention in agricultural research in the last decades although its deficiency can cause severe reductions in yield and quality of crops (Gransee and Führs, 2013; Neuhaus et al., 2014). Genetic responses to Mg starvation and restoration have been analyzed in the model species *Arabidopsis thaliana* (Hermans et al., 2010a,b), but Cakmak and Yazici (2010) called Mg 'a forgotten element in crop production.' The authors consider the deficiency of this nutrient to be a growing problem and limiting factor especially in intensive production systems, consequently also striking human and animal health (Cakmak, 2013).

Magnesium deficiency in plants may occur on soils even under high Mg content of parent soil material. Owing to its unique chemical properties, it is only weakly bound to negatively charged soil particles and is therefore easily replaced by other cations and consequently leached (Maathuis, 2009; Gransee and Führs, 2013). Hence, calcareous soils, acidic soil with excess of protons (H<sup>+</sup>), aluminium (Al) and manganese (Mn), and saline soils with high amounts of sodium (Na) are all prone to Mg deficiency (Mengel and Kirkby, 2001; Gransee and Führs, 2013). Also, elevated rates of NPK-fertilization in high-production systems harbor the risk of Mg deficiency, because strong reciprocal interactions regarding uptake exist among potassium (K) and Mg (Guiet-Bara et al., 2007; Cai et al., 2012). Mg is delivered to the rhizosphere mainly by mass flow (Lynch and St. Clair, 2004) and thus dry soil and low transpiration rates impair Mg uptake additionally. Taking together all these factors that limit Mg availability in soils, an application of Mg directly on leaves where the physiologically Mg demand is highest might be more advantageous than a Mg fertilization of soils with restricted potential of Mg uptake by plant roots. Moreover, such a foliar application allows a precise timing of Mg supply temporally and spatially to satisfy the varying demands of distinct plant organs at different growth stages. Positive effects of Mg foliar fertilization on plant growth have already been described for some crops for example soybean and faba bean (Vrataric et al., 2006; Neuhaus et al., 2013, 2014) but plant responses to foliar fertilizers differ immensely among plant species due to diverse leaf surface properties such as trichomes, cuticula chemistry and cell wall composition (Fernández et al., 2013) and can thus not be generalized. Although it is the most extensively cultivated cereal crop worldwide (Pechanova et al., 2013), the response of maize (*Zea mays* L.) to Mg fertilization is less recognized than for other cereals (Grzebisz, 2013) especially regarding foliar fertilization. To cover the growing worldwide demands, ranging from feed for humans and animals to biofuel production and energy generation, maize cultivation has expanded immensely in the last decade also on marginal soils, which harbor the risk of Mg deficiency (Oikeh et al., 2003).

The aim of this study was to examine whether leaf-application of  $MgSO_4$  in practically relevant amounts is as efficient as resupply of Mg via the roots in alleviating adverse effects of Mg deficiency in maize, namely low photosynthesis rate, disturbed ionic homeostasis and biomass reduction. A hydroponic cultivation experiment with four distinct experimental groups was conducted in the greenhouse and photosynthesis rate, SPAD values, biomass as well as [Mg], [K], and [Mn] in different organs were determined.

### **MATERIALS AND METHODS**

### PLANT GROWTH CONDITIONS

Maize (*Zea mays* L. cv. Susann, Nordsaat Saatzucht, Langenstein, Germany) plants were grown hydroponically in the greenhouse at 20–25°C and 30–50% relative humidity from June–July under natural light regime. Seeds were soaked in 2 mM aerated CaSO<sub>4</sub>

solution for 2 days and germinated in sterile quartz sand, which was kept moistened with 2 mM CaSO<sub>4</sub>. Seven-days-old seedlings were transferred into 9 L plastic vessels (four plants per vessel) with 25% strength nutrient solution (NS), which was raised stepwise to 100% strength within 4 days. The full-strength NS had the following composition: 1.3 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.7 mM NH<sub>4</sub>NO<sub>3</sub>, 2.0 mM CaCl<sub>2</sub>, 1.0 mM K<sub>2</sub>SO<sub>4</sub>, 0.2 mM KH<sub>2</sub>PO<sub>4</sub>, 200 µM Fe-EDTA, 5 μM H<sub>3</sub>BO<sub>3</sub>, 2 μM MnSO<sub>4</sub>, 0.5 μM ZnSO<sub>4</sub>, 0.3 μM CuSO<sub>4</sub>, 0.01 µM (NH<sub>4</sub>)<sub>2</sub>Mo<sub>7</sub>O<sub>24</sub>, and it was exchanged weekly. Four different experimental groups with distinct Mg treatments were cultivated in four independent biological replicates, including positive control with sufficient Mg and negative control with severe Mg deficiency (Figure 1). Vessels were arranged fully randomized. Plants of the positive control group were supplied with 0.5 mM MgSO<sub>4</sub> in the NS, whereas the other three groups grew under 0.02 mM MgSO<sub>4</sub> for 9 days to ensure proper seedling development. In order to induce clear Mg deficiency symptoms, [Mg] was further reduced to 0.01 mM MgSO<sub>4</sub> thereafter. After a growing period of 5 weeks, the NS [Mg] of one of the three deficiency groups was raised to 0.5 mM MgSO<sub>4</sub>. Another deficiency group was fertilized with Mg over the leaves while still growing in 0.01 mM MgSO<sub>4</sub> NS. This leaf-application treatment was conducted four times within 10 days by brushing 200 mM MgSO<sub>4</sub> on the whole plants, namely

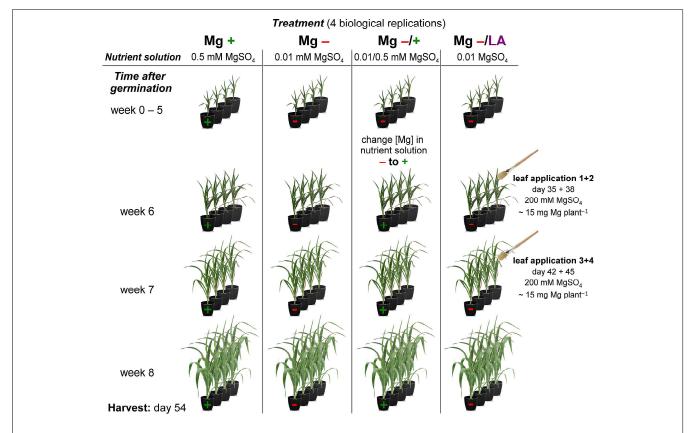


FIGURE 1 | Schematic view of experimental setup. Diagram of the hydroponic grown maize plants with four distinct Mg treatments. Mg+, 0.5 mM MgSO $_4$  in nutrient solution (NS); Mg-, 0.01 mM MgSO $_4$  in NS; Mg-/+, 0.01 mM MgSO $_4$  in NS for 5 weeks and 0.5 mM MgSO $_4$  in NS for 3 weeks; Mg-/LA, 0.01 mM MgSO $_4$  in

NS and leaf-application of 200 mM  $MgSO_4$  for four times. For each treatment group four pots with four maize plants each were installed (only one plant per pot displayed in the simplified diagram). For further details see descriptions in the text (Materials and Methods).

abaxial and adaxial leaf sides as well as the stems. By this special means the amount of Mg applied which directly reached the plants surface could exactly be quantified. Each plant received 60 mg Mg in total, which would correspond to an application rate of approximately 6 kg ha<sup>-1</sup> under field conditions. Leaf-application solution contained 0.1% Silwet as wetting agent and treatments took place in the morning to ensure opened stomata and to avoid leaf burnings by high irradiation. Plants were harvested after 54 days of growth.

### **SPAD VALUES AND GAS EXCHANGE MEASUREMENTS**

Changes in chlorophyll concentration of the 6th leaf were measured non-destructively using a portable chlorophyll meter (SPAD-502, Minolta, Japan) starting at day 26 after germination until harvest. In addition, the ninth leaf was analyzed at harvest. Measurements were performed on two plants per vessel of each of the four biological replicates, four readings per leaf were taken and values were averaged.

Rate of photosynthetic activity was measured regularly starting with the onset of leaf-application on day 35 of the experiment by a portable gas exchange system (LI-6400, LI-COR Biosciences, Lincoln, NE, USA). Measurements were conducted between 10 am and 14 pm on a central 6 cm² leaf segment next to the midrib of the sixth leaf. Measurements were taken at two plants of two biological replicates per treatment and values were averaged. Incoming photosynthetic photon flux density provided by a red/blue LED light source (6400-02B, LI-COR Biosciences, Lincoln, NE, USA) amounted to 600  $\mu$ mol mol $^{-2}$  s $^{-1}$  and ambient CO $_2$  concentration was adjusted to 400  $\mu$ mol mol $^{-1}$  by CO $_2$  injection (6400-01, LI-COR Biosciences, Lincoln, NE, USA) as described in detail by Geilfus et al. (2011).

### **ANALYSIS OF PLANT TISSUE**

Fresh weight (FW) of total shoot and several leaves (sixth to eight and youngest leaf) were determined by pooling all four plants per vessel and dividing the values by four.

For mineral analysis, plant tissue was dried at 60°C, ground and 100 mg were digested with 10 mL of 69% HNO3 (ROTIPU-RAN Supra for ICP, 69%) in a 1800 W microwave oven (MARS 6 Xpress; CEM, Matthews, MC, USA) with the following program: 2 min at 100°C, 1 min at 120°C, 20 min at 180°C, 20 min cooling time. Samples were diluted with distilled sterile water (18.2 M $\Omega$ cm conductivity) to 100 mL afterward and kept at 4°C until analysis. Concentrations of macro- and micronutrients were quantified using inductively coupled plasma-mass spectrometry (ICP-MS; Agilent Technologies 7700 Series, Böblingen, Germany). The analytical technique was standardized using NCS DC 73350, a certified reference material (leaves of poplar) from the China National Analysis Center for Ion and Steel (Beijing, China). Blank digestions were performed in the same way as for the samples. Every 20 samples, internal macro- and micronutrients standards, as well as reference material samples were analyzed as internal control.

### **STATISTICS**

Data was statistically analyzed using R statistical package software version 3.0.1 (The R foundation for statistical computing, Vienna, Austria, 2013). Effects of treatments were tested using

Student's *t*-test in case of two experimental groups or with one-way ANOVA and following Bonferroni correction in case of four different groups.

### **RESULTS**

### **SPAD MEASUREMENTS**

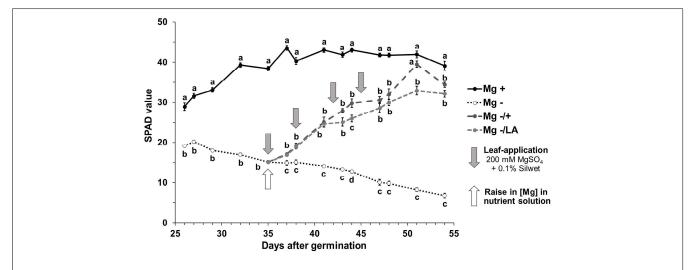
The comparison between the Mg sufficient positive control plants (0.5 mM MgSO<sub>4</sub> in NS) and the Mg deficient negative control plants (0.01 mM MgSO<sub>4</sub> in NS) revealed that Mg deficiency significantly influenced the chlorophyll concentrations in maize, as assessed by SPAD values (Figure 2). While SPAD values raised in leaves of well-nourished positive control plants until day 42 after germination and remained constant henceforth (black line), those of Mg deficient plants decreased until harvest (dotted line). With the onset of Mg resupply to deficient plants on day 35 after germination, SPAD values increased immediately in plants with root- and leaf-resupply to the same degree (light- and dark-gray dashed lines). Both treatments resulted in significantly elevated SPAD values compared to negative control plants after 2 days. At the end of the experiment, SPAD values in the sixth leaf of plants with Mg resupply did not reach the level of the positive control plants, regardless of whether Mg was given to the roots or the leaves. However, SPAD values in the ninth leaf were equal to the well-nourished positive control in both leaf-resupply and root-resupply treatment at harvest time (Table 1).

### PHOTOSYNTHESIS RATE AND TRANSPIRATION

Gas exchange measurements revealed significant differences in net CO<sub>2</sub>-assimilation and transpiration rates of the 6th leaf between Mg deficient negative control and well-nourished positive control plants 5 weeks after germination (Figures 3A,B). The 6th leaf of well-nourished control plants had stable assimilation rates of about 21 µmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> during the course of the experiment (black line) whereas a continuous decrease was observed under Mg deficiency (dotted line) from 6.8 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> at day 37 to 2.0 CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> at day 51 (dotted line). Transpiration rates of the 6th leaf of Mg deficient plants were significantly reduced compared to well-nourished plants throughout the whole measuring period. Resupply of Mg to the NS (dark-gray dashed line) or via leaf-application (light-gray dashed line) significantly raised both net CO<sub>2</sub>-fixation and transpiration rate within 2 days in comparison to the negative control. Assimilation rates continued to increase until they reached the level of positive control plants 6 days after Mg resupply via the roots and after 9 days, if MgSO<sub>4</sub> was applied onto leaves, respectively. The latter had been treated with 200 mM MgSO<sub>4</sub> solution for three times by then, which amounts to 45 mg Mg per plant. Transpiration rates equaled those of positive control plants after 6 days in both treated groups.

### **PLANT GROWTH**

Shoot FW of negative control plants was significantly reduced and amounted to 30.5 g shoot<sup>-1</sup> which accounted for 11.0% of adequately nourished positive control plants (**Figure 4A**). This reduction in FW of negative controls was also apparent in leaves number six, seven, eight, and in the youngest leaves (**Figure 4B**). Mg supply after 5 weeks of deficiency significantly raised shoot FW



**FIGURE 2 | Course of SPAD values of the sixth leaf over time.** Relative chlorophyll concentration in the sixth leaf assessed by SPAD measurements. Mg+, 0.5 mM MgSO $_4$  in NS; Mg-, 0.01 mM MgSO $_4$  in NS; Mg-/+, 0.01 mM MgSO $_4$  in NS for 5 weeks and 0.5 mM MgSO $_4$  in NS for 3 weeks; Mg-/LA, 0.01 mM MgSO $_4$  in NS and leaf-application of 200 mM MgSO $_4$  for four

times. Measurements were made on two plants of each of the four biological replicates per treatment; four individual SPAD values per leaf were averaged. Mean  $\pm$  SE; arrows show date of leaf-application with 200 mM MgSO4; small letters indicate significant differences between treatments for an observation date (ANOVA with Bonferroni adjustment,  $p \leq$  0.05).

Table 1 | SPAD values in the ninth leaf at day 54 after germination.

Treatment	SPAD	SE
(Positive control)	37.8ª	1.0
(Negative control)	16.0 <sup>b</sup>	0.5
(Root-resupply)	37.2ª	0.8
(Leaf-application)	35.7ª	1.5
	(Positive control) (Negative control) (Root-resupply)	(Positive control) 37.8a (Negative control) 16.0b (Root-resupply) 37.2a

Mg+,  $0.5\,mM\,MgSO_4$  in nutrient solution (NS); Mg-,  $0.01\,mM\,MgSO_4$  in NS; Mg-/+,  $0.01\,mM\,MgSO_4$  in NS for 5 weeks and  $0.5\,mM\,MgSO_4$  in NS for 3 weeks; Mg-/LA,  $0.01\,mM\,MgSO_4$  in NS and leaf-application of 200 mM  $MgSO_4$  for four times. Measurements were made on two plants of each of the four biological replicates per treatment; four individual SPAD values per leaf were averaged. Mean  $\pm$  SE; small letters indicate significant differences between treatments (ANOVA with Bonferroni adjustment,  $p \leq 0.05$ ).

to the same extent regardless of whether it was given to the NS or applied onto leaves (**Figure 4A**). Mean FW were 125.3 g shoot<sup>-1</sup> for root-resupply and 103.4 g shoot<sup>-1</sup> in plants with MgSO<sub>4</sub> leafapplication. Resupply of Mg increased FW of the eighth leaf to the largest extent (**Figure 4B**). In comparison to Mg-deficient plants, no increase in FW was observed in leaf number six upon Mg resupply via leaves or the NS. Mg addition to the NS raised the FW of leaf number seven and the youngest leaf compared to the negative control plants. Upon MgSO<sub>4</sub> leaf-application FW of these leaves were not significantly different from neither the negative control nor the plants with Mg-resupply via the roots.

### Mg, K AND Mn CONCENTRATIONS IN DISTINCT PLANT ORGANS

Positive control plants cultivated in NS with 0.5 mM MgSO<sub>4</sub> showed [Mg] between 1.5 g Mg kg<sup>-1</sup> dry weight (DW) in youngest and 2.7 g Mg kg<sup>-1</sup> DW in oldest investigated leaves; root tissue contained 2.7 g Mg kg<sup>-1</sup> DW (**Figure 5A**). Severe Mg starvation affected [Mg] significantly in the sixth leaf, in the youngest leaves

and in root tissue. Addition of 0.5 mM MgSO<sub>4</sub> to the NS after 5 weeks of Mg deficiency significantly raised [Mg] in all leaves and, predominantly, in the roots, which contained almost 70% more Mg in comparison to positive control plants. Resupply of Mg through leaf application resulted in a prominent increase of [Mg] in basal leaves. Leaves number six to eight contained 3.8 up to 12.6 g Mg kg<sup>-1</sup> DW, increasing with decreasing leaf number. Youngest leaves and roots, in contrast, were not affected and did not have a significantly higher [Mg] than Mg deficient negative control plants.

The dose and method of Mg nutrition also influenced [K] in the plant tissues (**Figure 5B**). Growth in NS with 0.01 mM MgSO<sub>4</sub> elevated [K] significantly in leaves number six to eight, but not in youngest leaves and roots. With 46.9 to 65.0 g K kg<sup>-1</sup> DW, [K] in basal leaves of negative control plants were up to 1.8 times as high as in positive control plants, which contained 32.3 to 38.7 g K kg<sup>-1</sup> DW. Re-nutrition with MgSO<sub>4</sub> via roots significantly reduced [K] in whole shoots and roots to levels of control plants, whereas [K] in the eighth leaf and roots remained elevated, if MgSO<sub>4</sub> leaf-application was performed.

Magnesium deficient negative control plants showed high [Mn] not only in leaves, but predominantly in roots. With 162.8 mg Mn kg<sup>-1</sup> DW they contained 13.4 times more Mn than positive control plants (12.1 mg Mn kg<sup>-1</sup> DW; **Figure 5C**). Increasing the MgSO<sub>4</sub> concentration in the NS led to a significant decrease of [Mn] in all examined plant parts to levels of the positive control group. Leaf-application of MgSO<sub>4</sub>, by contrast, only reduced [Mn] significantly in youngest leaves and roots, whereas they remained as high as in negative control plants in the older leaves.

### **DISCUSSION**

Magnesium deficiency is considered to be a growing problem throughout agriculture and it occurs on diverse soil types (Mengel and Kirkby, 2001). Since Mg availability in soil and uptake into

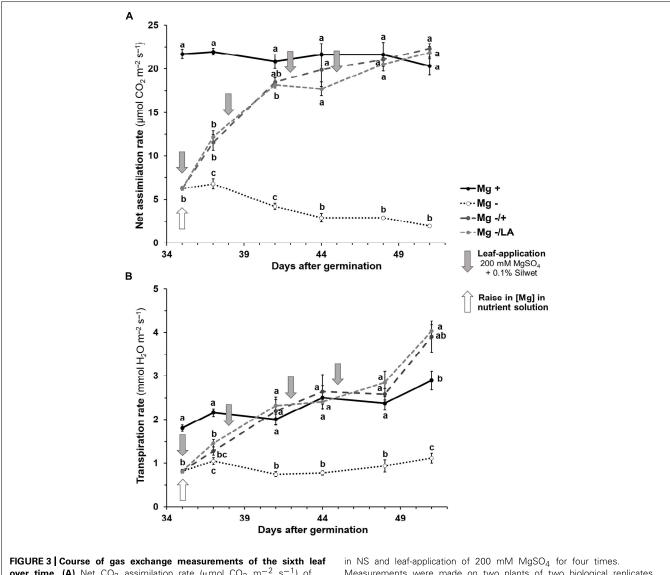


FIGURE 3 | Course of gas exchange measurements of the sixth leaf over time. (A) Net  $CO_2$  assimilation rate ( $\mu$ mol  $CO_2$   $m^{-2}$   $s^{-1}$ ) of the sixth leaf. (B) Transpiration rate (mmol  $H_2O$   $m^{-2}$   $s^{-1}$ ) of the sixth leaf. Mg+, 0.5 mM MgSO<sub>4</sub> in nutrient solution (NS); Mg-, 0.01 mM MgSO<sub>4</sub> in NS; Mg-/+, 0.01 mM MgSO<sub>4</sub> in NS for 5 weeks and 0.5 mM MgSO<sub>4</sub> in NS for 3 weeks; Mg-/LA, 0.01 mM MgSO<sub>4</sub>

in NS and leaf-application of 200 mM MgSO $_4$  for four times. Measurements were made on two plants of two biological replicates per treatment. Mean  $\pm$  SE; arrows show date of leaf-application with 200 mM MgSO $_4$ ; small letters indicate significant differences between treatments for an observation date (ANOVA with Bonferroni adjustment,  $p \leq 0.05$ ).

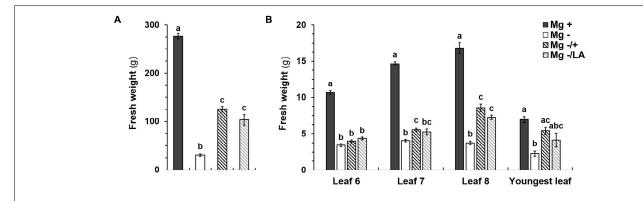
the plant often is limited foliar fertilization instead of Mg addition to the soil seems reasonable for meeting the crop's demands. Therefore, it was questioned, if foliarly applied Mg could alleviate Mg deficiency syndromes in vegetative maize plants as fast and efficient as a resupply via the roots. The amount of foliarly applied Mg was exactly quantified and amounted to 60 mg Mg per plant in total, which corresponds to a practically relevant application rate of approximately 6 kg Mg ha<sup>-1</sup>. Several physiological reactions of maize plants under severe Mg starvation and subsequent resupply of Mg via leaf- or root-fertilization were investigated by hydroponic cultivation.

Negative control plants grown in 0.01 mM MgSO<sub>4</sub> showed strong reductions in photosynthesis and biomass, which is in accordance with several studies on other crops (Gerendás and

Führs, 2013; Neuhaus et al., 2014). The negative effects on such yield-relevant physiological processes once again illustrate the importance of Mg as a plant macronutrient and underline the need for developing adequate Mg application strategies to ensure high plant productivity.

# LEAF-APPLICATION OF MgSO4 RAPIDLY INCREASES [Mg] IN Mg DEFICIENT MAIZE

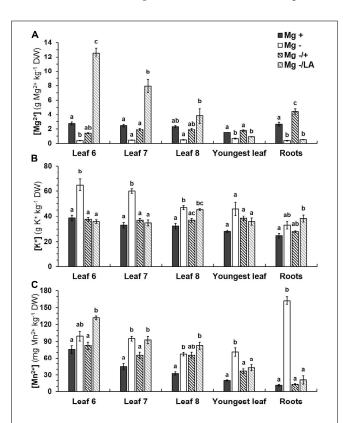
Bergmann (1983) states [Mg] in fully expanded maize leaves to range from 2.5 to 6.0 g kg<sup>-1</sup> DW. According to that threshold, positive control plants were sufficiently supplied with Mg whereas Mg deficient negative controls were growing under severe malnutrition (**Figure 6A**). Increasing [Mg] in the NS after 5 weeks of Mg restriction led to elevated [Mg] especially in newly developed



**FIGURE 4 | Fresh weights (FWs) of shoots and single leaves. (A)** FW (g) of shoots and of **(B)** leaves number six, seven, eight, and youngest leaves at harvest (54 days after germination). Mg+, 0.5 mM MgSO<sub>4</sub> in NS; Mg-, 0.01 mM MgSO<sub>4</sub> in NS; Mg-/+, 0.01 mM MgSO<sub>4</sub> in NS for 5 weeks and

0.5 mM MgSO $_4$  in NS for 3 weeks; Mg–/LA, 0.01 mM MgSO $_4$  in NS and leaf-application of 200 mM MgSO $_4$  for four times; mean (n=4 biological replicates)  $\pm$  SE; small letters indicate significant differences between treatments (ANOVA with Bonferroni adjustment,  $p \leq 0.05$ ).

leaves and to minor extent in those, which had already been built under deficiency conditions (**Figure 5A**). Furthermore, [Mg] in roots markedly increased, which might indicate elevated uptake after a time of nutrient deprivation. This would be in agreement

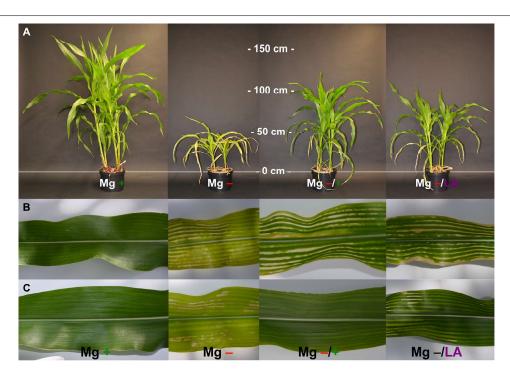


**FIGURE 5** | Ion concentrations in various leaves and roots. (A) [Mg], (B) [K], and (C) [Mn] were analyzed in different tissues at harvest (54 days after germination). Mg+, 0.5 mM MgSO<sub>4</sub> in NS; Mg–, 0.01 mM MgSO<sub>4</sub> in NS; Mg–/+, 0.01 mM MgSO<sub>4</sub> in NS for 5 weeks and 0.5 mM MgSO<sub>4</sub> in NS for 3 weeks; Mg–/LA, 0.01 mM MgSO<sub>4</sub> in NS and leaf-application of 200 mM MgSO<sub>4</sub> for four times; mean (n=4 biological replicates  $\pm$  SE; small letters indicate significant differences between treatments (ANOVA with Bonferroni adjustment, p<0.05).

with results from Cai et al. (2012), who found a putative Mg transporter gene to be up-regulated under Mg deficiency in rice. Nevertheless, our data do not reveal Mg uptake into the symplast of root cells since cation concentrations of the whole tissue has been analyzed. Leaf-application of 200 mM MgSO<sub>4</sub> remarkably increased [Mg] in leaves that had directly been treated by foliar application. Declining [Mg] in the upper leaves are attributable to the number of foliar application treatments. The sixth leaf was treated four times with MgSO<sub>4</sub> solution whereas leaf number eight only received one application. It is surprising that MgSO<sub>4</sub> leaf-application did not affect [Mg] in roots and youngest leaves, which did not receive MgSO<sub>4</sub> application, since Mg is known to be a highly phloem-mobile cation, which can be translocated within the plant to sites of high nutrient demand (Steucek and Koontz, 1970; White and Broadley, 2009). However, the Mg demand of roots is relatively low, given its primary role in chloroplasts (Maathuis, 2009) and [Mg] in youngest leaves obviously were high enough to significantly increase chlorophyll concentrations in youngest leaves (Table 1), as SPAD values were not different from well-nourished control plants at harvest.

# MAGNESIUM RESUPPLY VIA LEAVES RAPIDLY ENHANCES PHOTOSYNTHETIC PARAMETERS IN Mg DEFICIENT MAIZE

Chlorophyll concentrations estimated by SPAD measurements were markedly reduced under Mg deficiency (**Figure 2**, dotted line) and leaves showed deficiency symptoms being typical in cereals like interveinal yellowing, chlorosis, and necrosis (Figures 6B,C). Mg resupply elevated SPAD values significantly already within 2 days and application of 200 mM MgSO<sub>4</sub> to maize leaves turned out to be as efficient in raising SPAD values as addition of 0.5 mM MgSO<sub>4</sub> to the roots (Figure 2, dark-gray and light-gray dashed lines, respectively). Even one single leaf application treatment and hence 15 mg Mg per plant was sufficient for triggering significant effects. The efficient uptake of MgSO<sub>4</sub> solution into maize leaves can be ascribed to their amphistomatical anatomy and their wettability at both leaf sides (Urrego-Pereira et al., 2013). Raise in chlorophyll concentration by Mg foliar-application has also been shown for faba bean, oregano, and soybean before (Dordas, 2009; Teklic et al., 2009; Neuhaus et al., 2013). Mg not only serves as central atom of



**FIGURE 6 | Visual symptoms of Mg deficiency and effects of Mg resupply. (A)** Representative plants at harvest time (54 days after germination); **(B,C)** representative leaves of the sixth and seventh leaf respectively at day 44 after germination; plants with MgSO<sub>4</sub> leaf-application

had received three of four application-treatments by then. Mg+, 0.5 mM MgSO $_4$  in NS; Mg-, 0.01 mM MgSO $_4$  in NS; Mg-/+, 0.01 mM MgSO $_4$  in NS for 5 weeks and 0.5 mM MgSO $_4$  in NS for 3 weeks; Mg-/LA, 0.01 mM MgSO $_4$  in NS and leaf-application of 200 mM MgSO $_4$  for four times.

chlorophyll molecules but is also necessary in chlorophyll biosynthesis, activating Mg-chelatase, a complex enzyme which catalyzes the insertion of Mg into a chlorophyll precursor molecule (Reid and Hunter, 2004; Masuda, 2008). The high biological relevance of Mg for chlorophyll production hence explains the pronounced re-greening of all leaves already within a few days after Mg resupply (**Figures 6B,C**). Not only leaves that were directly treated with MgSO<sub>4</sub> showed higher SPAD values compared to Mg-deficient control plants, but the effects of the leaf-application also became visible in the youngest, untreated leaves, where SPAD values were not significantly different from control plants or plants with root resupply at harvest time (**Table 1**). This suggests the uptake of the applied Mg into the cytosol and is furthermore indicative for the systemic effect of MgSO<sub>4</sub> leaf-application in distant, untreated plant parts.

Increasing chlorophyll level assessed by SPAD measurements were certainly one reason why CO<sub>2</sub>-assimilation in the re-supplied treatments equaled to the level of the positive control plants after Mg had been restored via leaves or roots (**Figure 3A**). Similar to the SPAD values, the net CO<sub>2</sub>-fixation rate responded rapidly to Mg addition. Besides being the central atom of chlorophyll, Mg is furthermore involved in several other processes related to photophosphorylation and CO<sub>2</sub>-fixation (Lin and Nobel, 1971; Verbruggen and Hermans, 2013). For instance, Mg acts in charge balancing during the establishment of the proton motive force and activates several enzymes involved in photosynthesis, including RubisCO (Marschner, 2012). Also the transpiration rate rose upon Mg addition (**Figure 3B**), as was earlier observed in rice

plants under Mg deficiency (Kobayashi et al., 2012). According to the authors this might be caused by a relationship between the ABA signaling network and Mg deficiency. The results show that the adverse effects of Mg deficiency on plant metabolism can be amended by Mg leaf-application as efficiently as by root resupply in maize plants, since no considerable differences between both treatments were detected.

Magnesium deficient maize plants were sufficiently supplied with sulfur through the NS (1 mM sulfur) and showed no symptoms of sulfur deficiency throughout the whole growth period (**Figures 6B,C**). Hence, the described effects of MgSO<sub>4</sub> leafapplication on physiological processes can be attributed to the influence of Mg. Not only the added Mg directly improved photosynthetic processes through its participation in chlorophyll formation and enzyme activation, but a contribution of secondary effects is likely. To these belong for example, the restoration of ionic homeostasis especially regarding [Mn] in leaves because Mn stress was found to negatively impact the abundance of chloroplastic proteins important for CO<sub>2</sub> fixation (Führs et al., 2008; Millaleo et al., 2013).

# MAGNESIUM FOLIAR APPLICATION DECREASES THE RELATIVE UPTAKE OF OTHER NUTRIENTS AT THE ROOTSIDE

Ion analysis revealed that the Mg resupply not only affects [Mg] but also other cations such as K and Mn (**Figures 5B,C**). It has frequently been observed that variations in one nutrient is compensated by changing uptake of other cations instead (Morgan and Jackson, 1976; Mengel and Kirkby, 2001). Cations are in

competition for apoplastic binding sites, e.g., negatively charged pectins, as well as for further uptake for example through non-selective cation channels (NSCC), which can be passed by both monovalent and divalent cations (Shabala and Hariadi, 2005). Hence, reciprocal effects among cations with respect to uptake into and distribution within the plant exist and antagonistic interactions of Mg, Ca, and K have been demonstrated for example in onion (Kleiber et al., 2012). Interestingly, amelioration of Mg deficiency by root- or leaf-application reduced [K] and [Mn] in distinct plant tissues to a varying extent.

# ELEVATED [K] UNDER $M_g$ DEFICIENCY ARE DECREASED BY LEAF-APPLICATION OF $M_g$ IN MAIZE SHOOTS

Potassium concentrations, normally ranging from 30 to 45 g kg<sup>-1</sup> DW in maize leaves (Bergmann, 1983), were found to be unusually high in Mg deficient plants, especially in older leaves (Figure 5B). These results seem plausible since K is a strong competitor for other cations due to its efficient uptake systems (Mengel and Kirkby, 2001) and are in accordance with results from Cai et al. (2012), who measured much higher K concentrations in rice shoots under Mg starvation and a moderate up-regulation of the expression of a high affinity K transporter gene. Such a hyperaccumulation of K with a subsequent growth-reduction has also been shown for plants, which were genetically restricted in their ability to store Mg in the vacuole (Gilliham et al., 2011). Elevated [K] under Mg deficiency might not only be explained by increased relative uptake, but also decreased export out of leaves into physiological sinks, since this process depends on ATPase activity and is known to be disturbed under Mg deficiency (Cakmak et al., 1994a,b).

Potassium concentrations decreased to the level of control plants upon resupply of Mg to the NS both in shoots and roots of maize, illustrating again the competitive interaction between these cations. Decreased K flux through NSCC with increasing [Mg] has also been detected in broad bean (Shabala and Hariadi, 2005). In contrast to Mg resupply via roots, [K] remained high in roots, if MgSO<sub>4</sub> was foliarly applied to Mg deficient plants. This would be expected due to absence of Mg as a competitor in uptake at the rootside and the low [Mg] in the root tissue (**Figure 5A**). Nevertheless foliar treatment successfully lowered [K] in most investigated leaves significantly, not only in expanding tissue but also in mature leaves which have been built under deficient conditions.

# LEAF-APPLICATION OF $\text{MgSO}_4$ reduces high Mn concentrations in Mg deficient maize roots

Manganese is an essential micronutrient for plants and a major rival for Mg respecting uptake and transport due to their similar ionic radius and biochemical properties (Marschner, 2012). It occurs in different oxidation states and therefore plays a prominent role in redox processes, but can easily become phytotoxic if taken up in excess, which appears for example on soil with low pH or high redox potential (Kopittke et al., 2013). Effects of the competitive relationship between Mg and Mn vary between plant species, type of cultivation and nutrient concentrations in the medium. For example, elevated [Mn] in leaves of Mg deficient potato plants and wheat were measured (Cao and Tibbitts, 1992; Chatterjee et al., 1994), whereas the competitive effect of Mg on

the uptake of Mn was low in rapeseed (*Brassica napus*) and hardly existed in lucerne (*Medicago sativa*; Löhnis, 1960).

Maize grown under Mg deficiency showed high [Mn] not only in leaves but especially in roots (Figure 5C). This might partially be caused by the absence of Mg as a competitor and subsequently increased Mn uptake at the rootside. However, a dilution effect caused by impaired root growth under Mg deficiency, which has been observed in bean plants for example (Cakmak et al., 1994b), might have contributed to markedly increased [Mn] in root tissue. In fact, the total amounts of accumulated Mn were the same for Mg deficient and well-supplied control plants (data not shown). Mn is commonly known to be easily transported from roots to above-ground plant organs (Horst, 1983), but excess accumulation of Mn in roots has been observed in cultivars of grape (Mou et al., 2011) and certain maize varieties (Stoyanova et al., 2009). Since Mn is easily transported from roots to the shoot in the xylem (Page and Feller, 2005), strong depression of transpiration under Mg deficiency (Figure 3B) might have further added the high [Mn] in roots. Impaired nutrient transport activities upon Mg deficiency have also been found in rice and have been explained by reduced transpiration (Kobayashi et al., 2012). Mn concentrations in roots of Mg deficient plants had almost reached toxic levels. The normal range of [Mn] is stated to be 40 to 150 mg Mn kg<sup>-1</sup> DW in maize shoots (Bergmann, 1983) and the critical toxicity concentration amounts to approximately 200 mg Mn kg<sup>-1</sup> DW (Marschner, 2012), but clear differences in Mn tolerance exist among maize varieties (Stoyanova et al., 2009). Thus maize grown under severe Mg deficiency might already have experienced Mn toxicity at the rootside, being accountable for the observed shedding of fine roots during the experiment (data not shown). Such a loss of roots and reduced root growth in connection with increased [Mn] in root tissue has also been found in grape (Vitis vinifera) and was explained partially by oxidative stress leading to cellular damage (Mou et al., 2011). The phenomenon of root abscission due to oxidative stress has also been described for plants growing under heavy metal stress (Razinger et al., 2008; Xing et al., 2010).

Magnesium resupply to the NS successfully lowered [Mn] in both leaves and roots to the level of control plants, whereas Mg leaf-application reduced [Mn] only in youngest leaves and roots (Figure 5C). Reduction of [Mn] by increasing Mg supply to the roots has already been demonstrated (Löhnis, 1960; Elamin and Wilcox, 1985; Goss and Carvalho, 1992) but, to our knowledge, it was never shown before that this effect could at least partially be realized by Mg leaf-application. This finding is particularly interesting, since Mg deficiency often occurs on acid soils (Mengel and Kirkby, 2001), where an elevated risk of Mn toxicity exists due to reduction of MnO<sub>2</sub> to Mn, the plant-available form of this micronutrient. Consequently, Mg deficient plants growing in soil with low pH are especially prone to Mn toxicity. Lowering this risk by Mg addition to the soil has already been recommended (Löhnis, 1960) but leaf-application is much more favorable, since Mg is easily leached out in acid soils.

### **CONCLUSION**

Magnesium deficiency severely compromises physiological efficiency of maize plants. Foliar fertilization of maize plants with MgSO<sub>4</sub> turned to be an efficient means of Mg supply under severe Mg deficiency. Leaf-application of Mg proved to be as efficient as Mg resupply via the roots in ameliorating adverse effects of Mg deficiency, namely reduced photosynthetic capacity, high concentrations of other cations especially Mn in different plant tissues and reduced biomass. It might hence be of practical relevance to correct nutrient deficiencies during the growing season especially if Mg availability via root-uptake is limited due to poor soil properties or climatic conditions.

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4. Early-stage diagnosis of nutrient deficiency in maize leaves

# Chapter 4

# Early-Stage Diagnosis of Nutrient Deficiency by Chlorophyll Fluorescence in Maize Leaves

Mareike Jezek, Karl-Hermann Mühling and Christoph-Martin Geilfus

to be submitted

### **Abstract**

Rapid, non-destructive phenotyping for detection and specification of nutrient deficiency is of high interest in both plant research and agronomy. Chlorophyll fluorescence excited at different light wavelengths can provide information about the content of epidermal light-absorbing compounds such as flavonols and anthocyanins. Since anthocyanins accumulate in response to nitrogen (N) deficiency, Chl fluorescence measurements can be used for rapid, non-invasive detection of N deficiency. However, magnesium (Mg) shortage also results in increased anthocyanin synthesis in certain crops. This study was conducted in order to test the suitability of the "Dualex Scientific+TM" device, the latest generation of Chl meters, for early-stage identification of nutrient deficiency in maize seedlings by assessing levels of leaf chlorophyll and epidermal pigments via chlorophyll fluorescence.

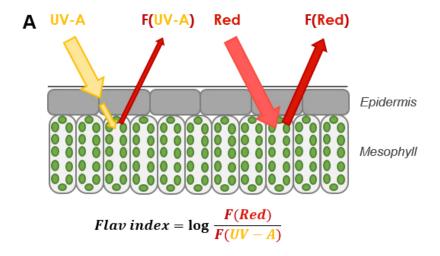
The results reveal that using Chl fluorescence excited at green and red light for measuring epidermal anthocyanins is generally not a suitable approach in N- or Mg-deficient maize leaves due to a methodical flaw, presumably caused by Chl fluorescence reabsorption effects. Decreasing Chl index and concomitant increasing flavonol (Flav) index were found in both nitrogen- (N) and magnesium- (Mg) deficient maize seedlings. However, Flav index and Chl index were not closely correlated in nutrient-deficient maize, indicating reasons for accumulation of epidermal UV-A light-absorbing compounds other than photoprotection. Analysis of soluble sugars in leaves did not provide conclusive information about a causal relationship between photoassimilate accumulation and nutrient deficiency-induced flavonoid synthesis. It was shown that the nitrogen-balance-index (NBI) may not be discriminative enough to identify early stage N deprivation in maize seedlings and may, therefore, lead to misdirected fertilization. However, monitoring the spatiotemporal progress of the Chl and Flav indices across the leaf lamina over time revealed differences between N- and Mg shortage. Low N supply resulted in a pronounced accumulation of epidermal UV-A absorbing compounds in upper leaves, whereas Flav index rise started at the tip of lower leaves in Mg-deprived plants. This knowledge might be useful for future optimisation of early-stage diagnosis of nutrient deficiency in maize seedlings using ChI fluorescence-based detection methods.

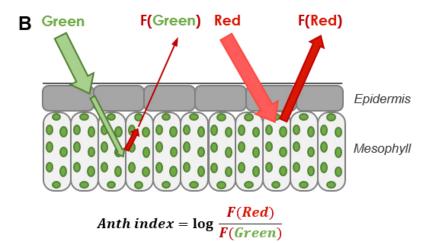
# Introduction

Modern agriculture faces the problem of declining fertility of arable land due to overuse and unsustainable management in the past resulting in soil compaction, erosion, and nutrient depletion. Even marginal nutrient deficiency can impair plant growth and development and consequently diminish crop yield. However, generous fertilization aiming at avoiding nutrient shortage is inefficient and unsustainable, whereas precise, resource-efficient on-demand crop nutrient management is economically profitable and ecologically favourable in the long-term. Best fertilizer management practice involves applying the right nutrient source, at the right rate, right time and right place, in order to unite good crop growth, soil health and decreased pollution of the environment via enhanced nutrient use efficiency (Senbayram et al., 2015). This strategy requires tools to non-destructively monitor the nutritional status of crops throughout the growing period to assist making fertilization decisions. Several non-destructive and rapid methods have been developed to assess crop nitrogen (N) status because of its major impact on yield formation and its high application rate in agriculture. Nitrogen is needed in photosynthesis and protein biosynthesis and therefore, N shortage impairs both crop yield and quality. The first visual N deficiency symptom is leaf chlorosis caused by declining chlorophyll concentrations and a strong linear relationship between leaf N and chlorophyll was found for many crops (Evans, 1989). Furthermore, a low N status increases the synthesis of carbon-based and N-free secondary compounds including flavonoids (Lea et al., 2007). Flavonoids such as flavonols and anthocyanins accumulate mainly in epidermal cells in response to abiotic stress and their ecophysiological significance regarding nutrient deficiency is not consensually resolved. Owing to their light-absorbing properties, flavonoids are supposed to serve as light attenuators in order to protect the photosynthetic e-transport chain in chloroplast from over-reduction under stressful conditions (Agati et al., 2011; Agati et al., 2013). Furthermore, specific flavonoids have antioxidant potential and might scavenge stressinduced reactive oxygen species (Pollastri and Tattini, 2011). The relationship between N availability, flavonoid synthesis and crop growth has also been explained in the 'carbonnutrient balance hypothesis' (Bryant et al., 1983) and the 'growth-differentiation balance hypothesis' (Herms and Mattson, 1992), which regard flavonoids synthesized under N deficiency as carbon storage compounds in times of restricted growth and simultaneously ongoing photosynthetic CO<sub>2</sub>-assimilation.

Both declining chlorophyll and increasing flavonoid concentrations display potential indicators for N deficiency (Cartelat *et al.*, 2005). Hand-held leaf-clip devices have been designed that estimate chlorophyll (Chl) and flavonoid content in leaves non-destructively by measuring Chl fluorescence. These instruments can be used for rapid phenotyping for screening purposes and early diagnosis of nutrient shortages in the field. The 'Dualex Scientific+TM' – the latest generation of Chl meters - simultaneously assesses the Chl content in leaves, and the epidermal contents of flavonols (Flav) and anthocyanins (Anth) on the same leaf spot by measuring near-infrared Chl fluorescence excited with light of different wavelengths. In this device, the Chl fluorescence excited with red light, which is not absorbed by flavonoids, serves as a reference and is compared to Chl fluorescence induced at wavelengths, which are absorbed by epidermal metabolites before reaching the Chl in the mesophyll (Fig. 1).

### 4. Early-stage diagnosis of nutrient deficiency in maize leaves





**Fig. 1:** Schematic drawing of the Dualex measuring principle for assessing epidermal light-absorbing metabolites by means of chlorophyll (Chl) fluorescence (adapted from Bilger *et al.*, 2001). (A) Determination of epidermal UV-A light-absorbing flavonols in leaves with high Chl content by measuring near-infrared (NIR) Chl fluorescence (F) excited with UV-A light and red light, which penetrates freely into the leaf and serves as a reference. The absorptivity of Chl for these wavelengths is similar. The Flav index calculated by the Dualex is defined as the logarithm of the ratio of Chl fluorescence at the reference wavelength and UV-A light. (B) Determination of epidermal green light-absorbing anthocyanins by measuring NIR Chl fluorescence excited with green light and red light as a reference. Due to the lower molar extinction coefficient of Chl for green light, the measuring beam penetrates deeper into the leaf and emitted Chl fluorescence is re-absorbed in upper chloroplast layers, thereby biasing the anthocyanin index calculations. This process is aggravated in leaves with low chlorophyll content. The Anth index calculated by the Dualex is defined as the logarithm of the ratio of Chl fluorescence at the reference wavelength and green light.

In theory, different concentrations of epidermal flavonols or anthocyanins influence near-infrared Chl fluorescence excited with UV-A or green light, respectively, whereas the reference excitation light can freely penetrate the epidermis and hence, this Chl fluorescence is independent of the concentration of epidermal secondary compounds. The logarithm of the ratio of these values is used to calculate Flav and Anth indices (Fig. 1; see also material and methods-section). Additionally, a nitrogen-balance-index (NBI) is computed from the Chl and Flav index, which is supposed to serve as a powerful N deficiency indicator (Cartelat *et al.*, 2005; Force-A, 2015). However, declining leaf Chl concentrations and up-regulation of the flavonoid biosynthesis pathway are not N-specific deficiency responses, but can also occur

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under magnesium (Mg) deficiency (Kobayashi *et al.*, 2013)(Jezek et al. 2015, *unpublished data*). This might lead to misinterpretation of deficiency symptoms and therefore, the suitability of Dualex measurements for making N-fertilization decisions at early stages of crop growth is questionable and might result in misguided nutrient application.

In this study, the suitability of the Dualex device for early-stage discrimination of nutrient deficiency was tested in hydroponically grown maize seedlings. This is not just of agronomic importance for fertilisation management, but also of interest in agricultural and biological research for unravelling the obscure ecophysiological function and adaptive significance of flavonoid biosynthesis in response to nutrient stress.

# **Objectives**

- 1.) Evaluate the discriminative power of the Dualex device for early-stage distinction between nutrient deficiencies with similar visual symptoms in maize seedlings.
- 2.) Visualize the ontogenetic progression of flavonoid synthesis in leaves of N or Mg deficient maize seedlings, respectively.
- 3.) Relate leaf concentration of sucrose and hexoses to epidermal flavonoid synthesis in nutrient-deficient maize seedlings.

### Material and methods

## Plant growth conditions

Maize (*Zea mays* L. cv. Susann, Nordsaat Saatzucht, Langenstein, Germany) plants were grown hydroponically in a climate chamber (16/8 h day/night; 25/18°C; 60% relative humidity; 200 μmol m<sup>-2</sup>s<sup>-1</sup> PAR). Seeds were soaked in 2 mM aerated CaSO<sub>4</sub> solution for one day and germinated in plastic foam sandwiches moistened with 2 mM CaSO<sub>4</sub>. Five-days-old seedlings were transferred into 5 L plastic vessels (four plants per vessel) with 25% strength nutrient solution, which was raised stepwise to 100% strength (Table 1) within three days. The nutrient solution was exchanged weekly in order to avoid nutrient depletion. Three experimental groups with different nutrient supply were cultivated in four independent biological replicates.

**Table 1:** Composition of the nutrient solution of hydroponically grown maize grown under optimal (control), nitrogen-deficient (N def.; 4% N of control) or magnesium-deficient (Mg def.; 4% Mg of control) conditions.

	Control	Mg def.	N def.
Nutrient salt	mmol L <sup>-1</sup>	mmol L <sup>-1</sup>	mmol L <sup>-1</sup>
Ca(NO <sub>3</sub> ) <sub>2</sub>	1.3	1.3	0
NH <sub>4</sub> NO <sub>3</sub>	0.7	0.7	0.08
CaCl <sub>2</sub>	2	2	3.3
K <sub>2</sub> SO <sub>4</sub>	1	1	1
KH <sub>2</sub> PO <sub>4</sub>	0.2	0.2	0.2
MgSO <sub>4</sub>	0.5	0.02	0.5
	μmol L <sup>-1</sup>	μmol L <sup>-1</sup>	μmol L <sup>-1</sup>
Fe-EDTA	200	200	200
H <sub>3</sub> BO <sub>3</sub>	5	5	5
MnSO <sub>4</sub>	2	2	2
ZnSO <sub>4</sub>	0.5	0.5	0.5
CuSO <sub>4</sub>	0.3	0.3	0.3
(NH <sub>4</sub> ) <sub>2</sub> Mo <sub>7</sub> O <sub>24</sub>	0.01	0.01	0.01

### Optical measurements of leaf pigments

Chlorophyll (Chl), flavonol (Flav), anthocyanin (Anth) and nitrogen-balance-index (NBI) measurements were conducted using the polyphenol and chlorophyll-meter 'Dualex Scientific+<sup>TM\*</sup>' (FORCE-A, Orsay, France). Readings were taken every other day, starting on the third emerging foliage leaf of eight-days-old seedlings three days after planting (DAP). Recordings on the fourth and fifth foliage leaf started five DAP and eleven DAP, respectively, according to their appearance (Fig. 5D, 6D). Four plants per biological control were analysed resulting in 16 analysed leaves per leaf level per experimental group. Five to six measurement – depending on the leaf age and size - were made along the leaf lamina avoiding the midribs (Fig. 5D, 6D). Readings were made on the adaxial leaf side. For analysis of temporal courses, measurements along the leaf blade were averaged.

# Dualex Scientific+<sup>™</sup> Technical specifications

The chlorophyll index (Chl) is determined by measuring the leaf transmission of red and near-infrared light. The latter is supposed to be not absorbed by chlorophyll and, therefore, can take leaf structure effects into account.

4. Early-stage diagnosis of nutrient deficiency in maize leaves

$$Chl\ index = \frac{NIR\ transmission\ -\ red\ transmission}{red\ transmission}$$

Near-infrared (NIR) chlorophyll fluorescence excited at two wavelengths, one within the UV range or green light range, which is absorbed by flavonols or anthocyanins, respectively, and one reference wavelength (red light) that crosses the epidermis without being absorbed before reaching Chl in the mesophyll, is used to calculate the indices of flavonols (Flav) and anthocyanins (Anth).

$$Flav\ index = log\ \frac{red\text{-}excited\ NIR\ chlorophyll\ fluorescence}}{UV\text{-}A\ light\ excited\ NIR\ chlorophyll\ fluorescence}}$$
 
$$Anth\ index = \frac{red\text{-}excited\ NIR\ chlorophyll\ fluorescence}}{green\ light\ excited\ NIR\ chlorophyll\ fluorescence}}$$

The nitrogen balance index (NBI) is defined as the ratio between the ChI index and the Flav index.

$$NBI = \frac{Chl \ index}{Flav \ index}$$

### Harvest

Analysed leaves of 18-days-old seedlings were harvested 15 days after planting. For sugar analysis, leaf material was dried at 60°C for two days and finely ground to a powder using a ball mill.

## Analysis of soluble sugars

Soluble sugars were extracted by hot water extraction. Five mg of finely ground leaf material was dissolved in deionised  $H_2O$  and boiled at  $100^{\circ}C$  for five minutes in a water-bath with proper mixing. Samples were cooled on ice for 30 min, centrifuged (4°C, 12,000 rpm, 10 min), the supernatant was collected and diluted 1:5 with deionised  $H_2O$ , if necessary. Samples were cleaned-up with chloroform and run over C18 columns (Strata spe. 8B-S001-DAK, Phenomex, Torrance, CA, USA). Sucrose, glucose and fructose concentrations were measured by anion exchange chromatography using an ICS-5000 system equipped with a Carbo Pac PA-100 column and an integrated amperometric detector (Dionex, Sunnyvale, CA, United States).

### **Statistics**

Data of Dualex measurements was statistically analyzed using R statistical package software version 3.0.2 (The R foundation for statistical computing, Vienna, Austria). Effects of treatments were tested by pairwise comparison using Student's t-test and following Bonferroni correction. For sugar analysis effects of treatments were tested by comparisons with the positive control group using Student's t-test. Coefficients of determination of simple linear regression (R²) for Chl, Flav, and Anth indices were calculated using Microsoft Excel 2013 (Microsoft Corporation, Redmond, Washington, USA).

# **Results**

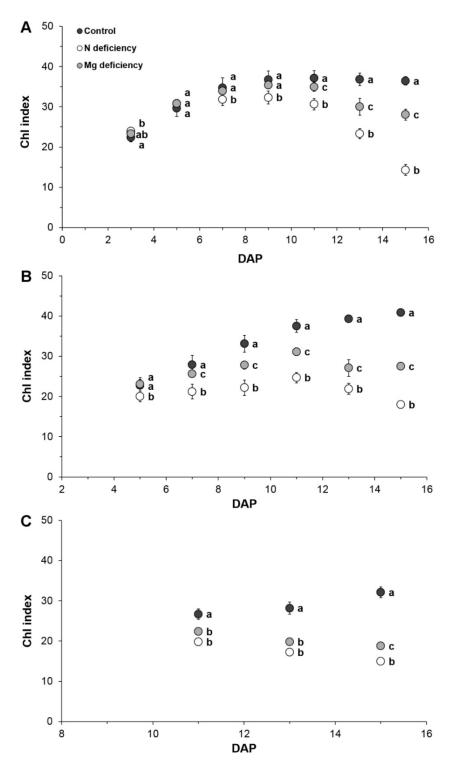
### **DUALEX** measurements

Figure 2 shows the course of the Chl index over time. Depending on the leaf age, five to six measurements were taken along the leaf blade and averaged. Increasing Chl index was measured in all leaves of control plants over time. In contrast, the Chl index increased in leaf 3 of N- and Mg-deficient plants during the first half of the experiment but stagnated and decreased in the following. Chlorophyll values were different from the control at day 7 and day 11 after planting for N and Mg deficiency, respectively. The Chl index in leaves 4 and 5 of nutrient-deficient plants was generally significantly lower than the control and declined until the end of the experiment. Chlorophyll decrease was more pronounced in N-deficient plants than in Mg-deprived maize.

Only minor changes were found in the Flav index averaged over the whole leaf blade in control plants in the course of the experiment (Fig. 3), whereas the Flav index increased markedly over time under N shortage and Flav values of all leaves were twice as high as in the control at harvest (Fig. 3). Low Mg supply also resulted in increasing Flav index, but less pronounced than in N-deficient plants (Fig. 3). However, Flav levels were significantly higher than in the control at the end of the experiment.

The NBI index is defined as the ratio of ChI and Flav index. Accordingly, the NBI in control plants increased in leaves 3 and 4 over time due to rising ChI and declining Flav indices, whereas the NBI index of nutrient-deficient plants showed similar courses like the ChI index over time, but differences between treatments were more pronounced (Fig. 4).

The spatiotemporal visualization of the measurements provided information about changes in the ChI and Flav indices along the leaf lamina over time (Fig. 5, 6). Independent of the nutritional status, longitudinal gradients of both ChI and Flav indices were measured along the leaf blade, increasing from the base to the tip and the ChI index was generally slightly lower in the uttermost leaf tip. Furthermore, it was revealed that the Flav index of Mg-deficient maize leaves increased predominantly at the tip in leaves 3 and 4 (Fig. 6C). This was also true for leaf 3 and 5 of plants with low N supply; however, the Flav index in leaf 4 of N-deficient plants was high at the leaf base as well (Fig. 6B).



**Figure 2:** Course of the Chl index of (A) leaf 3, (B) leaf 4 and (C) leaf 5. Control plants were grown under optimal nutrition; N and Mg deficiency was induced by reducing the N or Mg concentration, respectively, in the nutrition solution to 4% of the control. Measurements were taken on four plants of four biological replicates per treatment. Five to six measurement were taken along the leaf blade and averaged. Mean  $\pm$  SE; small letters indicate significant differences between treatments at a measuring date (pairwise t-test with Bonferroni adjustment, p  $\leq$  0.05).

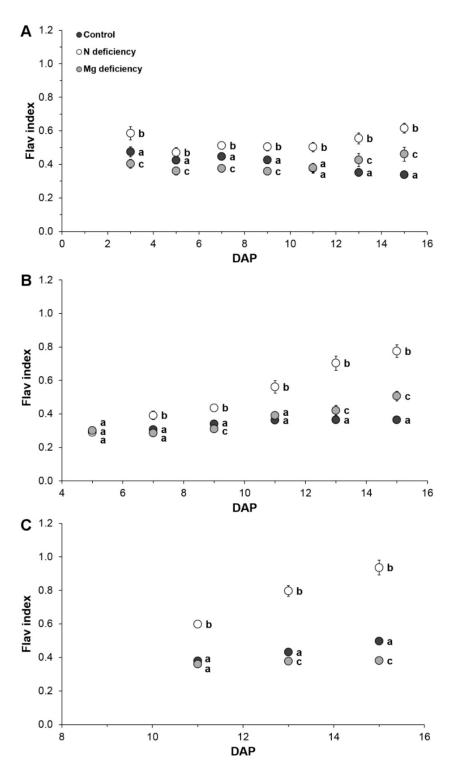


Figure 3: Course of the Flav index of (A) leaf 3, (B) leaf 4 and (C) leaf 5. Control plants were grown under optimal nutrition; N and Mg deficiency was induced by reducing the N or Mg concentration, respectively, in the nutrition solution to 4% of the control. Measurements were taken on four plants of four biological replicates per treatment. Five to six measurement were taken along the leaf blade and averaged. Mean  $\pm$  SE; small letters indicate significant differences between treatments at a measuring date (pairwise t-test with Bonferroni adjustment, p  $\leq$  0.05).

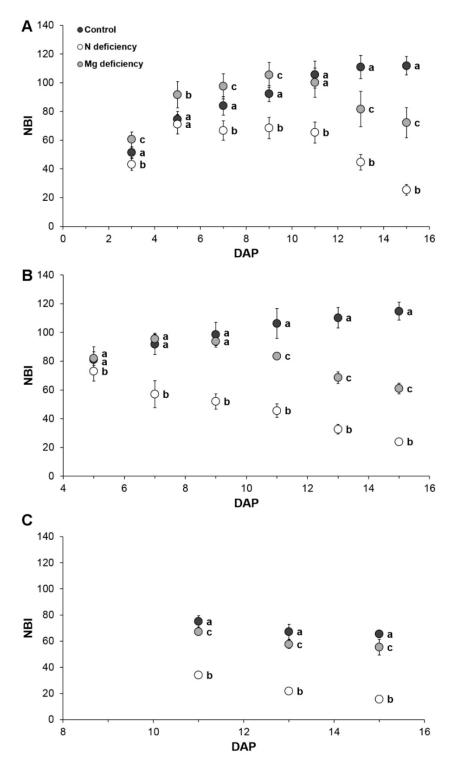


Figure 4: Course of the NBI index of (A) leaf 3, (B) leaf 4 and (C) leaf 5. Control plants were grown under optimal nutrition; N and Mg deficiency was induced by reducing the N or Mg concentration, respectively, in the nutrition solution to 4% of the control. Measurements were taken on four plants of four biological replicates per treatment. Five to six measurement were taken along the leaf blade and averaged. Mean  $\pm$  SE; small letters indicate significant differences between treatments at a measuring date (pairwise t-test with Bonferroni adjustment, p  $\leq$  0.05).

	Α			Contro	l			В		N o	deficier	ісу		
				Position							Position			
	DAP	1	2	3	4	5	6	DAP	1	2	3	4	5	6
	3	18.60	22.47	24.66	24.05	22.25		3	19.08	22.71	24.15	24.18	22.18	
	5	25.39	27.30	31.57	33.66	30.32		5	23.44	26.96	30.41	32.89	29.73	
Leaf 3	7	31.25	33.78	35.52	37.42	36.44	34.04	7	24.85	27.74	30.65	32.09	31.92	30.33
Leai 3	9	33.56	35.72	37.84	39.61	38.24	35.22	9	25.76	28.45	30.37	32.45	32.22	31.08
	11	34.62	36.33	37.36	38.02	38.43	37.83	11	24.40	27.10	28.59	30.38	31.00	29.54
	13	36.01	36.18	37.16	37.46	37.32	36.78	13	18.70	20.12	22.18	23.79	24.11	20.93
	15	35.75	34.94	35.88	36.88	37.64	37.60	15	12.95	13.25	14.71	14.65	13.86	9.64
1	DAP	1	2	3	4	5	6	DAP	1	2	3	4	5	6
	5	17.34	19.36	20.07	21.36	22.67		5	13.02	13.93	14.83	15.89	16.57	
	7	18.16	24.26	29.18	31.90	31.75	32.07	7	14.03	17.57	19.70	20.84	22.05	23.07
Leaf 4	9	22.63	29.19	34.54	37.74	38.00	36.45	9	10.98	15.22	19.33	24.25	26.20	26.73
	11	31.98	34.94	38.84	41.38	41.18	36.73	11	15.83	18.89	21.74	25.60	27.52	27.76
	13	34.85	37.50	40.47	42.50	41.31	39.03	13	14.80	17.65	20.34	22.82	23.38	22.71
	15	35.21	39.58	42.12	43.39	44.26	40.58	15	14.21	15.83	17.06	17.86	18.00	17.12
1						_	_				_		_	•
	DAP	1	2	3	4	5	6	DAP	1	2	3	4	5	6
Leaf 5	11	16.73	20.90	27.23	31.22	32.12	31.97 35.44	11 13	10.30 10.87	10.92 14.04	11.23 17.02	11.44 18.21	11.76 18.22	11.97
	13 15	11.75 18.73	19.24 24.34	27.72 31.07	36.27 37.84	38.70 41.46	39.19	15	5.51	8.76	13.06	17.07	17.02	18.43 16.24
J	13	10.73	24.34	31.07	37.04	41.40	39.19	13	5.51	0.70	13.00	17.07	17.02	10.24
	C		Ma	doficio	nov.				D		_	.,.		
	С		Mg	deficie	ncy				D			osition		
	С		Mg	deficie Position	ncy					1 2		osition 4	5	6
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]	DAP 3 5 7 9	19.69 25.36 29.53 31.29 32.87	2 23.34 29.94 33.44 33.93 35.33	3 25.52 33.52 35.93 36.72 36.79	4 24.88 34.77 36.97 37.55 37.28	23.16 30.54 35.63 38.58 35.39	32.05 34.26 31.90	39.61 M				4		
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Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11 13 15	19.69 25.36 29.53 31.29 32.87 30.45 29.32 1 17.40 16.71 20.77 28.13 25.69 29.46	2 23.34 29.94 33.44 33.93 35.33 29.83 28.80 2 19.21 20.89 25.94 30.97 26.68 28.58	Position  3 25.52 33.52 35.93 36.72 36.79 30.38 29.79  3 20.10 25.74 29.05 33.18 27.95 27.94	4 24.88 34.77 36.97 37.55 37.28 32.42 30.38 4 21.31 28.22 30.61 33.99 29.68 29.54	23.16 30.54 35.63 38.58 35.39 31.09 27.79 5 22.07 27.24 32.18 32.24 28.60 27.47	32.05 34.26 31.90 26.17 22.19 6 25.64 28.21 28.18 23.97 21.92	39.61 M 35.00 30.00 25.00 20.00 15.00 10.00	base		3	Lea	af 5	
Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11 13 15  DAP	19.69 25.36 29.53 31.29 32.87 30.45 29.32 1 17.40 16.71 20.77 28.13 25.69 29.46	2 23.34 29.94 33.44 33.93 35.33 29.83 28.80 2 19.21 20.89 25.94 30.97 26.68 28.58	Position  3 25.52 33.52 35.93 36.72 36.79 30.38 29.79  3 20.10 25.74 29.05 33.18 27.95 27.94	4 24.88 34.77 36.97 37.55 37.28 32.42 30.38 4 21.31 28.22 30.61 33.99 29.68 29.54	23.16 30.54 35.63 38.58 35.39 31.09 27.79 5 22.07 27.24 32.18 32.24 28.60 27.47	32.05 34.26 31.90 26.17 22.19 6 25.64 28.21 28.18 23.97 21.92	39.61 M 35.00 30.00 25.00 20.00 15.00 10.00	base		3	Lea	af 5	
Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11 13 15  DAP 11 13 15	19.69 25.36 29.53 31.29 32.87 30.45 29.32 1 17.40 16.71 20.77 28.13 25.69 29.46	2 23.34 29.94 33.44 33.93 35.33 29.83 28.80 2 19.21 20.89 25.94 30.97 26.68 28.58	Position  3 25.52 33.52 35.93 36.72 36.79 30.38 29.79  3 20.10 25.74 29.05 33.18 27.95 27.94 3 22.81	4 24.88 34.77 36.97 37.55 37.28 32.42 30.38 4 21.31 28.22 30.61 33.99 29.68 29.54 4 23.47	23.16 30.54 35.63 38.58 35.39 31.09 27.79 5 22.07 27.24 32.18 32.24 28.60 27.47 5	32.05 34.26 31.90 26.17 22.19 6 25.64 28.21 28.18 23.97 21.92 6 20.84	39.61 M 35.00 30.00 25.00 20.00 15.00 10.00	base		3	Lea	af 5	
Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11 13 15  DAP	19.69 25.36 29.53 31.29 32.87 30.45 29.32 1 17.40 16.71 20.77 28.13 25.69 29.46	2 23.34 29.94 33.44 33.93 35.33 29.83 28.80 2 19.21 20.89 25.94 30.97 26.68 28.58	Position  3 25.52 33.52 35.93 36.72 36.79 30.38 29.79  3 20.10 25.74 29.05 33.18 27.95 27.94	4 24.88 34.77 36.97 37.55 37.28 32.42 30.38 4 21.31 28.22 30.61 33.99 29.68 29.54	23.16 30.54 35.63 38.58 35.39 31.09 27.79 5 22.07 27.24 32.18 32.24 28.60 27.47	32.05 34.26 31.90 26.17 22.19 6 25.64 28.21 28.18 23.97 21.92	39.61 M 35.00 30.00 25.00 20.00 15.00 10.00	base		3	Lea	af 5	

# 4. Early-stage diagnosis of nutrient deficiency in maize leaves

**Figure 5:** Spatiotemporal courses of Chl index of leaf 3 – 5 from (A) control, (B) N-deficient and (C) Mg-deficient maize seedlings. Control plants were grown under optimal nutrition; N and Mg deficiency was induced by reducing the N or Mg concentration, respectively, in the nutrition solution to 4% of the control. Measurements were taken on four plants of four biological replicates per treatment. (D) Illustration of the measuring procedure. Dualex measurements were made at five to six leaf spots along the leaf blade – depending on the leaf age and size - starting from the leaf base. Leaves number 3 to 5 of maize seedlings were analysed.

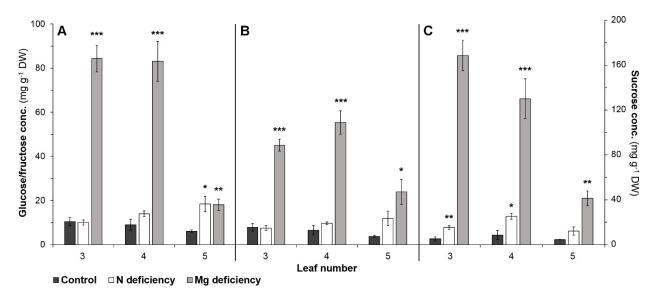
	Α			Contro	ļ			В		N o	deficier	тсу		
				Position							Position			
	DAP	1	2	3	4	5	6	DAP	1	2	3	4	5	6
	3	0.39	0.47	0.54	0.52	0.44		3	0.42	0.54	0.62	0.60	0.54	
	5	0.31	0.36	0.46	0.53	0.47		5	0.29	0.35	0.45	0.58	0.57	
Leaf 3	7	0.33	0.36	0.45	0.50	0.52	0.49	7	0.35	0.38	0.46	0.55	0.62	0.62
Leai 3	9	0.30	0.34	0.42	0.48	0.51	0.48	9	0.35	0.38	0.42	0.51	0.57	0.57
	11	0.29	0.32	0.35	0.40	0.44	0.45	11	0.36	0.39	0.42	0.49	0.58	0.62
	13	0.29	0.31	0.34	0.37	0.40	0.41	13	0.41	0.43	0.47	0.54	0.64	0.65
	15	0.28	0.31	0.33	0.36	0.37	0.37	15	0.45	0.47	0.50	0.59	0.72	0.76
	DAP	1	2	3	4	5	6	DAP	1	2	3	4	5	6
	5	0.18	0.23	0.27	0.29	0.32		5	0.17	0.20	0.23	0.25	0.26	
	7	0.21	0.26	0.30	0.32	0.34	0.36	7	0.29	0.34	0.37	0.39	0.40	0.41
Leaf 4	9	0.28	0.32	0.33	0.34	0.34	0.37	9	0.29	0.37	0.44	0.48	0.48	0.46
	11	0.35	0.35	0.37	0.36	0.38	0.37	11	0.55	0.55	0.53	0.54	0.52	0.49
	13	0.34	0.36	0.37	0.38	0.39	0.34	13	0.69	0.71	0.74	0.67	0.63	0.59
	15	0.34	0.37	0.38	0.38	0.38	0.34	15	0.73	0.75	0.78	0.75	0.71	0.66
	DAP	1	2	3	4	5	6	DAP	1	2	3	4	5	6
Leaf 5	11	0.18	0.34	0.43	0.44	0.44	0.44	11	0.25	0.31	0.35	0.37	0.37	0.38
Leai 3	13	0.24	0.39	0.48	0.50	0.48	0.50	13	0.54	0.69	0.77	0.83	0.84	0.83
	15	0.36	0.49	0.52	0.57	0.54	0.51	15	0.60	0.75	0.88	0.92	0.91	0.89
	C		Ma	deficie	ncv				D		Ъ	ocition		
	С		Mg	deficie	ncy				D	1 2		osition	_	6
i i				Position	•					1 2	P 3	osition 4	5	6
	DAP	1	2	Position 3	4	5	6			1 2			5	6
	<b>DAP</b> 3	0.32	2 0.41	Position 3 0.46	4 0.44	0.38	6						5	
	<b>DAP</b> 3 5	0.32 0.26	2 0.41 0.29	9 0.46 0.36	4 0.44 0.45	0.38 0.43							5	6 tip
Leaf 3	<b>DAP</b> 3 5 7	0.32 0.26 0.29	2 0.41 0.29 0.31	9 0.46 0.36 0.36	4 0.44 0.45 0.43	0.38 0.43 0.47	0.48						5	
	<b>DAP</b> 3 5 7 9	0.32 0.26 0.29 0.28	2 0.41 0.29 0.31 0.29	9 0.46 0.36 0.36 0.33	4 0.44 0.45 0.43 0.37	0.38 0.43 0.47 0.42	0.48 0.48						5	
	DAP 3 5 7 9	0.32 0.26 0.29 0.28 0.30	2 0.41 0.29 0.31 0.29 0.32	9 0.46 0.36 0.36 0.33 0.34	4 0.44 0.45 0.43 0.37 0.39	0.38 0.43 0.47 0.42 0.43	0.48 0.48 0.49	Flay index					5	
	<b>DAP</b> 3 5 7 9	0.32 0.26 0.29 0.28 0.30 0.31	2 0.41 0.29 0.31 0.29 0.32 0.34	Position  3  0.46  0.36  0.36  0.33  0.34  0.39	4 0.44 0.45 0.43 0.37 0.39 0.44	0.38 0.43 0.47 0.42 0.43 0.51	0.48 0.48 0.49 0.57					4		
	DAP 3 5 7 9 11 13 15	0.32 0.26 0.29 0.28 0.30 0.31 0.34	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45	0.38 0.43 0.47 0.42 0.43 0.51 0.55	0.48 0.48 0.49 0.57 0.65	0.89 0.80	base					
	DAP 3 5 7 9 11 13 15	0.32 0.26 0.29 0.28 0.30 0.31 0.34	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45	0.38 0.43 0.47 0.42 0.43 0.51 0.55	0.48 0.48 0.49 0.57	0.89	base		3	4		
	DAP 3 5 7 9 11 13 15  DAP 5	0.32 0.26 0.29 0.28 0.30 0.31 0.34	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40  3  0.29	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5	0.48 0.48 0.49 0.57 0.65	0.89 0.80 0.70 0.60 0.50	base		3	4		
Leaf 3	DAP 3 5 7 9 11 13 15  DAP 5 7	0.32 0.26 0.29 0.28 0.30 0.31 0.34 1 0.16	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37 2 0.24 0.23	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40  3  0.29  0.26	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45 4 0.31 0.30	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5 0.32 0.36	0.48 0.48 0.49 0.57 0.65	0.89 0.80 0.70 0.60 0.50 0.40	base		3	4		
	DAP 3 5 7 9 11 13 15  DAP 5 7 9	0.32 0.26 0.29 0.28 0.30 0.31 0.34 1 0.16 0.18	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37 2 0.24 0.23 0.24	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40  3  0.29  0.26  0.27	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45 4 0.31 0.30 0.32	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5 0.32 0.36 0.33	0.48 0.48 0.49 0.57 0.65	0.89 0.80 0.70 0.60 0.50 0.40	base		3	4		
Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11	0.32 0.26 0.29 0.28 0.30 0.31 0.34 1 0.16 0.18 0.23 0.31	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37 2 0.24 0.23 0.24 0.33	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40  3  0.29  0.26  0.27  0.37	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45 4 0.31 0.30 0.32 0.41	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5 0.32 0.36 0.33 0.45	0.48 0.48 0.49 0.57 0.65 6 0.41 0.40 0.48	0.89 0.80 0.70 0.60 0.50 0.40 0.30	base		3	Lea	af 5	
Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11 13	0.32 0.26 0.29 0.28 0.30 0.31 0.34 1 0.16 0.18 0.23 0.31 0.30	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37 2 0.24 0.23 0.24 0.33 0.33	Position  3  0.46 0.36 0.36 0.33 0.34 0.39 0.40  3  0.29 0.26 0.27 0.37 0.40	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45 4 0.31 0.30 0.32 0.41	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5 0.32 0.36 0.33 0.45 0.51	0.48 0.48 0.49 0.57 0.65 6 0.41 0.40 0.48 0.52	0.89 0.80 0.70 0.60 0.50 0.40 0.30	base		3	Lea		
Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11	0.32 0.26 0.29 0.28 0.30 0.31 0.34 1 0.16 0.18 0.23 0.31	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37 2 0.24 0.23 0.24 0.33	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40  3  0.29  0.26  0.27  0.37	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45 4 0.31 0.30 0.32 0.41	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5 0.32 0.36 0.33 0.45	0.48 0.48 0.49 0.57 0.65 6 0.41 0.40 0.48	0.89 0.80 0.70 0.60 0.50 0.40 0.30	base		3	Lea	af 5	
Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11 13 15  DAP	0.32 0.26 0.29 0.28 0.30 0.31 0.34 1 0.16 0.18 0.23 0.31 0.30 0.34	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37 2 0.24 0.23 0.24 0.33 0.33 0.38	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40  3  0.29  0.26  0.27  0.37  0.40  0.50	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45 4 0.31 0.30 0.32 0.41 0.47 0.58	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5 0.32 0.36 0.33 0.45 0.51 0.61	0.48 0.48 0.49 0.57 0.65 6 0.41 0.40 0.48 0.52 0.62	0.89 0.80 0.70 0.60 0.50 0.40 0.30	base		3	Lea	af 5	
Leaf 3 Leaf 4	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11 13 15  DAP 11 13 15	0.32 0.26 0.29 0.28 0.30 0.31 0.34 1 0.16 0.18 0.23 0.31 0.30 0.34	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37 2 0.24 0.23 0.24 0.33 0.38	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40  3  0.29  0.26  0.27  0.37  0.40  0.50	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45 4 0.31 0.30 0.32 0.41 0.47 0.58	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5 0.32 0.36 0.33 0.45 0.51 0.61	0.48 0.48 0.49 0.57 0.65 6 0.41 0.40 0.48 0.52 0.62	0.89 0.80 0.70 0.60 0.50 0.40 0.30	base		3	Lea	af 5	
Leaf 3	DAP  3 5 7 9 11 13 15  DAP 5 7 9 11 13 15  DAP	0.32 0.26 0.29 0.28 0.30 0.31 0.34 1 0.16 0.18 0.23 0.31 0.30 0.34	2 0.41 0.29 0.31 0.29 0.32 0.34 0.37 2 0.24 0.23 0.24 0.33 0.33 0.38	Position  3  0.46  0.36  0.36  0.33  0.34  0.39  0.40  3  0.29  0.26  0.27  0.37  0.40  0.50	4 0.44 0.45 0.43 0.37 0.39 0.44 0.45 4 0.31 0.30 0.32 0.41 0.47 0.58	0.38 0.43 0.47 0.42 0.43 0.51 0.55 5 0.32 0.36 0.33 0.45 0.51 0.61	0.48 0.48 0.49 0.57 0.65 6 0.41 0.40 0.48 0.52 0.62	0.89 0.80 0.70 0.60 0.50 0.40 0.30	base		3	Lea	af 5	

**Figure 6:** Spatiotemporal courses of Flav index of leaf 3 – 5 from (A) control, (B) N-deficient and (C) Mg-deficient maize seedlings. Control plants were grown under optimal nutrition; N and Mg deficiency was induced by reducing the N or Mg concentration, respectively, in the nutrition solution to 4% of the control. Measurements were taken on four plants of four biological replicates per treatment. (D) Illustration of the measuring procedures. Dualex measurements were made at five to six leaf spots along the leaf blade – depending on the leaf age and size - starting from the leaf base. Leaves number 3 to 5 of maize seedlings were analysed.

### Sugar measurements

Nutrient deficiency-induced flavonoid accumulation is assumed to result from shunting excessive photoassimilates into the phenylpropanoid pathway of secondary metabolism (Barker and Pilbeam, 2007; Bergmann, 1993; Schilling, 2000) and genetic upregulation of flavonoid synthesis is observed in response to external sugar feeding or inhibited phloem loading (Jeannette *et al.*, 2000; Lloyd and Zakhleniuk, 2004; Solfanelli *et al.*, 2006; Teng *et al.*, 2005). In order to reveal a potential causal relationship between N or Mg deficiency and accumulation of soluble sugars, the leaf concentrations of soluble sucrose, glucose, and fructose were analysed in this study.

Concentrations of soluble sugars were measured in extracts from whole leaves after harvest of plants 15 DAP. Especially low Mg supply resulted in markedly increased concentrations of soluble glucose, fructose and sucrose in all leaves (Fig. 7). Sucrose concentrations were up to 33-times as high as in the control in leaf 3, reaching almost 170 mg g<sup>-1</sup> DW (Fig. 7C). Nitrogen-deficient maize seedlings accumulated significantly more glucose in leaf 5 and sucrose in leaves 3 and 4 than the control (Fig. 7A, C). However, rises in sugar concentration were by far less pronounced than under Mg deficiency.



**Figure 7:** Concentrations of soluble (A) glucose, (B) fructose and (C) sucrose in maize leaves. Control plants were grown under optimal nutrition; N and Mg deficiency was induced by reducing the N or Mg concentration, respectively, in the nutrition solution to 4% of the control. Asterisks indicate significant differences between nutrient deficiencies and positive control at  $P \le 0.05$  (\*),  $P \le 0.01$  (\*\*) and  $P \le 0.001$  (\*\*\*).

### **Discussion**

# Critical evaluation of Dualex measurements for early-stage flavonoid detection

This study aimed at evaluating the suitability of the "Dualex Scientific+TM" (Dualex) – the latest generation of ChI meters – for early-stage discrimination between N and Mg deficiency in maize seedlings. The Dualex is promoted as a versatile measuring tool for simultaneous and accurate monitoring of chlorophyll content in leaves and flavonol and anthocyanin contents in the epidermis, which is useful in the field for rapid phenotyping and in the laboratory for scientific plant analysis (Force-A, 2015). According to the developers, the Dualex requires no prior calibration and can be used under all conditions of temperature and ambient light (Force-A, 2015). It is advertised as a promising technique in basic and applied research (Pfundel *et al.*, 2007). However, the latest Dualex model that has been introduced to the market - just as the previous versions - has not undergone extensive (published) calibration tests (Casa *et al.*, 2015).

The technique is based on Chl fluorescence measurements at different wavelengths, which lie within or outside of the absorption spectra of epidermal flavonoids, respectively. Chlorophyll in the mesophyll acts, thus, as a 'photosensor' (Goulas *et al.*, 2004) and Chl fluorescence is used to calculate Chl, Flav, Anth, and NB indices. Similar methods have previously been developed and proved to be useful for monitoring both the physiological plant state and the quality of crops (Bengtsson *et al.*, 2006; Bilger *et al.*, 2001; Bilger *et al.*, 1997; Hagen *et al.*, 2006). However, plant leaves consisting of cuticle, epidermis and mesophyll cells are highly complicated from an optical point of view, because of numerous boundaries and a large variety of light-absorbing metabolites (Gitelson *et al.*, 2003), and internal optics of leaves determine the penetration of excitation radiation and partial reabsorption of the emitted fluorescence (Buschmann *et al.*, 2000). Hence, non-invasive optical methods that analyse light-screening properties *in planta* have to be carefully validated and the applicability for specific purposes has to be assessed.

# General considerations

A general drawback of non-invasive optical methods is that only superficial layers can be analysed due to the limited penetration of radiation into the sample (Agati *et al.*, 2005). Light stress-induced flavonoids are known to accumulate mainly in the epidermis, the hypodermis and the cuticle (Dixon and Paiva, 1995; Fini *et al.*, 2011; Hernandez *et al.*, 2009). However, little is known about tissue-specific localization of flavonoid synthesis under nutrient deficiency. Since the ecophysiological significance of nutrient stress-induced up-regulation of the flavonoid biosynthesis pathway is not consensually resolved, it can only be assumed that they function as epidermal light-screens to protect chloroplasts in the mesophyll from excess excitation. However, due to the prevalent hypothesis that flavonoids act as carbon storage compounds in response to nutrient shortage (Coley *et al.*, 1985; Herms and Mattson, 1992) Bergmann, 1993), flavonoid accumulation in internal leaf tissue is also conceivable. Therefore, it is questionable, if they can be detected by this superficial optical approach.

Furthermore, the Dualex measurements are affected by leaf anatomical characteristics of monocotyledonous maize leaves with a high proportion of leaf veins. Also, anthocyanin synthesis in response to Mg shortage was found to be unequally spread across the leaf lamina in maize (Fig. S1). It is unknown if this patchy accumulation also holds true for flavonols. Thus, the natural heterogeneity of leaves leads to high variability of readings, because of the narrow Dualex measuring area, which is only 5 mm in diameter. Cerovic *et al.* (2012) found variances

of 10% and 40% in dicotyledons and monocotyledons, respectively, causing the necessity of high reading repetition, especially for scientific purposes.

### Anthocyanin measurement

The latest Dualex model "Dualex Scientific+TM" allows the measurement of epidermal anthocyanin content by calculating an Anth index from NIR Chl fluorescence at green and red light excitation. Since anthocyanins are synthesized in response to several nutrient deficiencies, their detection can provide valuable information for both fertilization management in the field and scientific analysis of plant responses to abiotic stress.

Chlorophyll and Anth indices were highly correlated in all leaves under optimal nutrition and in leaf 3 under N shortage (Fig. S4, Table S1). The dependency of Anth values on the Chl content might display light-induced physiological plant responses. For example, negative reciprocity between ChI and Anth was explained as a controlled physiological reaction during early seedling growth when seedlings are most susceptible to light-induced stress (Drummherrel and Mohr, 1985). However, decreasing ChI levels and simultaneously increasing Anth values were also recorded in leaf 3 in later stages of plant growth. Furthermore, the Anth index increased markedly under both N- and Mq-deficiency in this leaf (Fig. S2), but anthocyanins could only be visually detected in Mg-deprived plants (Fig. S3B), whereas N-deficient leaves showed no leaf purpling (Fig. S3A). Therefore, we reason that the recorded Anth values are biased by to a methodical problem created by the used excitation wavelengths and declining Chl contents under stress conditions. The applied technique of Chl fluorescence-based measurement of epidermal light-absorbing compounds can only be applied with excitation wavelengths for which the ratio of the ChI fluorescence at different wavelengths does not depend on leaf chlorophyll content, which is the case for excitation wavelengths with equal molar extinction coefficients of Chl and in leaves with high Chl content (Goulas et al., 2004; Ounis et al., 2001). The first precondition is fulfilled by the Dualex only for Flav index measurement, because of similar specific Chl absorptivity of UV-A light and red light (Cerovic et al., 1998; Lange, 1981). However, the molar extinction coefficients of Chl for green light is much lower than that for red light, which causes a dependency of the calculated Anth index on the Chl concentration. In fact, Nichelmann (2014) verified with diluted suspensions of isolated chloroplasts, that the ratio between Chl fluorescence excited at 545 nm and 650 nm depends on the ChI concentration. Green light is less absorbed by ChI than red light and therefore penetrates deeper into the leaf. Chlorophyll fluorescence coming from deeper mesophyll layers can be reabsorbed by chloroplasts, causing a decrease in the Chl fluorescence excited with green light (Fig. 1B). This effect increases with decreasing chlorophyll concentration and the green light beam can even transmit through when the Chl content is small (Ounis et al., 2001). Consequently, the calculated Anth index – defined as the logarithmic ratio of Chl fluorescence excited with red and green light, respectively (Fig. 1B) – overestimates the light-absorption by epidermal anthocyanins when the chlorophyll content falls below a certain concentration. In order to avoid this problem, Nichelmann (2014) expanded the anthocyanin-dependent Chl fluorescence excitation spectrum by blue light, thereby narrowing the region of dependency to very low Chl concentrations. Similarly, Hagen et al. (2006) used blue light at 470 nm for excitation of Anth-dependent Chl fluorescence and red light at 655 nm as a reference beam. Since the molar extinction coefficients of ChI are comparable for these wavelengths, the measurements are not biased by potential Chl fluorescence reabsorption effects. Consequently, this technique proved to accurately assess epidermal anthocyanins in fruits (Hagen et al., 2006), as verified by HPLC analysis, and can hence be used to monitor both physiological state and quality of crops. The coefficients of determination of simple linear

regression (R²) for Chl and Anth indices were low in leaves 3 and 4 or 3 to 5 of N- and Mgdeficient maize seedlings, respectively, probably indicating the onset of anthocyanin accumulation in response to nutrient stress (Table S1). However, correcting the results for the re-absorption effect was not possible from the dataset of this study.

In conclusion, estimating epidermal anthocyanins by measuring ChI fluorescence excited at red and green light does not meet the pre-conditions of applicability of the ChI fluorescence method formulated by Goulas *et al.* (2004), because the two excitation wavelengths have got distinct molar extinction coefficients of ChI and consequently, the calculated Anth index is dependent on the ChI concentrations (Fig. S4; Table S1). Therefore, the Dualex Scientific+<sup>TM</sup> does not represent a reliable instrument for measuring epidermal anthocyanins in N- or Mg-deficient plants, because both nutrient deficiencies are accompanied by declining ChI contents (Fig. 2, 5).

# Nitrogen-balance-index as an indicator of leaf N content

Next to providing indices for epidermal light-absorbing compounds, the Dualex calculates a so-called 'Nitrogen Balance Index' (NBI), which is the ratio of the ChI and Flav indices and is assumed to be related to metabolic Nitrogen/Carbon allocation (Cerovic *et al.*, 2012; Force-A, 2015). Nitrogen nutrition has got opposite effects on foliar flavonol and chlorophyll contents, accentuating differences of the NBI between N-sufficient and N-deficient plants than a single index alone (Cartelat *et al.*, 2005). Therefore, the NBI is conceived as a more precise method to get an earlier information about the N status of crops and is discussed to open an access to real-time site-specific N fertilisation (Cartelat *et al.*, 2005; Force-A, 2015). For example, Cartelat *et al.* (2005) measured ChI and Flav indices in the mature flag leaf of wheat in later developmental state and proposed using the NBI for determination of yield components after flowering, such as grain protein content to help in adjusting post-flowering rate of N fertiliser application.

However, using an indicator for N diagnosis requires specificity of the index, in order to avoid misdirected fertilization. The results of this study question the N-related uniqueness of the symptoms used to calculate the NBI. An inverse relationship between ChI and Flav indices was also found in leaves of Mg-deficient maize seedlings (Fig. 2 - 6). Consequently, the NBI decreased in the course of leaf maturation. Although the decline was less pronounced than in N-deprived plants, the NBI of leaf 3 and 4 differed significantly from the control plants. Hence, the NBI does not prove to be an adequate index for early-stage specification of nutrient deficiency in maize seedlings because it is not discriminative enough.

# Spatio-temporal distribution of chlorophyll and flavonols

The longitudinal gradient of flavonoids along the leaf blade and their progression over time provide valuable information about the onset and ontogenetic development of flavonoid biosynthesis in maize leaves under nutrient deficiency. This knowledge is of interest in ecophysiological plant research to unveil the physiological trigger and the function of flavonoid biosynthesis as an adaptive response to nutrient shortage. The suitability of the Dualex device for measuring leaf chlorophyll and flavonoid contents has been intensively tested and good correlations between the Flav index and extracted UV-absorbing phenolic compounds were found in diverse crops and under different N levels (Cartelat *et al.*, 2005; Cerovic *et al.*, 2012; Goulas *et al.*, 2004; Pfundel *et al.*, 2007). Due to the approved applicability of the Dualex for these measurements, the spatiotemporal development of Chl and Flav indices were determined in different leaves of maize grown hydroponically under optimal, N-deficient, and Mg-deficient conditions. Monocotyledonous leaves are optimally suited for this purpose

because of their basal growth with a narrow meristematic and elongation zone at the leaf base and cell age gradient up to the leaf tip (Cartelat *et al.*, 2005; Scanlon *et al.*, 2000).

As expected, chlorophyll contents in leaves of N- and Mg-deficient plants were basically lower compared to the control plants and declined in the course of leaf ageing (Fig. 2, 5B, C). Since the photosynthetic machinery accounts for more than 50% of the leaf N content (Evans, 1989), chlorophyll is degraded during N deficiency and N is translocated into growing tissue. Likewise, the photosynthetic apparatus including chlorophyll is dismantled under Mg deficiency, presumably in consequence of negative feedback inhibition of photosynthesis induced by accumulating photoassimilates (Hermans and Verbruggen, 2005). Due to the cell age gradient along the leaf blade, the Chl index generally inclined with distance from the leaf base in all experimental groups (Fig. 5). The lower Chl values in the uttermost leaf tip might be age-related (Wagner, 2003) or caused by lower light irradiance in this section due to the planophil leaf orientation.

Similar to the Chl index, the Flav numbers generally increased along the leaf lamina (Fig. 6), which is in agreement with measurements in wheat and barley (Cartelat et al., 2005; Wagner et al., 2003). Transiently higher Flav values were found in control leaf 3 (Fig. 6A). Such juvenile flavonoid accumulation is known from anthocyanins (Chalker-Scott, 1999; Murray and Hackett, 1991) and might display a photomorphogenic "high irradiance response" presumably for photoprotection of the developing photosynthetic apparatus (Chalker-Scott, 1999). The results of this study indicate that also UV-A absorbing compounds are increasing temporarily at the onset of leaf emergence in maize seedlings independent of the nutritional status. Decreasing Flav index over time has also been measured in flag leaves of field-grown wheat under optimal N conditions before the onset of senescence, indicating turnover of epidermal UV-A absorbing compounds (Cartelat et al., 2005). The Flav index of all analysed leaves in N-deprived maize increased strongly over time (Fig. 3, 6B). Genetic up-regulation of key enzymes of the flavonoid biosynthesis pathway in response to N depletion has been thoroughly documented in Arabidopsis thaliana and was shown to be under complex genetic control (Lillo et al., 2008). Tomato plants grown under limited N conditions show marked increase in foliar flavonols but not in fruits (Benard et al., 2011; Stewart et al., 2001). Flavonol accumulation was most pronounced in the uppermost leaf of maize seedlings under N deficiency, which may indicate a photoprotective light-screening function, because of increasing light irradiance with plant height.

Slight increases in the Flav values were also found in maize leaves 3 and 4 in response to Mg shortage (Fig. 3). Magnesium deficiency is known to induce foliar anthocyanin accumulation (Kobayashi *et al.*, 2013), but up-regulation of the flavonol biosynthesis pathway has hitherto not been shown. Cartelat *et al.* (2005) found a high correlation of ChI and Flav indices with N content in wheat leaves and consider both values as probes for crop N nutrition. However, the results of this study reveal that also Mg deficiency leads to a simultaneous decline in ChI values and increase in Flav index. In contrast to our experiment, the authors measured only the last fully developed leaf in mature wheat plants from tillering till flowering.

Interestingly, the spatiotemporal development of Flav values differed between maize seedlings grown under N- or Mg-deficiency, respectively. Increasing Flav index over time started at the leaf tip under Mg deficiency, which correlates with the supposed Mg gradient along the leaf blade in response to Mg deprivation (Fig. 6C). As a phloem-mobile nutrient, Mg is assumed to be translocated to growing tissue starting from the oldest part of source leaves (Senbayram *et al.*, 2015). Therefore, increasing Flav index at the leaf tip may display the onset of Mg remobilisation and a consequent physiological adaptation to Mg deficiency. The Flav index gradient in leaf number 3 was similar for both nutrient deficiencies but much more pronounced

under N limitation (Fig. 6B, C). In contrast, Flav values in leaf number 4 of N-deprived plants were highest at the leaf base, which is the ontogenetic youngest region in monocotyledonous leaves. Wagner *et al.* (2003) supposed that leaf gradients of epidermal UV-absorbing compounds might reflect different sun exposure of distinct leaf regions in barley under optimal nutrition. Hence, UV-screening compounds accumulate preferentially in the middle part of planophil barley leaves. The authors furthermore speculate, that older leaf regions with fully developed photosynthetic apparatus can invest more energy in flavonoid biosynthesis. Both assumptions cannot be the case in N-deficient maize seedlings since the highest Flav index was found in the vertically oriented youngest leaf (Fig. 3C, 6B). Chlorophyll and Flav indices were highly correlated in leaf number 4 and 5 of control plants (Fig. S4A; Table 1), which may reflect a concomitant age-dependent accumulation of the pigments. However, the Chl-Flav coefficients of determination of simple linear regression (R²) were lower under N- and Mg-deficiency (Table S1). This indicates that epidermal UV-absorbing compounds are accumulated independent of the Chl content and hence, might not solely be synthesized for photoprotective purposes under nutrient deficiency.

### Sugar induction of flavonoid synthesis

Accumulation of flavonoids in response to nutrient deficiency to sequester abundant carbon (C) is a prevailing hypothesis (Bergmann, 1993), because sugar accumulation is known to occur under a range of mineral depletions (Cakmak et al., 1994; Hermans et al., 2006; Pourtau et al., 2006; Verbruggen and Hermans, 2013). For example, N deficiency induced anthocyanin accumulation in crops has commonly been explained with high foliar sugar levels (Bergmann, 1993; Keller, 2005). The relationship between N availability, plant growth and flavonoid synthesis is explained in the carbon-nutrient balance hypothesis (Norbaek et al., 2003), which proposes that limited N availability restricts growth more than photosynthesis, resulting in an accumulation of C metabolites from photoassimilation. Consequently, excess C compounds are shunted into secondary metabolism, producing C-based, N-free compounds, in order to decrease the risk of negative feedback inhibition of photosynthetic CO2-assimilation and overreduction of the photosynthetic e<sup>-</sup> transport chain in the thylakoid membranes, which inevitably leads to ROS synthesis. This assumption is supported by the finding that also external sugar feeding or disruption of phloem loading in source leaves induce genes of the flavonoid biosynthesis pathway (Jeannette et al., 2000; Lloyd and Zakhleniuk, 2004; Solfanelli et al., 2006; Teng et al., 2005).

Soluble sugars were markedly increased in leaves of Mg-deficient maize seedlings (Fig. 7). Accumulation of photoassimilates is a known phenomenon in response to Mg shortage and may arise from impaired phloem loading (Cakmak *et al.*, 1994; Verbruggen and Hermans, 2013), or decreased metabolic activity in sink tissue (Fischer *et al.*, 1998). Although soluble sugars accumulated in all analysed leaves, the Flav index was only significantly different from the control in leaf 3 and 4 under Mg deficiency (Fig. 3, 6C). Therefore, no causal relationship between these parameters can be deduced from the results. Low N supply resulted in increased glucose and sucrose concentrations in leaf 5 and leaves 3 and 4, respectively (Fig. 7 A, C). Since the Flav index in all leaves was significantly higher than the control at harvest (Fig. 3), sugar induction of flavonoid synthesis in response to N deficiency is possible. The genetic and molecular regulation of nutrient deficiency-induced flavonoid synthesis is poorly understood, especially in crops, and reports about sugar effects on gene expression are contradictory (Lillo *et al.*, 2008). It has been suggested that sucrose is an important factor for the induction of both flavonol and anthocyanin synthesis-related genes (Lea *et al.*, 2007; Olsen *et al.*, 2008; Solfanelli *et al.*, 2006; Stewart *et al.*, 2001; Van den Ende and El-Esawe, 2014;

Zhang *et al.*, 2013), but also glucose-induction of major transcription factors for flavonoid biosynthesis has been reported (Pourtau *et al.*, 2006). Concentrations of soluble sugars increased up to 168 mg g<sup>-1</sup> DW sucrose, 84 mg g<sup>-1</sup> DW glucose and 45 mg g<sup>-1</sup> DW fructose in leaf 3 of Mg-deficient maize seedlings (Fig. 7). However, flavonols and anthocyanins in whole leaf extracts were in the range of few µg g<sup>-1</sup> DW or even below the detection limit for HPLC analysis (data not shown). Considering these quantitative differences in sugar accumulation and flavonoid synthesis in nutrient-deficient maize leaves, it can be assumed that flavonoids are not synthesized as C storage compounds in response to nutrient stress, and might fulfil different ecophysiological functions such as light-attenuation and/or ROS scavenging (Agati *et al.*, 2012; Agati *et al.*, 2013; Gould, 2004). Accumulated soluble sugars may, however, serve as important signalling elements in the genetic regulation of nutrient deficiency-induced flavonoid synthesis.

# Conclusion

This study revealed that epidermal accumulation of UV-A absorbing compounds is not a N deficiency-specific stress response in maize seedlings, but is also induced by Mg shortage. Spatiotemporal monitoring of the Flav index across the leaf lamina over time revealed differences between treatments, showing pronounced accumulation of UV-A absorbing compounds in upper leaves under N deficiency, whereas Flav index increase started at the tip of lower leaves in Mq-deprived plants. Since both nutrient deficiencies had similar effects on ChI and Flav index, the NBI may not be discriminative enough to distinguish between N and Mg deficiency in mature leaves of young maize plants. However, early-stage differentiation between N and Mg deficiency using the Dualex might be possible by measuring the Flav index in young, developing tissue because differences between treatments were most obvious in these leaves. Since the Flav and Chl indices were not closely correlated in nutrient-deficient plants, the biosynthesis of epidermal UV-A absorbing compounds in response to nutrient shortage might have reasons other than only photoprotection. Analysis of soluble sugars in leaves did not provide conclusive information about a causal relation between sugar accumulation and nutrient deficiency-induced flavonoid synthesis. Measuring Chl fluorescence excited at green and red light did generally not provide reliable information about the epidermal anthocyanin levels, presumably due to a methodical flaw caused by Chl fluorescence reabsorption.

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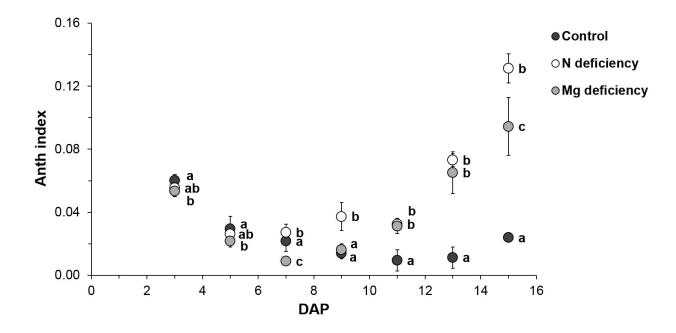
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# Supplementary material

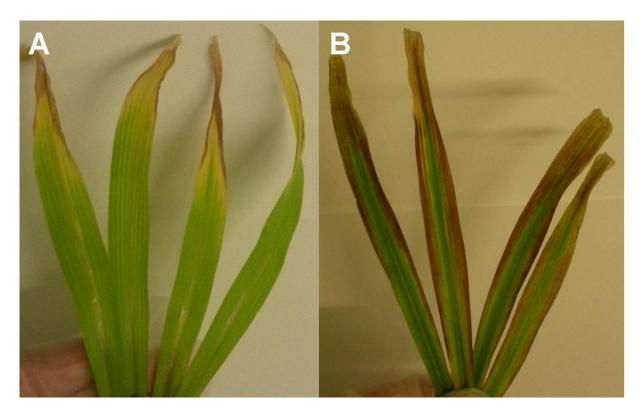


**Figure S1:** Leaf blade of maize seedling grown with reduced Mg supply. Leaf shows interveinal chlorosis and necrosis and unequal anthocyanin accumulation, which are typical Mg deficiency symptoms in monocotyledonous leaves.



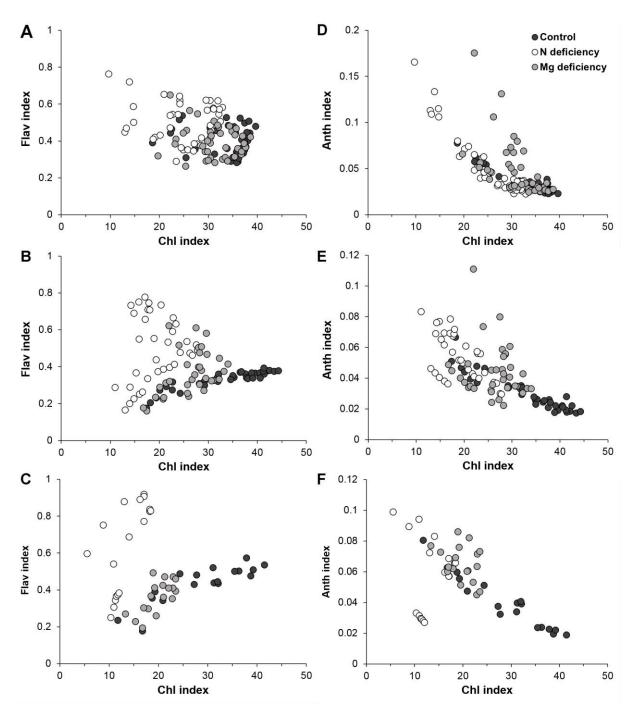
**Figure S2:** Course of Anth index of leaf 3. Control plants were grown under optimal nutrition; N and Mg deficiency was induced by reducing the N or Mg concentration, respectively, in the nutrition solution to 4% of the control. Measurements were taken on four plants of four biological replicates per treatment. Five to six measurements were taken along the leaf blade and averaged. Mean  $\pm$  SE; small letters indicate significant differences between treatments at a measuring date (pairwise t-test with Bonferroni adjustment, p  $\leq$  0.05).

# 4. Early-stage diagnosis of nutrient deficiency in maize leaves



**Figure S3:** Leaves number 3 of 18-days-old maize seedlings grown under (A) N deficiency (4% N of the optimal N supply) and (B) Mg deficiency (4% of the optimal Mg supply). Nitrogen-deficient leaves showed chlorosis and leaf tip necrosis but no signs of purpling, whereas additional anthocyanin accumulation was visible under Mg shortage.

# 4. Early-stage diagnosis of nutrient deficiency in maize leaves



**Figure S4:** Correlations between Chl index and Flav index in (A) leaf 3, (B) leaf 4 and (C) leaf 5 and correlations between Chl index and Anth index in (D) leaf 3, (E) leaf 4 and (F) leaf 5 of maize seedlings. Dualex measurements were taken along the leaf blade and values at individual measuring points were correlated (see Fig. 5 and 6 for Chl and Flav indices).

# 4. Early-stage diagnosis of nutrient deficiency in maize leaves

**Table S1:** Coefficients of determination of simple linear regression (R<sup>2</sup>) of Chl index and Flav index or Chl index and Anth index, respectively, of Dualex measurements taken along the leaf blade of maize seedlings grown under different nutrient regime (see Fig. S4).

	Chl in	dex - Flav in	dex	Chl index - Anth index			
Treatment	Leaf 3	Leaf 4	Leaf 5	Leaf 3	Leaf 4	Leaf 5	
Control	-0.15	0.90	0.86	-0.93	-0.93	-0.96	
N deficiency	-0.13	0.21	0.65	-0.93	-0.63	-0.03	
Mg deficiency	-0.18	0.43	0.69	-0.58	-0.17	-0.35	

5. Plant nutrition and anthocyanins

# **Chapter 5**

# The Relationship between Plant Nutrition and Anthocyanins and their Significance for Agriculture

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to be submitted

#### **Abstract**

Anthocyanins are water-soluble vacuolar plant pigments with red, purple or blue colouration. They are synthesized in different plant tissues such as fruits and leaves and they are of high significance in agriculture. On the one hand, transiently synthesized foliar anthocyanins indicate stressful growing conditions that can negatively affect crop yield and quality. On the other hand, anthocyanins are of importance because they raise the quality and market value of several agricultural commodities such as apples, grapes or other berries, because of their attractive colouration and health-promoting potential.

This review summarizes the current knowledge of the influence of plant nutritional aspects on anthocyanin synthesis and the concomitant physiological changes that alter crop quality and stress resistance. Here we review that a wide range of nutrient deficiencies or mineral toxicity also induces leaf purpling or affects the colouration of fruits and vegetables. This knowledge is of high importance, because farmers commonly, but often erroneously, associate transient foliar anthocyanin formation with a lack of phosphorus or nitrogen. Therefore, special emphasis will be put on the often-neglected multitude of nutrient disorders that cause anthocyanin accumulation and differences between nutrients are highlighted. Additionally, the underlying genetics of foliar anthocyanin formation as induced by nutrient deficiency is briefly explained. The ecophysiological function of anthocyanins under nutrient imbalances is critically discussed, because different concepts about their adaptive significance in response to nutrient disorders exist.

Furthermore, the impact plant nutrition on anthocyanin levels of apples, grapes and strawberries is shown and the potential for optimizing anthocyanin concentrations in fruits by fertilization is discussed. We highlight that agricultural fertilization practices have the potential to influence crop quality and stress resistance by regulating anthocyanin levels in fruits or leaves. This may increase the fruit quality and market value of agricultural goods on the one hand and the stress-resistance of crops in a challenging environment on the other hand.

# 5. Plant nutrition and anthocyanins

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# 1. Introduction

Anthocyanins are red, purple or blue water-soluble vacuolar plant pigments in plants. They belong to the class of flavonoids and are synthesized in the cytosol via the phenylpropanoid pathway. Anthocyanins are glucosides of anthocyanidins and their general structure consists of a flavylium ion backbone with variable numbers of hydroxyl and methoxy groups (Fig. 1). Conjugations with different sugar branches and further sugar acylation with organic acids increase the complexity of these secondary compounds and confer different properties such as colour, stability, and bioactivity (de Pascual-Teresa and Sanchez-Ballesta, 2008; Kovinich et al., 2014; Miguel, 2011). It has frequently been reported that the dietary intake of fruits that are rich in anthocyanins such as berries has got a profound health-promoting effect in humans (Cooke et al., 2005; Ghosh and Konishi, 2007; Kahkonen and Heinonen, 2003; Miguel, 2011; Seeram, 2008). Anthocyanins have attracted considerable attention in cancer prevention and they are used for phytotherapeutic applications (Chen et al., 2006). These beneficial effects are attributed to the antioxidant properties of anthocyanins, which are modulated by different modifications at the basic ring structure (Andersen, 2001; Manganaris et al., 2014; Wang et al., 1997). Anthocyanins can be present in various plant organs such as the leaf, stem, root, tuber, fruit, and seed. Both, endogenous as well as external factors, determine the anthocyanin level in different plant tissue and organs.

The ecophysiological function of anthocyanins depends on in their localization within the plant. For example, the primary function of constitutively expressed anthocyanins in fruits is to attract pollinators and seed dispersal agents. However, anthocyanins are also synthesized temporarily in leaves under environmental stresses, which implies a different functional role in response to stress. Understanding how environmental factors influence anthocyanin synthesis is of high interest in agricultural, horticultural, and ornamental plant production because such knowledge allows for the strategic anthocyanin enrichment in order to grow high-quality products with appealing colour and health-promoting properties. The effects of abiotic factors such as low temperature, high-light and UV irradiation on anthocyanin synthesis have been intensively investigated and thoroughly reviewed elsewhere (Chalker-Scott, 1999; Close and Beadle, 2003; Gould, 2004; Landi et al., 2015; Ramakrishna and Ravishankar, 2011; Steyn et al., 2002). This review will discuss the impact that nutrient deficiencies and mineral toxicities have on leaf purpling and the colouration of fruits and vegetables. In plant production, the occurrence of anthocyanins has a Janus-faced character: On the one hand, anthocyanins in leaves indicate stressful growing conditions that are associated with negative impact on yield. On the other hand, anthocyanins in plant-based products are a quality-determining attribute with the potential to prevent several chronic diseases, such as cardiovascular disease, diabetes and cancer (de Pascual-Teresa and Sanchez-Ballesta, 2008: Tsuda, 2012). Furthermore, the fruit colouration raises the market value of several agricultural commodities such as apples, grapes, and berries. We highlight that anthocyanin levels in fruits or leaves, and consequently crop quality and stress resistance, can be influenced by agricultural fertilization practices by controlling the availability of critical elements.

**Figure 1:** Basic chemical structure of the main anthocyanidins in nature with variable numbers of hydroxyl (-OH) and methoxy (-OCH<sub>3</sub>) groups including ring nomenclature of the flavylium core (A, B, C).

Here we review the current physiological and genetic knowledge of the influence of plant nutrients and toxic elements on anthocyanin synthesis and the concomitant physiological changes that can alter crop quality. Special emphasis is put on the multitude of nutrient disorders that cause anthocyanin accumulation, which is often overlooked. This is the key aspect of this review because even in scholarly literature it is forgotten that anthocyanin accumulation is not only the result of nitrogen or phosphate deficiency. We aim to draw the reader's attention to the subtle details of anthocyanin synthesis by pointing out nutrient-specific differences in respect to the anthocyanin localization and composition. Latest findings of the underlying genetics of leaf anthocyanin accumulation in response to specific plant nutrients or toxic elements are collected. This review further critically discusses the physiological and agricultural significance of anthocyanin synthesis under nutrient disorders. Furthermore, the impact that anthocyanins have on the quality of plant-based agricultural commodities will be exemplified through grapes, apples, and strawberries. Finally, the potential for optimizing the anthocyanin levels in crops through agricultural practices is discussed.

# 2. Foliar anthocyanins – Indicators for nutrient disorders

The symptom of leaf reddening under nutrient deficiency has already been described centuries ago (Bergmann, 1993). In agricultural practice, anthocyanin formation in leaves is usually, but in some cases erroneously, ascribed to a lack of nitrogen or phosphorus. Intriguingly, a wide range of other nutritional disorders can lead to increased anthocyanin synthesis - a fact that is less appreciated and often results in misdirected fertilization in practice. Although being a common phenomenon in the field, unravelling the mechanistic and molecular details of anthocyanin synthesis in leaves induced by nutrient stress have received only very little scientific attention. In this chapter, we focus on the diverse element stresses that can cause anthocyanin accumulation in vegetative parts of agricultural crops. Furthermore, the phenotypic differences between nutrient disorders and species-specific symptomatic variations will be explained. The genetic and molecular regulation of nutrient-induced upregulation of the anthocyanin biosynthesis pathway in crops is still not well understood. The genetic background of this process has mainly been investigated in Arabidopsis thaliana under nitrogen or phosphorus deficiency, and these studies will briefly be explained. Finally, the current knowledge regarding the ecophysiological significance of colour change in nutrient stressed plants will be outlined.

#### 2.1 Nutrient deficiency induces anthocyanins in crop leaves

## Nitrogen deficiency

Reddening or purpling of leaves is a well-known symptom under nitrogen (N) deficiency and has been observed especially in species that are naturally high in anthocyanins like *Brassica* species (Fig. 2), fruiting trees and berry bushes (Bergmann, 1993; Hodges and Nozzolillo, 1996). However, foliar anthocyanin accumulation under N deficiency was also reported in other crops such as cotton, spinach and maize (Bergmann, 1993; Bongue-Bartelsman and Phillips, 1995; Bumgarner *et al.*, 2012; Gade, 2013; Khavari-Nejad *et al.*, 2009; Larbat *et al.*, 2012; Lawanson *et al.*, 1972; Lovdal *et al.*, 2010). Blades, veins, and petioles of older leaves turn purple and this symptom is often preceded by leaf chlorosis (Bergmann, 1993; Gade, 2013). Concomitant chlorophyll breakdown may occur but this is not always the case as the genetic up-regulation of anthocyanin genes has also been measured in stay-green cultivars of rapeseed under N starvation). QTL mapping in N-deficient plants suggests that anthocyanin synthesis and leaf yellowing are two genetically separate pathways and plants respond to N limitation by promoting both processes independently (Diaz *et al.*, 2006).

Most often, anthocyanin accumulation is not uniform in different tissues and little is known about cell-specific anthocyanin synthesis under N deficiency. In N-deficient Arabidopsis thaliana leaves, anthocyanins accumulate mainly in the epidermis (Shi and Xie, 2010). Likewise, Hodges and Nozzolillo (1996) observed in Brassica seedlings that red pigment accumulation starts predominantly in the leaf epidermis and the sub-epidermal layer of hypocotyls. Mesophyll cells of these plants also showed pigmentation when the anthocyanin content was high. The authors report that anthocyanins accumulated mainly in so-called anthocyanoplasts or anthocyanin vacuolar inclusions (AVIs). These sub-vacuolar pigment bodies are formed under certain conditions and they may sequester specific anthocyanin species (Chanoca et al., 2015; Pourcel et al., 2010). For example, in grapevine cell cultures, AVIs mainly contained acylated (specifically p-coumaroylated) anthocyanin species (Conn et al., 2003). In Arabidopsis, the formation of AVIs is promoted by an increase in decorated cyanidin 3-O-glucoside derivatives (Chanoca et al., 2015). These findings imply the enrichment of other anthocyanin species in N-deficient Brassica seedlings compared to phosphorus deficiency, where less AVI formation was observed although similar total anthocyanin amounts were detected (Hodges and Nozzolillo, 1996). This assumption is in accordance with investigations in Arabidopsis thaliana, where N deficiency increased the molecular diversity of the anthocyanin profile (Shi and Xie, 2010; Zhou et al., 2012). It is notable that under Ndeficient conditions, the absence of anthocyanins is not necessarily associated with growth reduction or more severe senescence symptoms. For example, several Arabidopsis thaliana transparent testa-mutants that are defective at various steps in the anthocyanin biosynthesis pathway accumulated comparable or even slightly more biomass than the wild-type under N deficiency (Misyura et al., 2013). This, however, was only true for mutants that lack components in the later steps of the anthocyanin synthesis pathway (Fig. 5), whereas mutants deficient in the early steps of anthocyanin biosynthesis (Fig. 5) grew more poorly relative to the wild type control under limiting N conditions. Furthermore, there was no noticeable effect of these mutations on the senescence process or the capacity to remobilize nutrients from chlorophyll breakdown, which raises the question of the physiological function of N stressinduced anthocyanins. The finding that anthocyanin-free Arabidopsis mutants can even accumulate more biomass than the wild type under N limitation raises the question whether anthocyanin production is per se beneficial under these conditions or if anthocyanin-producing plants suffer from metabolic disadvantages that are the result of or are indirectly associated

with an increased anthocyanin synthesis. The assumption that an increased synthesis negatively impacts plant performance in terms of biomass production seems likely because anthocyanins have been shown to reduce the maximum quantum yield for photosynthetic  $O_2$  evolution (Burger and Edwards, 1996). Consequently, the photosynthetic efficiency of red leaves is often lower than that of acyanic leaves under low-light conditions (Burger and Edwards, 1996; Hughes *et al.*, 2014).

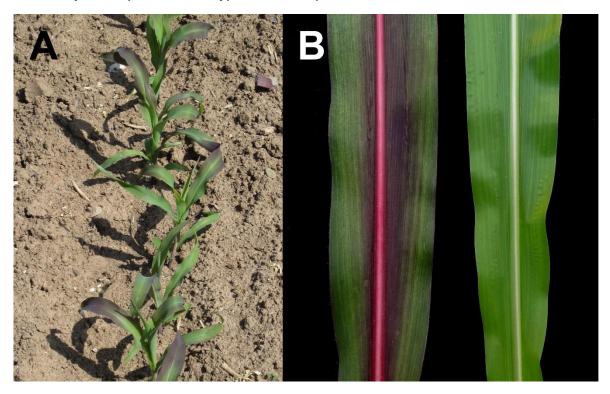


Figure 2: Anthocyanin accumulation in nitrogen-deficient rapeseed.

#### Phosphorus deficiency

Besides N deficiency, phosphorus (P) deficiency is the most well-known elicitor for nutrient stress-induced anthocyanin synthesis. However, both nutrient deficiencies can occasionally be distinguished by contrasting anthocyanin symptoms. For example, under P-deficient conditions particularly the stems, petioles, leaf bases, and leaf veins turn red or purple (Fig. 3) (Bergmann, 1993; Mengel and Kirkby, 2001). Red colouration can also spread across the leaf blade beginning from the edges and in the case of *Poaceae* the leaf ears can turn purple as well (Bergmann, 1993). In contrast to N deficiency where anthocyanin accumulation usually comes along with chlorophyll breakdown, P starvation often causes a growth reduction that results in an increased chloroplast density and the leaves appear blueish-green (Fig. 3A). Furthermore, these two nutrient deficiencies may be distinguished at the leaf veins: during P deficiency, the reddening emerges between the veins whereas under N deficiency it appears along the veins (Barker and Pilbeam, 2007). Interestingly, anthocyanin accumulation was reported to be limited by temperature in P-deficient tomato leaves and anthocyanins were not synthesized in summer when temperatures rose above 30°C (Ulrychova and Sosnova, 1970). Chen et al. (2013) found that anthocyanin production in the flower stalk epidermis of Chinese kale might be regulated by the epidermal pH and the activities of the enzymes phenylalanine ammonia-lyase (PAL) and chalcone isomerase (CHI). The pH measured in the epidermal press sap gradually decreased with decreasing P levels, whereas the enzyme activities increased. However, a general functional or causal relationship between these parameters was not demonstrated.

Cell-type- and tissue-specific accumulation of anthocyanins has been found in P-deficient C<sub>4</sub> species, but little is known about a preferential accumulation of anthocyanins in different tissues under P deficiency in general (Henry *et al.*, 2012; Hernandez and Munne-Bosch, 2015). In contrast to N limitation, only minor accumulation of anthocyanins in vacuolar anthocyanoplasts was found in P-starved *Brassica* species (Hodges and Nozzolillo, 1996). This suggests that N and P deficiencies induce the synthesis of different anthocyanin species because the occurrence of these sub-vacuolar inclusions strongly correlates with specific anthocyanin species (Pourcel *et al.*, 2010). Similar to the results in N-deficient *Arabidopsis* mutants, no differences related to photosynthesis or growth under P deficiency could be found in genetically similar maize and coleus varieties with contrasting anthocyanin content. Therefore, it remains unknown if foliar anthocyanin production has a certain ecophysiological function under P deficiency (Henry *et al.*, 2012) or whether the accumulation of those polyphenols is a secondary effect that results from other metabolic mechanisms that ensue under nutrient deprivation and is therefore a secondary effect. Considering the huge diversity of anthocyanins species, both hypotheses are plausible.



**Figure 3:** (A) Anthocyanin accumulation in phosphorus-deficient maize leaves. (B) Leaves of maize grown in phosphorus-deficient nutrient solution (left) and control leave grown under optimal nutrition (right).

## Magnesium deficiency

In some species such as maize (*Zea mays* L.) foliar anthocyanin accumulation is also a typical response to magnesium (Mg) limitation. Moreover, leaves of several fruiting trees and certain grape varieties with blue fruits (Jacob, 1955). Reddening of cotton leaves has also been attributed to Mg limitation (Chimmad and Panchal, 2001; Gade, 2013; Santos *et al.*, 2010). Furthermore, under experimental conditions with severe Mg restriction, leaf purpling was observed in cowpea, fava bean, rice, roses and maize (Hariadi and Shabala, 2004a, b; Jezek, *unpublished data;* Kobayashi *et al.*, 2013; Premkumar and Kulandaivelu, 2001; Yeo *et al.*,

2013). Mg deficiency induced anthocyanin accumulation occurs usually in older leaves, starts at the leaf margins and is most often preceded or accompanied by chlorophyll breakdown (Fig. 4) and, dependent on the severity of the Mg-deficiency, by necrosis. This gradual appearance coincidences with the Mg depletion in plant tissue under Mg-deficient conditions because Mg is translocated acropetally from older to younger leaves to enable plant development. In Mg-deficient maize, these symptoms differ from P deficiency-induced leaf purpling, where anthocyanin accumulation is predominantly observed in and near the midrib of the leaf (Fig. 3B). Although anthocyanin synthesis under Mg deficiency has often been described, the underlying biochemical mechanisms remain unclear (Kobayashi *et al.*, 2013)(Kobayashi, 2012) and further research is needed on this topic.



**Figure 4:** Leaves of maize (*Zea mays* L.) grown hydroponically with optimal Mg supply (A; 0.5 mM MgSO<sub>4</sub> in the nutrient solution) or under Mg-deficient conditions (B; 0.1 mM MgSO<sub>4</sub> in the nutrient solution).

#### Sulphur deficiency

Sulphur (S) is another plant nutrient that, upon deficiency, has been shown to induce leaf purpling in e.g. rapeseed (Bergmann, 1993; D'Hooghe *et al.*, 2013; Schnug, 2004). In contrast to N and Mg limitation, S deficiency-induced anthocyanin formation first appears in younger leaves (Bergmann, 1993; D'Hooghe *et al.*, 2013). Controversial findings regarding S deficiency-induced anthocyanin synthesis exist in *V. vinifera* (Tavares *et al.*, 2013). Several genes of the anthocyanin synthesis pathway were up-regulated and anthocyanins accumulated in S-deficient *V. vinifera* plantlets but these findings could not be confirmed in cell cultures. Very small increases in the anthocyanin concentration were detected in S-deficient rice but mRNA levels of anthocyanidin reductase genes were not increased (Lunde *et al.*, 2008). The discrepancy in these results could be caused by species-specific differences, experimental setups, and/or a more complex regulation where carbon to nitrogen balance is involved (Gao *et al.*, 2015).

#### Other mineral elements

Anthocyanins were also reported to accumulate during the deficiencies of micronutrients. For example, *Brassica* and *Trifolium* species and red salad varieties showed red-purple leaf

phenotypes upon boron deficiency (Bergmann, 1993; Hajiboland and Farhanghi, 2010). Copper deficiency lead to anthocyanin accumulation in roots of common bean (*Phaseolus vulgaris* L.) (Naya *et al.*, 2014) and Rahimi and Bussler (1975) showed evidence of red leaf colouration in zinc deficient maize plants. To our best knowledge, no current studies have investigated the underlying genetic regulations of micronutrient deficiency-induced anthocyanin biosynthesis.

# 2.2 Leaf-purpling as a sign of toxicity

Not only deficiency but also surplus of plant nutrients or metal toxicity is known to elicit physiological stress and induces anthocyanin synthesis in crops. Consistent with nutrient deficiency, this symptom has been particularly observed in the leaves of species that already have relatively high anthocyanins concentrations. For example, tomatoes show increased leaf purpling under molybdenum toxicity (Bergmann, 1993). The content of anthocyanins increases in red cabbage with rising copper concentration (Posmyk et al., 2009) and tended to increase upon combined application of the heavy-metals arsenic, cadmium and lead in red basil (Ruzickova et al., 2015). Likewise, purple-leafed cultivars of sweet basil accumulated more anthocyanins when grown under boron stress (Landi et al., 2014). Boron toxicity is known to inhibit photosynthesis, probably caused by a combination of factors such as oxidative damage, reduced photosynthetic enzyme activities and impaired electron transport capacity (Han et al., 2009). Acyanic basil plants showed a significantly lower maximum potential quantum efficiency of PSII as estimated by fluorescence measurements than pigmented plants after high-light treatment. Covering the green basil plants with a purple polycarbonate filter, whose optical properties approximated those of anthocyanins, during the high-light exposure abolished differences in the decline of the maximum PSII quantum efficiency between the two cultivars were abolished. Therefore, the author conclude that anthocyanins in red basil have a lightscreening and hence photoprotective function that mitigates boron toxicity.

High concentrations of aluminum in nutrient solution caused enhanced accumulation of anthocyanins in hypocotyls of buckwheat seedlings (Horbowicz et al., 2011). Experiments with diverse non-crops such as Arabidopsis, Azolla imbricate, poplar and several metal hyperaccumulator Thlaspi species revealed that the heavy metal elements lead, mercury and cadmium and excess levels of manganese, copper and zinc-induced anthocyanin synthesis (Baek et al., 2012; Dai et al., 2012; Pongrac et al., 2009; Zhang et al., 2014a). The supposedly ameliorating function anthocyanins fulfil under nutrient toxicity is not fully elucidated, but there is emerging evidence that they chelate metals and/or increase the stress-related antioxidant capacity (Baek et al., 2012; Glinska et al., 2007; Landi et al., 2015). The molecular structure of anthocyanins enables them to form complexes with metal ions and the formation of so-called metallo-anthocyanins with central Fe<sup>3+</sup>, Ca<sup>2+</sup>, Mg<sup>2+</sup> or Al<sup>3+</sup> ions are even responsible for the blue colour of several flower petals (Ellestad, 2006; Takeda et al., 1990). Although metalloanthocyanins naturally occur in reproductive organs such as flowers, there is little experimental evidence that these complexes also function as chelators for toxic elements in vegetative tissue. It has been shown in vitro, that both synthetic and natural anthocyanins are capable of forming complexes with the phytotoxic elements aluminium, gallium and cupric ions in aqueous solution (Buchweitz et al., 2012; Elhabiri et al., 1997; Noda et al., 2000; Somaatmadja et al., 1964), but this has not yet been confirmed in planta. Noda et al. (2000) revealed that anthocyanins can efficiently inhibit ROS stress by chelating iron. The authors studied the inhibitory effect of lipid peroxidation of nasunin, an anthocyanin derivate found in eggplants. They found a potent scavenging activity of  $O_2$  but no scavenging activity of OH. However, nasunin was shown to chelate free Fe3+ ions and therefore, the authors conclude that the

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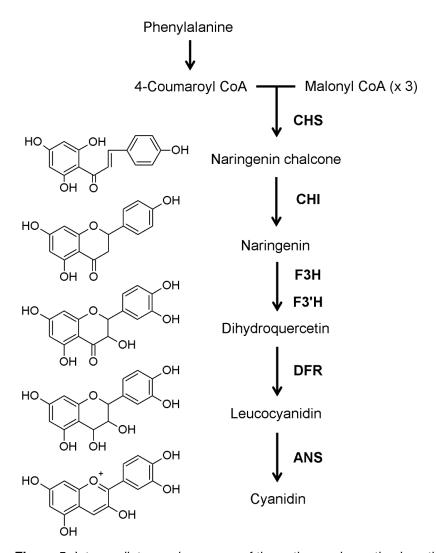
anthocyanin nasunin inhibits 'OH formation by chelating iron. Several other studies indicate that anthocyanins function as chelators to immobilize and sequester toxic ions in the vacuole. For example, *Brassica* species supplied with excess molybdate accumulated blue compounds in the vacuoles of peripheral cell layers (Hale *et al.*, 2001). Similar results were obtained in *Brassica* species supplied with tungsten and the blue compounds that were formed were identified as tungsten-anthocyanin complexes. However, accumulation of anthocyanins in response to tungsten supply had no effect on the root growth under the prevailing experimental conditions, indicating that the anthocyanin synthesis was not directly correlated with tungsten tolerance (Hale *et al.*, 2002). Chelation by a ligand and subsequent compartmentalization is a general mechanism for immobilizing heavy metals or xenobiotica in plants (Hasan *et al.*, 2009). However, the hypothesis that anthocyanins may function in such a way *in planta* needs further experimental validation.

It has been proposed that plant species rich in anthocyanins could be used for metal phytoremediation to purify contaminated environments. For example, anthropogenic soil contamination with cadmium has become a serious environmental challenge that negatively impacts human health worldwide (Davis, 1984; Ravera, 1984). Zhang *et al.* (2014a) suggest using fast growing coloured-leaf trees like red poplar cultivars for purification of contaminated soil from cadmium. According to the authors, this would be an aesthetically pleasing, low-cost alternative to traditional technology of soil remediation with beneficial environmental and landscape effect. However, when applying this strategy when using deciduous trees such as poplars, it is highly likely that wind distributes the metal-containing leaves throughout ecosystems. Hale *et al.* (2002) proposed that existing species that are already used for metal phytoremediation could be engineered to produce more anthocyanins by overexpressing enzymes of the anthocyanin biosynthesis pathway in order to improve their efficiency in metal sequestration.

Experimental evidence showed that the leaf anthocyanin concentration might reflect the selenium toxicity degree in maize plants, and hence be potentially useful as bioindicators of selenium stress (Hawrylak-Nowak, 2008). However, as outlined above, anthocyanin accumulation can be induced by a multitude of nutritional disorders and therefore the exact differentiation of nutritional disorders by anthocyanins remains an ambitious future target.

# 2.3 Genetic regulation of foliar anthocyanin synthesis induced by nutritional disorders

In the recent years, genetics has revealed new insight into the specific transcriptional regulation of the anthocyanin biosynthetic pathway in *Arabidopsis thaliana* as influenced by the nutrient status. A detailed overview of the effects of N and P deficiency on the expression levels of all major enzymes of the shikimate and flavonoid pathway including the structural genes encoding the enzymes relevant for anthocyanin synthesis in vegetative plant parts was presented by Lillo *et al.* (2008). Besides the early genes of the phenylpropanoid pathway that encode enzymes required to synthesize anthocyanin intermediates, N and P deficiency strongly induce the genes of late enzymes of the anthocyanin synthesis pathway, namely dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) (Fig. 5). Moreover, genes responsible for anthocyanidin glycosylation and sequestration in the vacuole are highly up-regulated.



**Figure 5:** Intermediates and enzymes of the anthocyanin synthesis pathway illustrated for the example of cyanidin. 'Early steps' (according to Misyura, 2013) – CHS: chalcone synthase, CHI: chalcone isomerase; 'late steps' (according to Misyura, 2013) – DFR: dihydroflavonol 4-reductase, F3H: flavonon 3-hydroxylase, F3'H: flavonoid 3-hydroxylase, ANS: anthocyanidin synthase.

Besides the structural genes encoding enzymes of the anthocyanin synthesis pathway a range of regulatory factors are involved in the anthocyanin formation upon N deficiency and have been characterized recently. A complex hierarchical network of diverse transcription factors is thought to determine the class and localization of the flavonoid derivatives produced (Broun, 2005; Gonzalez *et al.*, 2008; Tsay *et al.*, 2011; Zhou *et al.*, 2012; Zimmermann *et al.*, 2004). Together, these so-called MBW-complexes (named after the transcription factor families MYB, bHLH and WD40) activate the expression of several structural anthocyanin genes and they are differently induced in response to a particular stimulus (Gonzalez *et al.*, 2008; Tsay *et al.*, 2011). Not only the total level but also the molecular diversity of anthocyanins is controlled by the action of these transcription factors (Zhou *et al.*, 2012). Particularly the Myb transcription factor PAP2 (production of anthocyanin pigment2) and the bHLH (basic helix-loop-helix) transcription factor GL3 (GLABRA3) are of major importance for the induction of anthocyanin synthesis in *Arabidopsis* under nutrient depletion (Bi *et al.*, 2007; Krapp *et al.*, 2011; Lea *et al.*, 2007a; Lillo *et al.*, 2008; Peng *et al.*, 2007a; Scheible *et al.*, 2004). Besides inducing structural anthocyanin genes, GL3 is involved in the negative feedback-regulation that leads to a halt in

N starvation-induced anthocyanin production as soon as the level of anthocyanins has reached its optimum (Nemie-Feyissa *et al.*, 2014). Furthermore, N-induced transcription factors from the LATERAL ORGAN BOUNDARY DOMAIN (LBD) gene family act as negative regulators of anthocyanin synthesis in *Arabidopsis thaliana* (Rubin *et al.*, 2009; Zhou *et al.*, 2012). Next to repressing many other known N-responsive genes, these transcription factors suppress the key regulators of anthocyanin synthesis *PAP1* and *PAP2* in the presence of N. Wang *et al.* (2015) report that ROOT HAIR DEFECTIVE3, an ER-membrane localized protein, negatively regulates N deprivation-induced anthocyanin synthesis presumably in consequence of ethylene signaling. The gaseous plant hormone ethylene is transiently produced in response to N starvation and negatively impacts anthocyanin formation in Arabidopsis (Wang *et al.*, 2015). Similarly, P starvation-induced bHLH transcription factor has been characterized that might act as a negative regulator of anthocyanin formation (Chen *et al.*, 2007). The existence of such feedback inhibitions strongly indicates that anthocyanins are produced in a fine-tuned manner as a result of a metabolic adaptation to nutrient stress. It is therefore possible that anthocyanin accumulation does confer a physiological advantage to the plant.

Peng et al. (2007b) ascertained that the ubiquitin ligase gene NLA (nitrogen limitation adaptation) is an essential molecular regulator in the specific reaction to N deficiency. Nitrogen-deprived nla mutants do not show the essential adaptive responses including anthocyanin synthesis (Peng et al., 2007a; Peng et al., 2007b). Obviously, repression of the flavonoid branch in the phenylpropanoid pathway in N-deficient nla mutants channels the metabolic flux towards lignin synthesis instead (Peng et al., 2008). Interestingly, anthocyanin synthesis induced by P deficiency is not impaired in nla mutants. Therefore, the nla gene seems to be particularly involved in the specific acclimatization reaction in response to N deficiency (Peng et al., 2008). The genetic regulation of nutrient deficiency-mediated anthocyanin synthesis is even more complicated by the finding that phytohormones modulate the anthocyanin synthesis during stress (Jiang et al., 2007; Trull et al., 1997; Zhang et al., 2014b). For example, abscisic acid is required for full activation of the anthocyanin biosynthesis pathway under P deficiency in Arabidopsis (Trull et al., 1997) and also gibberellic acid modulates P starvation-induced changes in anthocyanin accumulation (Jiang et al., 2007). The vast majority of findings that describe the details of the anthocyanin biosynthesis pathway and its regulation under nutrient imbalances are based on Arabidopsis thaliana and only few studies examined this response in agricultural crops. An up-regulation of several key enzymes and transcription factors of the flavonoid metabolism has been documented in N-deficient tomato leaves (Bongue-Bartelsman and Phillips, 1995; Lovdal et al., 2010) and transcriptomic analysis of winter oilseed rape also revealed increased expression of anthocyanin synthesisrelated genes during N deprivation (Koeslin-Findeklee et al., 2015). Furthermore, the anthocyanin accumulation in apple leaves under N and P deficiency was attributed to a reduction of PAL-IS (phenylalanine ammonium lyase-inactivating system) and a concomitant increase in PAL (phenylalanine ammonium lyase) activity. The latter catalyzes a key reaction in the synthesis of phenylpropanoids, including anthocyanins (Tan, 1980). However, the underlying genetic mechanisms of nutrient-induced anthocyanin synthesis are poorly understood in crops until now and need thorough exploration (Bongue-Bartelsman and Phillips, 1995; Lei et al., 2011; Lillo et al., 2008). A better understanding of anthocyanin-based processes in crops is urgently needed because a direct transfer of findings in Arabidopsis to crop plants might be complicating not just because of general differences among species. Moreover, Arabidopsis has a lower requirement for the nutrients that are known to induce anthocyanin accumulation as compared to crop plants that are bred for high biomass and yield formation. Therefore, the underlying genetic mechanisms that are responsible for the

anthocyanin biosynthesis may be partially different from those in *Arabidopsis*, especially when it comes to colour formation in fruits such as apples and berries. This is the case for example for ethylene induced anthocyanin synthesis. This gaseous plant hormone promotes the anthocyanin accumulation in grapes (El-Kereamy *et al.*, 2003) but suppresses anthocyanin formation in *Arabidopsis* leaves (Das *et al.*, 2012).

# 2.4 Physiological functions

The physiological function of leaf purpling in nutrient stressed plants is not consensually resolved and remains to some extent obscure (Lillo *et al.*, 2008). There is a growing interest in understanding the mechanisms by which anthocyanins help plants to adjust their metabolism to abiotic and biotic stress, because it might enable crop breeders to select for traits that are advantageous under changing environmental conditions. As outlined in the previous sections, foliar anthocyanin accumulation is observed under a range of diverse nutritional disorders that impact various metabolic processes. Therefore and because of the fact that evolution gave rise to a structural diversification of anthocyanins in response to various biotic and abiotic factors it is assumable that anthocyanins confer different functions, although a unified function in photoprotection has also been proposed (Steyn *et al.*, 2002). In this chapter, potential roles of anthocyanins that are synthesized in response to nutritional disorders are discussed with special regard to differences between nutrient stresses.

## Sugar sequestration

Nutrient deficiencies often induce growth inhibition with simultaneously ongoing Subsequently accumulated photoassimilates photosynthesis. negatively impact photosynthesis (Martin et al., 2000). Since anthocyanins can be highly decorated with sugar moieties (Zhao et al., 2014), they represent ideal alternative carbon sinks for sugar sequestration, when source carbon levels exceed utilization and/or transport, as is the case under several nutrient deficiencies (Cakmak et al., 1994a, b; Hermans et al., 2006; Pourtau et al., 2006). Moreover, anthocyanins could conceivably act as temporary carbon stores until nutrient limitation and growth retardation are amended, which would lead to an immediate degradation of anthocyanins again (Hughes et al., 2005). In this view, nutrient deficiency induced leaf purpling in crops has commonly been explained with high foliar sugar levels (Bergmann, 1993). This assumption is based on the carbon-nutrient balance (CNB) hypothesis, which states that in case of high internal carbon-nutrient ratios, photosynthates are primarily used for the production of C-based metabolites such as phenylpropanoids, including anthocyanins (Bryant et al., 1983; Larbat et al., 2014). Accordingly, anthocyanin biosynthesis serves as an 'energy overflow' mechanism by diverting photosynthates and cellular reducing power into stable product pools in order to sustain photosynthesis without sequestering any N or P (Grace and Logan, 2000; Hernandez and Van Breusegem, 2010). Indeed, anthocyanin levels have been shown to be correlated to soluble sugar concentrations under N and P deficiency (Khavari-Nejad et al., 2009). Further evidence supporting this theory is that anthocyanin synthesis can also be induced by external sugar feeding (Kumar and Sharma, 1999; Murakami et al., 2008; Rajendran et al., 1992) or artificial disruption of phloem loading in source leaves (Botha et al., 2000; Jeannette et al., 2000; Lloyd and Zakhleniuk, 2004) and the major regulating transcription factors for anthocyanin synthesis respond positively to increased sucrose concentrations (Lloyd and Zakhleniuk, 2004; Solfanelli et al., 2006; Teng et al., 2005). This indicates a direct linkage between nutrient deficiency induced sugar accumulation and anthocyanin synthesis. The assumption that anthocyanins function as alternative metabolites for C sequestration has especially been put forward for N deficiency. In plants growing in highly diluted nutrient solution anthocyanin formation is inhibited by urea or ammonium nitrate addition in a dose-dependent manner, revealing that anthocyanin formation is mainly a response to a high C-N ratio (Kumar and Sharma, 1999). According to the Protein Competition Model (PCM), protein and phenolic synthesis are inversely correlated because they compete for the limiting resource phenylalanine (Jones and Hartley, 1999; Phillips and Henshaw, 1977). Under N limitation, leaf proteins are degraded and leaf N is transported to the roots in the phloem for enhancing root growth and increasing N uptake (Marschner et al., 1996). The excess C skeletons that are produced when phenylalanine is deaminated in the leaves are shunted to flavonoid formation, including anthocyanins (Bonque-Bartelsman and Phillips, 1995; Hodges and Nozzolillo, 1996; Margna, 1977; Margna et al., 1989; Stewart et al., 2001). In this light, Rajendran et al. (1992) explained anthocyanin accumulation in N- and P-deficient Daucus carota callus cells to be a consequence of reduced growth, resulting in mobilization of precursors such as phenylalanine from primary metabolism to secondary metabolism. Following this, anthocyanins would not be produced because of their advantageous properties under stress but simply for maintaining the chemical equilibrium between phenylalanine and its deaminated precursor.

An additional advantage of phenylpropanoid synthesis has been suggested under P deficiency (Grace and Logan, 2000). Usually, phosphorylated photosynthetic C intermediates such as triosephosphate are used for the synthesis of monomeric carbohydrates that sequester internal phosphate pools. However, when phosphorylated intermediates are used for the synthesis of phenylpropanoid precursors, P is released inside the chloroplast before the products are exported into the cytosol. Therefore, phenylpropanoid synthesis under P deficiency features the dual benefits of C sequestration and P recycling in the chloroplast. Carbon skeletons are removed which avoids feedback inhibition of photosynthesis and inorganic P is released which allows its recycling in phosphorylation cycles that take place in the chloroplast.

However, several objections challenge the function of nutrient deficiency induced anthocyanins as C sinks. Considering the quantitative differences in anthocyanin production and sugar accumulation in leaves under nutrient deficiency, the extent to which anthocyanins can contribute to sugar sequestration is presumably inconsiderable in most species. Sugar levels increase by several mg per g fresh weight when plants grow under nutrient stress (Cakmak et al., 1994a; de Groot et al., 2003; Hermans et al., 2004; Jezek, unpublished data), whereas anthocyanin concentrations rise by only a few hundred ug per g fresh weight in nutrient deficient leaves (Juszczuk et al., 2004; Larbat et al., 2012; Lovdal et al., 2010). Thus, based on a C balance calculation, Henry et al. (2012) considered anthocyanins not to be significant C sinks under P deficiency. Moreover, anthocyanins that accumulated upon nutrient deficiency are not readily degraded after amelioration of the nutrient deficiency (Kumar and Sharma, 1999). Thus, they are unlikely to serve as reversible C stores. Furthermore, the assumption that the synthesis of N-free anthocyanins instead of N-containing metabolites under N deficiency ignores the N demand for the synthesis of the several anthocyanin-synthesizing enzymes. Moreover, anthocyanins increase disproportionately to other flavonoids in Nstressed tomato leaves (Bongue-Bartelsman and Phillips, 1995), which points to a more specific adaptive significance than only C sequestration. Therefore, increased internal sugar concentration could be necessary for anthocyanin gene expression by acting as a signaling element (Hughes et al., 2005; Koeslin-Findeklee et al., 2015), but the direct functional relationship between carbohydrate and anthocyanin accumulation in terms of C storage remains unlikely given the arguments above. Furthermore, differences exist in the transcriptional regulation of sugar-induced or nutrient deficiency-induced anthocyanin

synthesis. The sucrose-induced anthocyanin synthesis pathway is controlled by the transcription factor PAP1 (Lloyd and Zakhleniuk, 2004; Peng *et al.*, 2008; Solfanelli *et al.*, 2006; Teng *et al.*, 2005), whereas under N deficiency the transcription factor PAP2 is mainly responsible for structural gene induction (Lea *et al.*, 2007b; Peng *et al.*, 2007a) These findings reveal a more complex regulation of nutrient deficiency induced anthocyanin gene expression, reaching beyond sugar accumulation. The finding that anthocyanin patterns synthesized under P deficiency differ from those induced by external sugar-feeding supports this assumption (Kovinich *et al.*, 2014). Remarkably, sugar accumulation is also a common symptom of K deficiency (Cakmak *et al.*, 1994a; Gerardeaux *et al.*, 2010; Hermans *et al.*, 2006; Lavon *et al.*, 1995). However, K deficiency is usually not accompanied by leaf purpling (Hodges and Nozzolillo, 1996). This inconsistency questions the hypothesis of anthocyanins being a sink for excess C under nutrient deficiency.

# Osmotic regulation

Closely related to the C sink hypothesis is the assumption that anthocyanins are produced as osmotic metabolites under nutrient deficiency. Chalker-Scott (2002) hypothesizes that many nutrient deficiencies are either directly or indirectly associated with water uptake or water retention, leading to the development of drought-associated characteristics such as anthocyanin accumulation. However, the anthocyanin concentration is presumably far too low to fulfil such role (Hughes *et al.*, 2013). Compatible osmolites such as proline accumulate in the order of mg per g fresh weight (Verbruggen *et al.*, 1996) but anthocyanins increase only by a few hundred µg per g fresh weight (Juszczuk *et al.*, 2004; Larbat *et al.*, 2012; Lovdal *et al.*, 2010).

## Signalling

Pourcel *et al.* (2013) reported that the anthocyanin intermediate naringenin induces a gene encoding a high-affinity nitrate transporter. Speculatively, high or fast accumulation of certain anthocyanin intermediates may carry sensory information to signal low N. Hence, specific anthocyanins may be formed to modulate the stress response pathway under N starvation. According to Van den Ende and El-Esawe (2014), the intriguing hypothesis stating that intermediates or final products of anthocyanin synthesis function as "secondary" signals under nutrient stress opens a new era of research in plant stress physiology.

## Photo-protective function

Anthocyanins are believed to provide photoprotection via light attenuation and/or antioxidant activity under several conditions that imply energetic imbalances such as high-light stress, UV-radiation or low temperatures (Burger and Edwards, 1996; Close and Beadle, 2003; Grace and Logan, 2000; Landi *et al.*, 2015; Steyn *et al.*, 2002). Anthocyanins have the potential to mitigate photo-oxidative injury in leaves, both by shielding chloroplasts from excess high-energy quanta, and by scavenging reactive oxygen species (Neill *et al.*, 2003). Anthocyanins absorb visible light mainly in the green region of the light spectrum. They can efficiently protect leaves from light stress by absorbing excess photons that would otherwise be intercepted by chlorophyll (Gould, 2004; Hughes *et al.*, 2014; Nichelmann, 2014). Anthocyanin-containing leaves often have a reduced pool of xanthophyll-cycle components which indicates a lower amount of non-photochemical quenching (NPQ) (Kytridis *et al.*, 2008; Manetas *et al.*, 2002). Non-photochemical quenching is a photoprotective mechanism converting excess excitation energy into heat that is harmlessly dissipated. Reducing the amount of NPQ under high-light

conditions can also be achieved by shielding acyanic leaves with a purple polycarbonate filter that imitates the light-absorption of anthocyanins (Landi *et al.*, 2012). The finding that anthocyanins are mainly localized in epidermis cells or mesophyll cells below the epidermis supports the assumption that anthocyanins function as light-shields.

Nutrient deficiency often induces chlorophyll breakdown in leaves and therefore nutrient stress-induced anthocyanin synthesis for light-attenuation in order to protect the sensitive photosystems from energetic over-excitation is highly reasonable. However, studies that exclusively link nutrient deficiency with anthocyanin-mediated photoprotection are scarce especially in crops. The photoprotective function of anthocyanins in combination with low N concentrations has been described in the research of winter-reddening or autumnal purpling of trees preceding leaf abscission (Carpenter et al., 2014; Kytridis et al., 2008; Nikiforou et al., 2011; Schaberg et al., 2003). These studies suggest that photoprotective anthocyanins might extend the lifespan of senescing leaves and thus enable extensive nutrient retrieval, which is also advantageous under nutrient deficiency. However, these investigations are partly influenced by other anthocyanin-inducing factors such as cold temperature. The finding that sufficient light intensity is a prerequisite for N deficiency induced anthocyanin synthesis might be evidence for a light-attenuating function under nutrient limitation (Lea et al., 2007b; Shi and Xie, 2010). However, no noticeable effect on the senescence process or the capacity to remobilize nutrients could be found in N-deficient Arabidopsis mutants defective at various steps in the anthocyanin biosynthesis pathway (Misyura et al., 2013). These findings question the hypothesis, that the photoprotective function of anthocyanins is essential for a tightly controlled and efficient chlorophyll breakdown and nutrient retrieval during leaf senescence induced by N starvation (Koeslin-Findeklee et al., 2015). The functional significance of anthocyanins as light-attenuators under nutrient stress seems likely especially if leaf purpling occurs simultaneously with chlorophyll degradation for example under N or Mg deficiency. However, in case of P deficiency, which leads to a spatial chlorophyll accumulation, this protective effect seems unlikely. Accordingly, no consistent evidence for a photosynthetic advantage of anthocyanin production could be found in P-deficient maize (Henry et al., 2012). Additionally to their potential role in light attenuation, anthocyanins can scavenge reactive oxygen or nitrogen species (ROS, RNS) because of their antioxidant activity (Gould et al., 2002; Kytridis and Manetas, 2006). The relative significance of anthocyanins for ROS scavenging has been outlined in detail elsewhere (Agati et al., 2013; Gould, 2004; Gould et al., 2002; Grace and Logan, 2000; Landi et al., 2015; Yamasaki, 1997). Nutrient deficiency or toxicity most often results in limited capacity for energy utilization and is usually accompanied by ROS production due to incomplete or blocked light reaction. Hence, anthocyanins might alleviate photo-oxidative damage and photo-inhibition in response to nutrient stress by scavenging reactive oxygen species. For example, increased anthocyanin accumulation and enhanced peroxidase activity were explained as an adaptation to ROS stress in P-starved tomato leaves and bean plants (Juszczuk et al., 2004; Khavari-Nejad et al., 2009). However, anthocyanins often reside only in epidermis cells and therein they are exclusively found in the vacuole. Therefore, the pigments are not optimally localized to scavenge ROS that are produced primarily in other subcellular compartments such as chloroplasts and mitochondria. Hence, the relevance of anthocyanins as scavenging agents is thought to be of minor relevance (Hernandez et al., 2009).

It is unlikely that anthocyanins quench ROS that have a short lifetime, such as hydroxyl radicals and singlet oxygen, because of the discrepancy between the primary site of their formation inside the chloroplast and the anthocyanin localization inside the vacuole (Nichelmann, 2014). However,  $H_2O_2$  has got a longer lifetime and hence a wider radius of damage. Severe stress

such as nutrient deficiency or metal toxicity can lead to massive generation of H2O2 and considerable amounts of H<sub>2</sub>O<sub>2</sub> can enter the vacuole either by passive diffusion or through tonoplast intrinsic proteins (TIPs) (Agati et al., 2013; Bienert et al., 2006; Fini et al., 2011). Hence, a vacuolar scavenging system could still be important under these conditions in order to increase the detoxification capacity (Bienert et al., 2006; Gould et al., 2002; Yamasaki, 1997). This "secondary antioxidant system" is supposed to be activated upon the depletion of primarily defenses in other cell compartments such as the glutathione-ascorbate cycle (Agati et al., 2013; Bienert et al., 2006). This secondary system comprises vacuolar peroxidases that reduce entering H<sub>2</sub>O<sub>2</sub> using flavonoids such as anthocyanins as electron donor (Bienert et al., 2006). Subsequently, flavonoid radicals are recycled back to their original form by ascorbate (Agati et al., 2013). Agati et al. (2013) stress that even the reduction of small amounts of H<sub>2</sub>O<sub>2</sub> is of high physiological importance because H<sub>2</sub>O<sub>2</sub> is a signaling molecule capable of inducing programmed cell death. It has been shown that anthocyanins can scavenge H<sub>2</sub>O<sub>2</sub> in the presence of a peroxidase in vitro (Yamasaki, 1997) and comparisons between the H<sub>2</sub>O<sub>2</sub> scavenging efficiency of cyanic and acyanic leaf mesophyll cells indicate that this is also the case in planta (Gould et al., 2002). Likewise, it has been shown that mesophyll-localized anthocyanins increase the resistance to methyl viologen, which induces ROS formation at the PSI (Kytridis and Manetas, 2006; Nichelmann, 2014). Hence, anthocyanins may be efficient ROS scavenger in vivo, provided that they are in close vicinity with the source of ROS production. Furthermore, Arabidopsis mutants defective in anthocyanin synthesis under N limitation show up-regulation of genes that are involved in ROS and radical scavenging (Peng et al., 2007a), implying that other detoxification mechanisms are induced to compensate for the lack in anthocyanins. However, we note that not all anthocyanins exhibit the same potential to act as ROS scavengers, because this greatly depends on their chemical structure. Hydroxylation and/or methoxylation on the C ring and glycosylation patterns affect the antioxidant activity of anthocyanins. This structure-activity relationship has been explained in detail by Rice-Evans et al. (1996) and Miguel (2011), and individual anthocyanins derived from several plant foods were analyzed and ordered by their antioxidant capacity (Baskan et al., 2015). These findings reinforce the aforementioned necessity to investigate the very complexity of anthocyanin profiles that are formed under different nutrient imbalances in order to unravel their physiological function in nutrient stressed plants.

#### 2.5 Interim conclusion

The purple colouration of crop leaves can be used for diagnosis of nutrient deficiencies if other abiotic factors such as suboptimal growing temperature can be precluded as causes for anthocyanin synthesis. However, in contrast to common belief, foliar anthocyanin accumulation is not only caused by insufficient N or P availability but by a multitude of nutrient deficiencies or element toxicities. This has to be considered when anthocyanins are used as indicators for the type of nutrient stress. Organ- and tissue-specific differences in anthocyanin accumulation were observed under N or P deficiency, but other nutrient deficiencies partly affect the same organs. Therefore, focusing on more subtle details such as "anthocyanin fingerprints" that describe various anthocyanin varieties in different organs and tissues is necessary to better ascribe anthocyanin occurrence to a specific element stress. Investigating the very complexity of anthocyanins profiles that are formed under different nutrient disorders can also help to unravel the physiological function of the observed leaf purpling in nutrient stressed plants, which is not consensually resolved yet. There is increasing evidence that anthocyanins play more important roles in stress adaptation or even stress signaling. Taking into account the structure-activity relationship it is suggested that anthocyanin production is

## 5. Plant nutrition and anthocyanins

more than just an efficient energy outlet under limited growing conditions (Kovinich et al., 2015; Kovinich et al., 2014). Under photooxidative conditions it is highly likely that anthocyanins act simultaneously as light screening compounds and as antioxidative agents in order to protect the photosynthetic apparatus (Nichelmann, 2014). However, differences in the structure of anthocyanins highly impact their properties and functionality, which suggest that they have different biological functions, or that decoration patterns of each anthocyanin are used for unique purposes during stress (Kovinich et al., 2014). However, little is known about the synthesis of distinct anthocyanin patterns and their tissue-specific expression in response to individual nutrient disorders. Therefore, more experimental verification is needed in order to comprehensively understand the process of anthocyanin expression ecophysiological function under nutrient stress. More detailed knowledge about the genetic and molecular nature of nutrient-stress related anthocyanin synthesis should also unveil, if the different abiotic stresses share a common characteristic that leads to anthocyanin synthesis induction. We primarily suspect sucrose accumulation and/or oxidative stress to be a universal signaling element that induces anthocyanin synthesis under nutrient stress. In combination with other metabolic adjustments that occur in response to different nutritional disorders this common trigger could lead to the fine-tuned synthesis of stress-specific anthocyanin patterns. It is highly likely that phytohormones such as ethylene and gibberellic acid are involved in the complex system of anthocyanin accumulation, because they are differentially synthesized in response to different nutrient stresses and are known to modulate anthocyanin patterns under other abiotic stresses as well (Das et al., 2012; El-Kereamy et al., 2003; Jiang et al., 2007; Trull et al., 1997; Zhang et al., 2014b). Comprehensive knowledge about stress-specific modulation of anthocyanins would allow their utilization as biomarkers for certain nutrient disorders with the goal to optimize fertilization strategies and improve plant fitness.

Furthermore, understanding the genetic and molecular background that links nutrient availability with anthocyanin synthesis might enable anthocyanin enrichment in both fruits and vegetative plant parts by directed fertilization strategies, in order to improve crop quality by triggering the accumulation of health-promoting compounds in crop-based commodities. This aspect will be addressed in the following.

# 3. Anthocyanin management

Increasing the anthocyanin content in fruits and vegetative parts of crops is a highly exciting topic for improving the quality of agricultural goods. An attractive colouration does not only increase consumer acceptance and the market value of fruits, but most importantly, enriching fruits and vegetables with these bioactive compounds is of benefit for human nutrition. The dietary consumption of health-promoting anthocyanins has been associated with protection against some types of cancer and cardiovascular disease (Cooke et al., 2005; Kong et al., 2003; Wallace, 2011). Furthermore, there is evidence that anthocyanins have antiinflammatory activity, scavenge free radicals, promote visual acuity and hinder obesity and diabetes (Butelli et al., 2008). Anthocyanins not only contribute to the colour, market value and wholesomeness of fresh or processed fruits and vegetables, they furthermore represent source for the production of natural food colours using plant cell cultures in food industry (Deroles, 2009; Francis, 1989; Miguel, 2011; Rao and Ravishankar, 2002; Sahai and Knuth, 1985). Increasing concern over the use of artificial food colourants has increased the demand for anthocyanins as a healthier alternative with higher consumer acceptance (Ananga, 2013; Deroles, 2009). Therefore, especially anthocyanins from grapes and other berries are a favoured food additive because of their attractive colour (Francis, 1989; Jeppsson, 2000). Modification of anthocyanin levels in crops can be achieved by conventional breeding or genetic engineering (Butelli et al., 2008; Holton and Cornish, 1995; Manganaris et al., 2014; Petroni et al., 2014). We emphasize that adjusting controllable environmental factors such as nutrient availability has the potential to shift the plants metabolism towards an increased production of these health-promoting polyphenols. Likewise, the expression level of anthocyanins in plant cell cultures can easily be optimized by adjusting the nutrient concentrations in the growing medium. Especially N and P levels seem to be highly important growth factors in controlling the synthesis (Dedaldechamp et al., 1995; Do and Cormier, 1991; Rajendran et al., 1992; Yamakawa et al., 1983) and Mg in increasing the stability of anthocyanins (Sinilal et al., 2011). As outlined above, limitation of major plant nutrients like N and P foster anthocyanin synthesis. Therefore, excessive fertilizer can have detrimental effects on the quality of fruits by lowering the anthocyanin content. Currently, little is known about the use of agronomic fertilization practices to increase plant phenolics including anthocyanins (Tomas-Barberan and Espin, 2001) although they are of high importance in commercially grown fruits and vegetables. Detailed strategies to enrich plant-based foods with beneficial compounds are needed. Apples, grapes and strawberries are the anthocyanin-containing fruit varieties with the highest share of global export value (Huang, 2004). Therefore, the majority of studies on anthocyanin enrichment under varying nutrient regime were conducted in these fruits. This aspect will be addressed in the following. Furthermore, the idea of taking advantage of the protective functions of anthocyanins in leaves by increasing the anthocyanin content via short-term nutrient restriction has been put up for debate and will briefly be summarized here.

#### 3.1 Anthocyanin enrichment in fruits by deliberate fertilization

#### Anthocyanins in grapes

Grape (*Vitis vinifera* L.) is an important crop grown worldwide to produce wine, fresh fruit and derived products (Reisch, 2012). Grape-derived commodities contain polyphenols such as anthocyanins that possess health-promoting activity (Cramer, 2010). Anthocyanins and their oxidation products are antioxidants that are highly abundant in red grape and wine (Mattivi *et al.*, 2002). They are extracted or even enriched during the winemaking process conferring

bifunctional properties to grape and red wine by contributing to the prevention of a number of human illnesses (Giovinazzo and Grieco, 2015). Anthocyanins are among the most important compounds for the quality of the red wine, since they are responsible for major characteristics of this beverage such as hue and colour stability (Keller and Hrazdina, 1998). Their biosynthesis depends on the berry development starting at the onset of ripening (véraison) and reaching maximum level at berry maturity (Zhu *et al.*, 2012). Malvidin derivatives (Fig. 6B) are often the major anthocyanins in grapevines (Reynolds, 2015) but each grape species and variety has got a unique set of anthocyanins (Table 1) (Zhu *et al.*, 2012). They accumulate mainly in the berry skin, but in some other cultivars anthocyanin pigments accumulate in the flesh of the berry (Giovinazzo and Grieco, 2015).

Anthocyanin patterns in grapes are of economic value and for this reason target for enrichment by breeding or by agricultural management. Despite high light or temperature, the nutrients N and K present controllable environmental factors that can be adjusted with the aim to enrich the beverage in anthocyanins. It was reported that it is possible to control anthocyanin concentration in the field by controlling plant N status. Excess N supply promotes excessive vegetation, delayed fruit maturation and also delays the quantitative and qualitative biosynthesis of anthocyanins and enhances their degradation during the final steps of berry maturation (Delgado et al., 2006; Hilbert et al., 2003). In terms of K fertilization however, balanced K supply appears to lessen the negative effects of excessive N fertilization, which was shown in Tempranillo grapes (Delgado et al., 2006). When K in the growing medium was high, the treatments with maximum N had higher polyphenols and anthocyanins. Vice versa a high K application reduced anthocyanins, but when the high K was balanced with sufficient N, no reductions in anthocyanins was detected. Therefore, a balance between N and K seems to be crucial for the production of high anthocyanins (Delgado et al., 2006). Low N supply in the form of NH<sub>4</sub>NO<sub>3</sub> (Soubeyrand et al., 2014) as well as foliar application of urea (Portu et al., 2015) caused a significant increase in anthocyanin levels at two ripening stages (26 days postvéraison and maturity). Delphinidin and petunidin derivatives were the most affected compounds. Transcript levels of both structural and regulatory genes involved in anthocyanin synthesis confirmed the stimulation of the phenylpropanoid pathway (Soubeyrand et al., 2014). Keller and Hrazdina (1998) found that the N availability at bloom changes the anthocyanin profile in Cabernet Sauvignon grapes at maturity. Among the five measured anthocyanins, the accumulation of cyadinin-3-glucoside was most strongly reduced by high N nutrition, in contrast to malvidin-3-glucoside, whose percentage of the total anthocyanin content increased with high rates of N fertilization. This is of high importance for red winemaking, because the composition of individual anthocyanins determines the hue and colour stability of the wine. In general, the redness increases with the degree of anthocyanin methylation, while the blueness increases with the number of anthocyanin methylation (Keller and Hrazdina, 1998). Therefore, the authors conclude that a low N status results in the highest wine quality, leading to a deeply coloured Cabernet Sauvignon wine showing a well-balanced crimson to purple hue.

It is of high importance to finely orchestrate N fertilization with the developmental stage since anthocyanins and flavonols are accumulated after véraison (Portu *et al.*, 2015). Furthermore, the effect of organic nutrient management in viticulture is discussed in the light of a changed wine quality. Because organic management frequently involves a reduction of N input, this might be one reason for increased production of high anthocyanins and polyphenols in grapes. Malusa *et al.* (2004) showed that the overall polyphenol concentration was higher in organic grapes, resulting in a higher protection from oxidation. However, it is not easy to disseminate a single process of such a complexity which may sum up in these different management

systems, therefore we will not focus on the difference of organic management systems in detail.

The underlying mechanism for anthocyanin accumulation in berries and the question how changing environmental conditions integrate with anthocyanin biosynthesis still awaits clarifications. It has been shown that Gamay Red cell suspensions derived from fruits possess the potential to mimic the behaviour of genes or regulatory sequences from grape berries with respect to anthocyanin biosynthesis (Gollop et al., 2002; Gollop et al., 2001). Therefore, cell cultures may provide an opportunity for studying regulatory cellular processes that ensue during stress treatments and might furthermore represent controllable systems for producing high-value metabolites such as anthocyanins in wine berries (Ananga, 2013; Sharathchandra et al., 2011). Vitis vinifera cell cultures have been suggested for the production of natural red food colourings and therefore systematic studies have been carried out to increase the efficiency of anthocyanin production by improving the composition of the culture medium (Do and Cormier, 1991). Especially low external N and P concentrations stimulate the anthocyanin synthesis (Dedaldechamp et al., 1995; Do and Cormier, 1991; Yamakawa et al., 1983). The authors suggest that these metabolic changes result from growth inhibition and hence an antagonistic relationship between the primary and secondary metabolism (Dedaldechamp et al., 1995; Do and Cormier, 1991). Dedaldechamp et al. (1995) found a good correlation between anthocyanin synthesis and the activity of dihydroflavonol reductase, which catalyses the reduction of dihydrokaempferol to leucoanthocyanidin, in grape cells cultured in medium with low P levels. Sinilal et al. (2011) revealed that a treatment with Mg increases the anthocyanin concentration produced by red grape cells. No substantial induction of the biosynthetic genes were measured, but Mg inhibited pigment degradation, especially that of anthocyanin species with low stability. Consequently, the ratio between different anthocyanins and the colour of the cultured cells changed.

Table 1

Anthocyanins and their derivatives in the skin of blue vine berries: Alphonse-Lavallee; Muscat-Bleu; and red wine, Regent. Berries and wine were collected at the Hohenheimer Schlossberg and measured; n=8; Data were measured by the use of a HPLC (according to Kammerer *et al.* (2005) by a Merck-Hitachi HPLC series 7000 (Merck, Darmstadt, Germany); D-7000 HSM software, L-7612 degasser, L-7200 ternary gradient pump, L-7450A diode array detector, and a Knauer Jetstream column oven. The separation was performed with a Phenomenex, (Torrance, CA) Aqua C18 column (250x4.6 mm; 5  $\mu$ m particle size), with a C18 ODS guard column (4.0x3.0 mm) at 25 °C.

[mg kg <sup>-1</sup> ]	delphinidine	kuromanine	oenin	petunidine	paeonidine	malvidine	Σ
Alphonse- Lavallee	868	332	2322	620	1001		5144
Muscat- Bleu	1115	289	2777	786	869		5835
Regent (wine)	5804	1074	4932	5479	1197	7317	25803

#### Anthocyanins in apples

The colouration of red or bicoloured apples such as 'Delicious', 'Gala' and 'Jonagold' is an important qualitative trait for farmer and consumer. The extent and intensity of fruit colouration determine the time of fruit harvest and the red skin colour together with the fruit size define the basis for the grading standards that influence the commercial value (Iglesias et al., 2008). Apples with an intense colouration attain the highest visual consumer acceptance and command better prices (Ritenour, 1997). Moreover, the content of anthocyanins in the fruit skin influences the antioxidant activity and consequently the potential beneficial health effects for the consumer and, owing to their ecophysiological properties as described above, are critical for plant fitness. Attaining the optimal colouration in apples at harvest is often a serious problem in southern European countries because the prevalent climatic conditions with hot dry summers do not favour fruit colour development (Iglesias et al., 2008). Consequently, the harvest is delayed in order to attain a certain degree of colour, but this has a negative effect on other quality parameters like firmness and storability and increases the incidence of fruit cracking for example in 'Gala' apples (Iglesias et al., 2008). Therefore, the potential of directed anthocyanin management by strategic fertilization is of high interest in apple orchards, because it would enable the achievement of optimal cosmetic and internal qualitative fruit properties at the ideal harvest time.

The colour of apples is the combination of several plant pigments, including chlorophylls, anthocyanins, and carotenes (Reay et al., 1998), with cyanidin 3-galactoside (Fig. 6C) being the main anthocyanin representative (van der Sluis et al., 2002). In orchards, N uptake has been linked to fruit skin colour and excessive N fertilization is commonly known as an inhibitory factor for the red pigmentation of apples (Treutter, 2010). Increasing N supply decreases both anthocyanin synthesis and chlorophyll degradation in the fruit skin but accelerates flesh starch degradation in 'Gala' apples (Wang and Cheng, 2011). Awad and de Jager (2002) found that the fruit N concentration was the most important variable in predictive models for the anthocyanin concentration in 'Elstar' apples, which is in accordance with other studies that found negative effects of high N fertilization on fruit colouration in the apple cultivars 'Aroma', 'Fuji', 'Goldrush', 'Cox's Orange Pippin' and 'Gala' (Kaether, 1965; Nava et al., 2008; Raese and Drake, 1997; Reay et al., 1998; Tahir et al., 2007; Wargo et al., 2003). The skin anthocyanin concentration can either be directly or indirectly be influenced by the N status of the tree. An indirect relationship can be assumed because higher N fertilization increases leaf growth and consequently shading of apples. Hence, the light-induced anthocyanin production in the skin would decrease with increasing N status. Furthermore, high N can lead to higher chlorophyll concentration in the fruit skin and increased greenness of fruits. For example, Reay et al. (1998) showed that foliar urea-application of 'Gala' apple trees leads to increased chlorophyll concentration on the back of the fruit and lessened the increase of anthocyanins in the blush side skin. Consequently, the harvest was delayed for two weeks because the colour scores, which are used as indicators for fruit picking and grading, were greener. However, controlled experiments with Vitis cell cultures revealed a direct antagonistic relationship between the N concentration in the medium and the anthocyanin content (Yamakawa et al., 1983). Therefore, anthocyanins in the apple skin might be directly influenced by N and not just indirectly by light effects (Awad and de Jager, 2002). Next to N fertilization, Tahir et al. (2007) found that K fertilization positively affected fruit colour in 'Fuji' apples, and CaCl<sub>2</sub> foliar application improved the fruit skin redness in 'Jonathan', 'Red Delicious' and 'Golden Delicious' apples (Kadir, 2004; Raese and Drake, 2000), presumably by changing the balance of other minerals in fruit and leaf tissue (Kadir, 2004).

Controlling the anthocyanin concentration in the fruit skin by plant nutrition is not just relevant in red coloured apples, but N fertilization may also be used for suppressing anthocyanin formation in green apple cultivars such as 'Granny Smith' where red blush is undesirable (Saure, 1990).

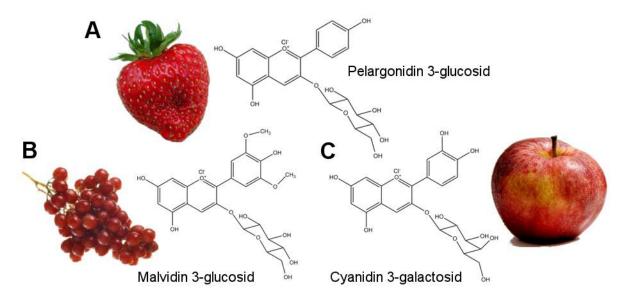
Since the N status influences apple skin colouration and consequently the fruit attractiveness, it is concluded that the consumer acceptance and marketability can be increased by optimizing fertilization, especially that of N (Awad and de Jager, 2002). Not just the amount but also the timing of N fertilization has to be taken into account in order to attain optimal fruit attractiveness. High N levels are most detrimental to colour formation if maintained late in the season (Saure, 1990). Nitrogen fertilization according to monthly soil analysis and fruit development phase instead of regular seasonal N supply could be an effective method to improve fruit quality (Tahir *et al.*, 2007). By this means, other qualitative fruit parameters like fruit firmness and sugar concentration can simultaneously be improved (Awad and de Jager, 2002; Raese and Drake, 1997; Wang and Cheng, 2011).

## Anthocyanins in strawberries

Strawberries are one of the most popular fruit crops from a commercial and economic point of view, and colour of the berries is an important quality parameter (Valentinuzzi et al., 2015). Increasing demand for strawberries, not only because of flavour but also due to their nutritional value has increased the economic importance of strawberries (Fan et al., 2012). Owing to their high content of compounds with oxygen radical absorbance activity, strawberries are highly nutritious fruits with profound health benefits (Kahkonen and Heinonen, 2003; Manganaris et al., 2014; Seeram, 2008; Wang et al., 1996). The attractive redness of strawberries is due to anthocyanins and pelargonidin 3-glucoside (Fig. 6A) is their predominant anthocyanin type (D'Evoli et al., 2010; Moor et al., 2005; Valentinuzzi et al., 2015; Wang et al., 2002) and moreover an important contributor to the antioxidant activity of strawberries (Wang et al., 2002). Fertilization of strawberry plants is likely to influence the anthocyanin content in the fruits, but contradictory results about the effects of nutrient supply on the colour and anthocyanin concentration in the berries have been reported (Pincemail et al., 2012). Wang and Lin (2003) tested the two strawberry cultivars 'Allstar' and 'Honeoye' and found that strawberry plants that were cultivated with high nutrient levels yielded fruits with the highest levels of phenolics, flavonols, and anthocyanins. Likewise, a negative impact of N deficiency on the anthocyanin concentration was reported in other strawberry cultivars (Yoshida et al., 2002). Different combinations of multicomponent fertilizer plus ammonium nitrate application were tested by Chelpinski et al. (2010) but neither the type of fertilizer nor the rate affected the concentration of total polyphenols in the fruits of field-grown strawberries at harvest. Similar results were obtained from hydroponic pot experiments (Castellanos-Morales et al., 2010), where the effect of different N nutrition on the quality of strawberry fruits was investigated. No significant changes in the concentration of the major strawberry anthocyanin pelargonidin 3glucoside were found among the different experimental groups, presumably because the N levels tested were not low enough to induce any stress response. However, a couple of studies pointed out that reduced nutrient application represents a proper way to obtain strawberries with increased anthocyanin concentrations. For example, 'biodynamic cultivation' that is characterized by a total abandonment of synthetic fertilizers and pesticides resulted in strawberry fruits that had a higher content of pelargonidin 3-glucoside (D'Evoli et al., 2010). D'Evoli et al. (2010) attribute these differences partly to the utilization of compost in the biodynamic growing system, which implies that N is not easily plant-available and induces the synthesis of non-nitrogen-containing compounds like anthocyanins. Similarly, several studies

revealed higher anthocyanin content and antioxidant activity in organically grown strawberries compared to conventionally grown fruits (Crecente-Campo et al., 2012; Jin et al., 2011). However, in most cases this increase cannot exclusively be ascribed to the plant nutrition because also the abandonment of pesticides might have contributed to the induction of the plant stress response by causing pathogen stress. Moor et al. (2005) found that strawberries grown on unfertilized plastic mulch yielded the highest concentration of fruit anthocyanins compared to plants on fertilized plastic mulch or straw mulch-grown strawberries. Presumably, the strawberry plants experienced nutrient stress in the unfertilized substrate and hence produced defence compounds. Furthermore, a negative correlation between the soil calcium content and the anthocyanin concentration was revealed in this study. The authors speculate that fruits grown under lower calcium conditions are more susceptible to pathogens and consequently increase their anthocyanin synthesis because calcium is known to strengthen the fruit cell walls and thus to increase the pathogen resistance (Moor et al., 2005). Activating the plant defence mechanism can also be achieved by soaking strawberry plants in phosphitecontaining fertilizer solution before planting (Estrada-Ortiz et al., 2013; Moor et al., 2009). This treatment showed to be effective in increasing the concentration of anthocyanins in the fruits. Phosphite is the salt of phosphorous acid and is absorbed and translocated in the plant in a similar way like inorganic P<sub>i</sub> but phosphite does not metabolically replace P<sub>i</sub> within the plant (Varadarajan et al., 2002). Applying phosphite does not significantly affect the yield but increases fruit quality in terms of bioactive compounds such as anthocyanins. This increase in anthocyanins in the fruits was not caused by physiological P deficiency because phosphite application did not negatively impact the P concentrations in leaves. Therefore, the authors recommend the usage of phosphite as a plant defence stimulator in order to induce the formation of bioactive compounds, rather than a P fertilizer in strawberry production. Hydroponically grown strawberries produced under P and iron deficiency showed remarkably higher concentrations of fruit anthocyanins (Valentinuzzi et al., 2015). Interestingly, both low P and low iron nutrition reduced the shoot growth of strawberry plants but the fruit yield per plant and the average fruit number per plant were increased compared to fertilized control plants (Valentinuzzi et al., 2015). Both the yield and other quality parameters such as acidity, firmness, and soluble solid content were not seriously negatively affected. However, these results must be handled with care, because sufficient P nutrition has been shown to be pivotal for the soluble solid content, which is an important parameter in determining the strawberry flavor and nutritive value (Cao et al., 2015). Wold and Opstad (2007) revealed that not only the total amount but also the timing of nutrient application is essential for both the yield and quality parameters of strawberries. In a field trial they fertilized half the field with NPK (nitrogenphosphate-potassium) plus micronutrients at planting in August with a low nutrient rate from spring, while the second half was unfertilized at planting but received a relatively high nutrient rate in spring. Pre-planting fertilized plots receiving the lowest fertilizer rate during the harvesting season had the darkest fruit colour and yielded berries with higher weight compared to late fertilization. Therefore, the authors recommend pre-planting fertilization and the avoidance of additional nutrient application before harvest, which is in agreement with Moor et al. (2005), who state that 'the best treatment for the health of the consumer based on increased anthocyanin content would be growing strawberries with plastic mulch with only pre-planting fertilization without additional application of liquid fertilizer during the growing season'. A number of studies showed that the antioxidant activity and hence the nutritional value of fresh or processed strawberries increases concomitant with the fruit anthocyanin content (Anttonen et al., 2006; D'Evoli et al., 2010; Jin et al., 2011; Valentinuzzi et al., 2015; Wang and Lin, 2003). Therefore, the adjustment of the nutrient application can be an effective means to produce

high-quality strawberries with the desired amount of bioactive compounds such as anthocyanins (Anttonen *et al.*, 2006; Valentinuzzi *et al.*, 2015). However, strawberry is one of the most sensitive plants in horticultural production and the nutrient application has to be managed with care in order to increase not only colour and nutritional value of fruits but to guarantee high yields and other fruit quality parameters at the same time (Trejo-Tellez, 2014). For example, N has a pivotal role in plant growth and affects fruit attributes such as fruit size, firmness, taste, and shipping and storage qualities. However, Trejo-Tellez and Gomez-Merino (2014) gave a review about the impact of nutrient management in strawberries and conclude that excessive N fertilization does enhance the content of compounds related to the sensorial fruit quality, such as sugars and organic acid. Furthermore, lower N fertigation rates promote longer shelf life of strawberries and can be applied without negative effects on marketable yield. In conclusion, adjusting strawberry fertilization may provide a potential means to increase the colour and nutritional value of strawberries by altering the anthocyanin content in the fruit.



**Figure 6**: Fruits and molecular structure of their main containing anthocyanin species. (A) Strawberry and pelargonidin 3-glucoside; (B) red grapes and malvidin 3-glucosid; (C) apple and cyaniding 3-galatosid.

## 3.2 Anthocyanin-mediated increased stress resistance

Owing to their coveted bioactive properties, directed up-regulation of foliar anthocyanins by agronomic means has been proposed in order to increase the plant tolerance to abiotic and biotic stress. For example, *Eucalyptus nitens* seedlings nutrient starved in the nursery were pre-conditioned to photoinhibition by cold and sunny conditions experienced soon after planting (Close *et al.*, 2000). Therefore, this treatment was suggested as a useful risk-management tool where cold-induced photoinhibition is likely to occur during seedlings establishment. Besides the above outlined physiological functions, anthocyanins are discussed as aposematic repellents against herbivores and parasites (Carpenter *et al.*, 2014; Gould, 2004; Lev-Yadun and Gould, 2009). This is of particular interest in organic farming, which relies mainly on natural plant defence mechanisms. These cropping systems do not primarily seek to maximize growth and yield. Therefore, rational N fertilization or short-term nutrient deprivation in order to increase internal defence compounds was suggested as an

## 5. Plant nutrition and anthocyanins

environmentally friendly strategy to control herbivory and pathogen infection (Benard *et al.*, 2011; Larbat *et al.*, 2012; Larbat *et al.*, 2014). Larbat *et al.* (2012) investigated the effects of decreased N fertilization on the concentration of phenolic compounds including anthocyanins in tomato leaves in order to increase the plant resistance to pathogens. Since long-term N restriction negatively affects plant growth and yield, the tomato plants were subjected to repeated short-term N limitations. Anthocyanin concentrations in the tomato leaves increased manifold especially during the first low N treatment, but high anthocyanin levels could not be maintained during the following full nutrient supply. Therefore, the potential to increase the anthocyanin-mediated stress resistance in crops by fertilization remains speculative and more work is needed to elucidate this topic.

# 4. Concluding remarks and perspectives

Anthocyanins are colourful secondary plant metabolites of high importance for plant fitness and human nutrition. Anthocyanins levels in leaves of crops have the potential to indicate nutritional deficiencies or metal toxicities and the variety of nutrients associated with anthocyanin synthesis is much more diverse than commonly acknowledged. Detailed knowledge of the subtle details that exist among different nutrient stresses such as the organor tissue-specific synthesis of specific anthocyanin profiles may allow the utilization of anthocyanins as biomarkers for certain nutrient deficiencies or element toxicities with the goal to optimize fertilization strategies and improve plant fitness. However, diagnosis of nutrient disorders by leaf colouration is only applicable if other abiotic factors such as suboptimal temperature can be precluded as causes for anthocyanin synthesis. Future research must focus on unravelling the genetic and molecular details of abiotic stress-induced anthocyanin formation. This can also contribute to a more profound understanding of the ecophysiological background of leaf purpling in plants under element stress.

Furthermore, anthocyanins highly impact the quality and thus the economic and nutritional value of fruits and vegetables. Consumers are increasingly concerned about the health implications of their nutrition. Therefore, anthocyanin containing fruits are of massive consumer interest because of their high health-promoting antioxidant activity. This review shows that fertilization management can be a powerful tool to increase the anthocyanin concentration in fruits with major global trading value. This is particularly true in the glasshouse where external factors can be controlled more easily. Especially a fine-tuned N fertilization has got the potential to increase the colouration and antioxidant capacity of fruits by shifting metabolism towards an increased anthocyanin synthesis without negatively affecting fruit yield. Only time will tell, if this strategy will be applied in the field in the future.

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# 6. General Discussion

Magnesium deficiency is becoming an increasing agricultural problem in both industrialized crop production systems and subsistence agriculture, elicited by a variety of soil- and weatherborn factors. However, being known as the 'forgotten nutrient' in plant nutrition research (McHoul, 2006), the effects of Mg shortage on crop growth have rarely been investigated.

This work aimed at providing new knowledge about the impact of Mg deficiency on certain aspects of maize metabolism, namely foliar ammonium assimilation potential, leaf protein concentration, and foliar flavonoid biosynthesis. Furthermore, leaf-application as an alternative means of fertilization and the suitability of a ChI fluorescence-based leaf-clip device for rapid, non-destructive diagnosis of nutrient stress were evaluated in maize (*Zea mays* L.).

In this general discussion, certain aspects of physiological responses of maize to Mg deprivation will be discussed based on the collected data and will be brought into context with the latest state of research. The results of the different experiments will be approached on a holistic level by relating them to each other. Furthermore, the potential of foliar fertilization for ameliorating acute Mg deficiency in maize will be evaluated on the basis of the analysed parameters. Potential drawbacks of this alternative fertilization strategy will also be addressed.

# 6.1 Effects of Magnesium Deficiency on Photosynthesis

Magnesium fulfils several function in photosynthesis. As the central atom of chlorophyll, it is essential for light harvesting, it activates enzymes of the Calvin-Benson cycle including RubisCO and it functions in charge balancing inside the chloroplast upon illumination. Also, the enzyme phosphoenolpyruvate carboxylase (PEP carboxylase), acting in CO<sub>2</sub>-fixation in C<sub>4</sub> plants such as maize, uses Mg as a cofactor (O'Leary *et al.*, 2011).

As expected, the photosynthesis rate was markedly decreased in maize grown under severe Mg deficiency (Chapter 3, Fig. 3A). Reduced photosynthetic capacity in response to Mg shortage has been documented in other crops such as sugar beet before (Hermans et al., 2004). One reason for this is the lower chlorophyll content in the leaves (Chapter 3, Fig. 2), which causes the characteristic chlorotic phenotype under Mg deficiency (Chapter 3, Fig. 6). It may be presumed that a lack of Mg for chelating chlorophyll and hence inhibited chlorophyll synthesis is the critical factor under Mg deficiency. This assumption is supported by the finding that the transcript abundance of a subunit of the Mg-chelatase, the enzyme catalysing Mg insertion into the chlorophyll precursor molecule, is down-regulated under Mg deficiency in Vicia faba (Neuhaus et al., 2013). However, Hermans and Verbruggen (2005) revealed, that this is not the primary cause for photosynthesis inhibition under Mg deficiency. In order to unravel the hierarchy of physiological perturbations upon Mg depletion, the authors recorded several physiological parameters in Arabidopsis thaliana at regular intervals during the induction of Mg deficiency. They showed that sucrose and starch accumulation started some days prior to decrease in chlorophyll concentration, which is in accordance with findings in sugar beet (Hermans et al., 2005; Hermans et al., 2004; Hermans and Verbruggen, 2005). The authors conclude that the decline in photochemical activity is elicited by increased sugar concentrations and that low chlorophyll concentrations in Mg-deficient plants is the result of chlorophyll catabolism rather than inhibited chlorophyll synthesis due to a lack of Mg atoms for chelating chlorophyll molecules (Hermans and Verbruggen, 2005; Verbruggen and Hermans, 2013). Controlled chlorophyll degradation is a nutrient deficiency-induced process for N recycling because the chlorophyll-protein complexes are major N resources in chloroplasts (Shimoda *et al.*, 2012). Additionally, chloroplast destruction and chlorophyll degradation in response to Mg shortage can be exacerbated by ROS stress, which is induced by impaired phloem loading (Cakmak *et al.*, 1994).

Cakmak et al. (1994) found sugar accumulation at early stages of Mg deficiency in Phaseolus vulgaris leaves and proposed that inhibition of phloem loading is the primary effect in the hierarchy of physiological reactions to Mg shortage and that photosynthesis is less sensitive to Mg deficiency. Feedback-inhibition of photosynthesis in consequence of increased sugar concentrations in Mg-deficient leaves is highly likely. For example, the transcript abundance of Cab2, which encodes a chlorophyll a/b binding protein in photosystem II, decreases prior to a marked decline in total chlorophyll concentration under Mg deficiency and reduced Cab2 expression consequently leads to chlorophyll breakdown (Hermans and Verbruggen, 2005). Van Oosten et al. (1994) showed that the abundance of transcripts of cab genes are also reduced in response to increased CO<sub>2</sub> concentration or application of external hexoses. Therefore, the genetic down-regulation of photosynthesis under Mg deficiency may be the result of sugar-induced feedback-inhibition. Detailed transcriptomic analysis in Arabidopsis thaliana showed that some genes related to the photosynthetic apparatus maintenance are readily down-regulated already 28 hours after the onset of Mg depletion (Hermans et al., 2010a). The acclimatisation of the photosystems (PSs) I and II to low Mg availability was presented in sugar beet (Hermans et al., 2004). Presumably, PSII photochemistry declines due to a loss of light-harvesting complexes (LHC II) and PSI is down-regulated primarily through a loss of reaction centres, thereby reducing the linear e transport between the photosystems to prevent over-reduction of the e<sup>-</sup> transport chain during conditions of restricted sugar export. It is highly likely, that the phloem transport was also inhibited in Mg-deficient maize in this work. Both soluble sugars and the amino acids glutamine and asparagine, which are the organic N transport forms in most crops (Lea et al., 2007), were markedly increased under Mg deficiency (Chapter 4, Fig. 7; Chapter 2, Table 2). Therefore, it can be concluded, that negative feedback-inhibition contributed to the measured depression of photosynthetic CO<sub>2</sub> assimilation in maize under Mg deficiency. The findings that the chlorophyll content significantly increased and the net CO<sub>2</sub>-assimilation rate even doubled within only two days upon application of Mg to Mg-deficient maize plants (Chapter 3, Fig. 2, 3A) implies that the described metabolic adaptation to Mg deficiency is readily reversible. In fact, phloem loading is restored already within several hours after Mg resupply (Cakmak et al., 1994) and transcription analysis in Arabidopsis thaliana showed that the expression of half of the genes that are altered under Mg deficiency is restored within 24 hours after Mg resupply (Hermans et al., 2010a).

# **6.2 Magnesium Deficiency and Magnesium Restoration affect Cation Homeostasis in Maize**

#### 6.2.1 Magnesium concentration

Growing maize in low Mg nutrient solution resulted in extremely low Mg concentrations in both shoot and root (Chapter 2, Fig. 2; Chapter 3, Fig. 5A). Resupply of Mg to the nutrient solution restored the Mg levels in both leaves and roots and this process must have happened rapidly, because increased chlorophyll content and net CO<sub>2</sub>-assimilation rate were already recorded two days after Mg resupply (Chapter 3, Fig. 2, 3A). In fact, experiments in *Arabidopsis thaliana* showed that the Mg concentrations were increased by 125% in roots, 75% in the rosette and 127% in the xylem sap already 24 hours after Mg resupply to Mg-deficient plants (Hermans *et al.*, 2010a). Applying Mg to the nutrient solution of Mg-deficient maize raised the Mg root

#### 6. General Discussion

concentration above the level of the positive control (Chapter 3, Fig. 5A). It might be assumed that this displays a 'luxury consumption' strategy after a time of nutrient deprivation. In fact, wild plant species adapted to low N and P conditions absorb these nutrients in excess of immediate growth requirements during nutrient flushes (Chapin, 1980). However, this strategy has not been observed for Mg before and little is known about the adaptability of the Mg uptake capacity in response to Mg deficiency. Surprisingly, genes encoding putative Mg transporters such as the MRS2/MGT family (mitochondrial RNA splicing 2/magnesium transporter) or MHX1 (magnesium proton exchanger) were not found to be up-regulated under Mg deficiency in Arabidopsis thaliana (Hermans et al., 2010b) and also the abundance of the proteins were not altered in Mg-deficient citrus shoot and root (Peng et al., 2015). Hermans et al. (2010b) state that this is a unique response, because deficiencies of other nutrients such as N, P or S trigger the expression of genes encoding nutrient transporters in order to increase the root uptake capacity. In contrast to these observations, Mao et al. (2014) report high expression of MGT6 – a putative Mg transporter that is mainly expressed in aerial tissues under optimal Mg nutrition - in Arabidopsis thaliana roots during Mg deficiency. Further evidence for an upregulation of genes encoding proteins potentially mediating Mg transport was found in shoots of rice in response to Mg shortage (Cai et al., 2012). Tracer studies with the radioactive <sup>28</sup>Mg isotope in rice indicate that the Mg uptake rate is indeed promoted by Mg deficiency (Tanoi et al., 2014). However, these experiments also revealed that the higher uptake activity fell to the basal level within 5 minutes after Mg resupply. Therefore, the increased Mg concentrations in maize roots in response to Mg resupply to the nutrient solution, which were found in this work. cannot be accounted for by a long-lasting adaptation effect in response to Mg shortage. According to expectations, the Mg concentration in Mg-deficient plants were raised by foliar fertilization (Chapter 3, Fig. 5A). Geilfus et al. (2016) showed in Vicia faba that the applied Mg is not only bound in the leaf apoplast, but that a fraction of the Mg also enters the symplast. The immediate physiological responses of maize after MgSO<sub>4</sub> leaf application, which were recorded in this work, demonstrates that foliar applied Mg can efficiently and efficiently become biologically active at the site of application. Magnesium – as a phloem-mobile element – can be distributed basipetally within the plant (Karley and White, 2009; Steucek and Koontz, 1970; White and Broadley, 2009). Therefore, foliar-applied Mg can potentially be translocated from treated leaves into untreated plant parts. However, no significant increases in the Mg concentrations of roots and the youngest leaves, which received no Mg application, was observed in maize (Chapter 3, Fig. 5A). The same phenomenon was reported in Vicia faba, where no translocation into growing leaves or the seeds was registered (Neuhaus et al., 2014). Magnesium translocation from source to sink tissue in the phloem is mainly driven by the sink strength. Therefore, it can be speculated that the Mg demand in the youngest leaves and the roots of maize did not exceed the present Mg concentrations. This is corroborated by the fact that physiological processes such as root growth and chlorophyll concentration in the youngest leaves were not limited by a lack of Mg (Chapter 3, Fig. 4, Table 1). The Mg in leaves and roots of maize plants that receive Mg via the nutrient solution is delivered mainly through acropetal movement within the xylem. Hence, the Mg concentrations in these plants is determined not only by the actual physiological Mg demand but also by the transpiration-driven water flow rate. Therefore, differences between the two fertilization strategies in terms of Mg concentrations in plant tissue may be the result of distinct transport systems that are involved in Mg distribution within the plant. From the results of this work it can be deduced that Mg leaf-application has got a systemic effect in distant, untreated plant parts in terms of physiological responses such as chlorophyll content and growth.

#### 6.2.2 Potassium concentration

Under ideal nutrition, the uptake, distribution and storage of the essential nutrients is highly regulated and elements are taken up in defined ratios to each other, to facilitate optimal functioning of metabolic processes. However, the shortage of one nutrient leads to an imbalance of the ion homeostasis and modifies the internal concentration of other elements as well. A mutual relationship between Mg and K uptake, respectively, was found in Mg-deficient maize leaves and roots in this work (Chapter 3, Fig. 5B). These cations compete for transport through non-selective cation channels, which show low selectivity towards both monovalent and divalent cations (Demidchik et al., 2002). In the absence of Mg a compensatory increase of other cations occurs, in order to maintain osmolality and balanced uptake of cations and anions. The K concentrations in maize grown under Mg deficiency were markedly increased in mature leaves compared to the control plants (Chapter 3, Fig. 5B). This agrees with findings in Citrus sinensis, where Mg-deficient leaves had higher K levels (Ma et al., 2016), and an antagonistic relationship between Mg and K was also found in rice shoots (Cai et al., 2012). Knowledge about the impact of Mg nutrition on K transporters is scarce and inconsistent. Conn et al. (2012) found that two members of the KUP family, that facilitate K uptake, were negatively correlated with Mg accumulation in Arabidopsis thaliana, which corroborates the assumption of a reciprocal relationship between both cations. However, Ma et al. (2016) report about an inhibition of K<sup>+</sup> uptake transporter 3 (KUP3) in Mg-deficient citrus leaves. The authors explain that lowering K uptake under Mg deficiency may be an adaptive strategy in order to maintain nutrient balance. Cai et al. (2012) found up-regulation of a high affinity K transporter gene (OsHAK1) in roots of rice under Mg deficiency. The K concentration in the roots of maize grown in low Mg nutrient solution - treatments 'Mg -' and 'Mg -/leaf application' - were tendentially and significantly increased (Chapter 3, Fig. 5B), indicating a compensatory increase in K under Mg deficiency.

Potassium is taken up into the roots against the K concentration gradient, because of a high cytosolic K concentration of 100 mM (Wang and Wu, 2013). Therefore, the uptake into the root and also the further accumulation in the shoot is driven by the electrochemical membrane potential, which is established and maintained primarily by H+-ATPase activity. However, Cakmak *et al.* (1994) proposed that the activity of the proton pump is the primary target of Mg deficiency due to a lack of Mg-ATP and stands at the beginning of the hierarchy of metabolic responses to Mg shortage. This hypothesis is in conflict with the high K concentrations in Mg-deficient maize that were found in this work.

Apart from a modified K uptake caused by low Mg concentrations in the nutrient solution, decreased reallocation of K from older leaves into growing leaves because of impaired phloem loading may have contributed to the high K levels found in the lower leaf levels in Mg-deficient plants (Chapter 3, Fig. 5B). Accordingly, not only photoassimilates and amino acids but also K concentrations were found to be reduced in the phloem sap of *Vicia faba* under Mg shortage (Cakmak *et al.*, 1994). Magnesium application to Mg-deficient maize plants basically lowered the K concentrations to levels of the positive control (Chapter 3, Fig. 5B). This presumably resulted from decreased uptake at the roots and enhanced redistribution from lower into upper leaves, by restoration of the phloem loading capacity, which seems to be an immediate response to Mg resupply and happens already within a few hours (Cakmak *et al.*, 1994).

Potassium concentrations in leaves with Mg leaf-fertilization did not decrease below the control levels, revealing that even application of even high Mg concentrations did not have displacement effects on K. This is in contrast to root fertilization methods, where a significant antagonistic effect of increasing Mg application can reduce plant K concentrations (Kleiber *et al.*, 2012).

#### 6.2.3 Manganese concentration

Potassium is the main osmoticum in plant cells and therefore replaces Mg in its role in turgor regulation. However, K cannot substitute Mg in other physiological functions due to profoundly different chemical properties. The opposite is the case for manganese (Mn), whose concentration was also found to be increased in Mq-deficient maize (Chapter 3, Fig. 5C). Manganese and Mg are divalent cations with similar biochemical characteristics and Mn can substitute Mg in ligand binding (Bock et al., 1999). A competitive relationship in regard to uptake has been found in other crops such as wheat as well (Goss and Carvalho, 1992), and it is known that adequate Mg nutrition can mitigate the risk of Mn toxicity especially on acidic soils (Goss and Carvalho, 1992; Le Bot et al., 1990). The Mn concentration was particularly increased in the roots of Mg-deficient maize plants (Chapter 3, Fig. 5C). This might result from decreased xylem transport into the shoot caused by low transpiration (Chapter 3, Fig. 3B), but may also display a strategy to avoid Mn toxicity in the shoot. Reduced transport of Mn from roots to leaves has been shown in maize before and was explained to be the reason of the superior Mn tolerance of maize compared with other crops (Foy et al., 1978). Within the cell, Mn can be detoxified by sequestration into the vacuole or chelation in the cytosol (Pittman, 2005). Despite predominant accumulation of Mn in the root, Mn toxicity might have contributed to the observed phenotype in leaves of Mq-deficient maize, such as reduced photosynthesis rate and transpiration, and leaf chlorosis and necrosis (Chapter 3, Fig. 3, 6B, C), because toxic concentrations of Mn cause comparable symptoms (Nagajyoti et al., 2010; Stoyanova et al., 2009).

Because of similar liganding properties of Mn and Mg, Mn can replace Mg in the active site of Mg-utilizing enzymes without basically losing the catalytic activity of the enzyme (Bock *et al.*, 1999). This is also the case for the ammonium-assimilating enzyme glutamine synthetase (GS), whose activity was analysed in this work. The *in vivo* GS efficiency is known to be decreased when chelated with Mn, because Mn binding shifts the pH optimum of the enzyme to more acidic levels (Hirel and Gadal, 1980; O'Neal and Joy, 1974). However, it was shown in this work that the total capacity of the GS-mediated ligation of free ammonium and glutamate to form glutamine in the leaves of maize plants was not impaired upon severe magnesium starvation in maize (Chapter 2, Fig. 3, Table 2). Therefore, a significant inhibition of this reaction due to replacement of Mg by Mn at the active site of GS can be ruled out.

# 6.3 Role of Magnesium in Nitrogen Metabolism

# 6.3.1 Glutamine synthetase

One of the reasons for the observed growth depression under Mg deficiency might be inhibited N metabolism, because Mg is significantly involved in primary N assimilation, amino acid formation and protein biosynthesis. Grzebisz (2013) proclaimed that Mg is the basic nutrient supporting N uptake and utilization and that sufficient Mg supply raises the N use efficiency (NUE). However, it remains to be elucidated, if this is a secondary effect caused by high photosynthetic capacity and unimpaired assimilate distribution under adequate Mg nutrition, or if Mg deficiency directly impedes and reduces N metabolic processes.

One of the key enzymes of primary N assimilation is glutamine synthetase (GS), which catalyses the ligation of inorganic ammonium (NH<sub>4</sub><sup>+</sup>) and the amino acid glutamic acid (Glu) to form glutamine (Gln) (Cowan, 2002). Ammonium-ligation and the subsequent transfer of the amino-group from Gln onto organic acids is the essential starting point for the synthesis of all other N-containing compounds within plants. Since the GS-catalysed Gln synthesis is the only

#### 6. General Discussion

reaction allowing assimilation of inorganic N into organic molecules (Pratelli and Pilot, 2014), proper GS activity is of paramount importance for plant growth and yield formation.

Magnesium is pivotal for the GS activity in several regards. It is both an integral catalytic cofactor of the enzyme and Mg-ATP serves as a co-substrate in the GS-catalysed reaction (Cowan, 2002). Above that, post-translational GS activation via phosphorylation and binding of a 14-3-3 protein are Mg-dependent reactions as well (Finnemann and Schjoerring, 2000; Lima *et al.*, 2006; Riedel *et al.*, 2001). Consequently, decreased GS activity is expected to occur under Mg deficiency. However, the relationship between the plant Mg status and GS activity is rarely investigated and controversial findings exist in different species. Both spinach and rice showed impaired GS activity under Mg deficiency (Ding *et al.*, 2006; Yin *et al.*, 2009), whereas an influence of the Mg supply on GS activity could not be found in both leaves and roots of tea plants (Ruan *et al.*, 2012).

Spectrophotometric determination of the GS activity and immunoblotting analysis in different leaf levels of Mg-deficient maize were conducted in this work. It was revealed that neither the in vitro GS activity nor the GS abundance was negatively impacted under Mg deficiency (Chapter 2, Fig. 3, 4). To corroborate these results, the leaf concentrations of both substrate and product of the GS enzyme were analysed. It can be assumed that a decreased GS activity would result in an accumulation of Glu and a decrease in Gln compared to the positive control. However, neither Glu accumulation nor Gln depression was found (Chapter 2, Table 2). These results show that even severe Mg deficiency is not consequently accompanied by decreased NH<sub>4</sub><sup>+</sup> assimilation capacity and hence, is not a growth limiting step in Mg-deficient maize. In fact, the Gln concentrations were even markedly elevated in Mg-deficient leaves and increased together with asparagine (Asn) disproportionately in relation to other amino acids. This can be interpreted as a result of impaired phloem loading, because both Gln and Asn display the major organic transport form of N within the plant owing to their low molecular C to N ratio (Lea et al., 2007). It is highly likely, that phloem loading was impaired in Mg-deficient maize in this work, because the increased K concentration (Chapter 3, Fig. 5B) and particularly the pronounced accumulation of sucrose and hexoses in lower leaf levels (Chapter 4, Fig. 7) are strong indicators for hampered internal distribution of nutrients and assimilates. Accumulation of free amino acids, in particular those, which are Gln-dependent, are known to decrease GS activity (Eisenberg et al., 2000). However, such a feedback-inhibition was not found in Mgdeficient maize in this work. In fact, the GS activity is not only controlled by its product abundance, but genes involved in N assimilation are regulated by a complex network of factors to coordinate C and N metabolism. Since organic acids are prerequisite for amino acid syntheses, N uptake and NH<sub>4</sub><sup>+</sup> assimilation are controlled by the relative abundance of carbon skeletons versus amino acids (Oliveira and Coruzzi, 1999; Thomsen et al., 2014). Sucrose acts as a signal molecule in this regulating network and the expression of GS is enhanced by sucrose (Cren and Hirel, 1999; Oliveira and Coruzzi, 1999). Sucrose-induced changes in GS mRNA levels can also be observed at the level of GS enzyme activity (Oliveira and Coruzzi, 1999). Therefore, sucrose accumulation in Mg-deficient leaves, which were found in this work (Chapter 4, Fig. 7C), might have contributed to the observed constant GS activity and GIn synthesis under Mg deficiency.

The discrepancy between these findings and results in spinach and rice (Ding *et al.*, 2006; Yin *et al.*, 2009) might result from species-specific differences. Hirel *et al.* (2005) found constantly high GS activity in maize throughout the whole plant life-cycle, as well as in different leaf stages, which contrasts with other species such as tobacco, where the total leaf GS activity falls drastically during leaf senescence (Masclaux *et al.*, 2000). Hirel *et al.* (2005) speculate that a species-specific GS expression and activity pattern maintains a constant export of

organic N forms from ageing into developing leaves for protein synthesis, or translocation into the grain. Therefore, it can be speculated that the decreased GS activity in Mg-deficient spinach and rice display nutrient deficiency-induced senescence processes rather than primary metabolic consequences of Mg deficiency. Zhao *et al.* (2012) supposed that reduced N assimilation might be related to the increase of sucrose and soluble sugars in maize leaves under Mg starvation since they found a reduction in nitrate reductase activity as well as reduced ammonium and protein concentrations in Mg-deficient maize leaves. However, this cannot be confirmed from the results of this work.

Both the GS activity in leaves number six and seven and the relative GS abundance in leaf number six were elevated in maize plants that received Mg leaf-application after 27 days of Mg starvation (Chapter 2, Fig. 3a, 3b, 4). As outlined before, both the GS activity and the enzyme abundance are presumably regulated by a large number of factors and internal concentrations of specific metabolites. Therefore, it can only be speculated about the reason for the observed high GS capacity in these plants. Conceivably, a switch of metabolism from catabolic processes during Mg deficiency to anabolic processes upon Mg resupply with concomitant increased NH<sub>4</sub><sup>+</sup> assimilation upon Mg resupply might have led to increased GS activity, which is known to be elevated during the N-assimilatory phase in maize (Hirel *et al.*, 2005). Therefore, the increased capacity to assimilate NH<sub>4</sub><sup>+</sup> in these plants cannot directly be attributed to the fertilization method, meaning that from the results of this work it cannot generally be deduced that Mg leaf-fertilization increases the GS activity in maize.

#### 6.3.2 Protein biosynthesis

Magnesium mediates the association of ribosomal subunits (Petrov et al., 2012) and is involved in amino acid activation for incorporation into polypeptides during translation (Cole and Schimmel, 1970). Regarding the fact that up to 75% of the leaf Mg is associated with protein synthesis via its role in ribosomal structure and function (Waters, 2011; White and Broadley, 2009), it is widely assumed that Mg deficiency inhibits protein synthesis, probably caused by dissociation of the ribosomes into their subunits in the absence of Mg (Chatterjee et al., 1994; Kirkby and Mengel, 1976; Mengel and Kirkby, 2001). Mengel and Kirkby (2001) even proposed that a disturbed polypeptide synthesis in the ribosomes is the primary effect of Mg undersupply, leading to restricted protein production and growth inhibition. In fact, there is evidence that protein biosynthesis is impaired by Mg deficiency. The proportion of protein N decreases in favour of non-protein N under Mg shortage (Kirkby and Mengel, 1976) and accumulation of free amino acids has often been recorded in the absence of Mg (Cakmak et al., 1994; Fischer et al., 1998; Ruan et al., 2012). Furthermore, decreased total soluble protein concentrations were found in leaves of Mg-deficient spinach, rice, and citrus (Ding et al., 2006; Peng et al., 2015; Yin et al., 2009). Moreover, Mg fertilization was found to be positively correlated with increased protein concentrations in soybean and fava bean seeds (Neuhaus et al., 2014; Vrataric et al., 2006) and grains of winter wheat, rye, and barley (Gerendas and Fuehrs, 2013). However, low protein concentrations in leaves under Mg deficiency can also result from enhanced protein degradation caused by nutrient deficiency-induced senescence processes, and accumulation of free amino acids can be attributed to inhibited phloem loading under Mg deficiency. Likewise, the positive impact of Mg fertilization on grain and seed protein concentration may be the effect of enhanced assimilate export from source leaves into sink tissue facilitated by adequate leaf Mg concentrations. Therefore, the prevailing assumption that low internal Mg levels directly impact protein biosynthesis by leading to ribosome dissociation is questionable.

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In fact, the protein concentration in leaf number 7 of Mg-deficient maize was not different from the positive control (Chapter 2, Fig. 5b), although the leaf Mg concentration was markedly decreased (Chapter 2, Fig. 2). These results are in accordance with findings in leaves of other crops such as *Triticum aestivum* (Andrews *et al.*, 1999; Lasa *et al.*, 2000; Peng *et al.*, 2015; Polle *et al.*, 1994; Tewari *et al.*, 2006) and in roots of *Citrus sinensis* (Peng *et al.*, 2015). Andrews *et al.* (1999) even recorded increased protein concentrations in leaves of *Pisum sativum* and *Phaseolus vulgaris* under Mg shortage. It is highly likely that the low protein concentrations in leaf number six of Mg-deficient maize (Chapter 2, Fig. 5a) arose from nutrient deficiency-induced senescence, because other physiological parameters such as decreasing chlorophyll content in leaf number six (Chapter 3, Fig. 2) and preferential synthesis of the organic N-transport form Gln and Asn (Chapter 2, Table 2) are indicative of catabolic processes with concomitant protein degradation. In fact, an increased abundance of proteins of the proteasome and ubiquitination pathway were found in Mg-deficient maize and citrus (Li *et al.*, 2015; Peng *et al.*, 2015), indicating that proteolysis might be enhanced under Mg deficiency, thus lowering the concentration of total soluble proteins (Peng *et al.*, 2015).

#### 6.3.3 Conclusion

In conclusion, the results of this work show that severe Mg deficiency is not consequently accompanied by impaired GS-mediated Gln formation or protein biosynthesis in maize leaves. The results indicate that the relationship between Mg nutrition and NUE is an indirect one in maize. By increasing the photosynthesis rate (Chapter 3, Fig. 3A) and facilitating the transport of photoassimilates, amino acids and nutrients (Chapter 4, Fig. 7; Chapter 2, Table 2; Chapter 3, Fig. 5) between plant organs, adequate Mg nutrition promotes the overall growth (Chapter 3, Fig. 4, 6A) and can consequently also promote N uptake and distribution within the plant. However, a direct effect of Mg deficiency on basic processes of N metabolism such as ammonium assimilation or protein biosynthesis was not found.

# 6.4 Flavonoid Metabolism as influenced by Magnesium Deficiency

#### 6.4.1 Anthocyanin Synthesis

Next to chlorosis and necrosis (Chapter 3, Fig. 6B, C), leaf purpling was shown to be a visual symptom of Mg deficiency in maize, indicating accumulation of anthocyanins (Chapter 4, Fig. S1, S3B; Chapter 5, Fig. 4). This phenomenon has also been described in leaves of fieldgrown fruiting trees and shrubs with high anthocyanin content in fruits, such as apples, cherry, black currants and blue grape varieties (Jacob, 1955), and was shown under experimental conditions in leaves of rice, cowpea, fava bean and roses (Hariadi and Shabala, 2004; Kobayashi et al., 2013; Premkumar and Kulandaivelu, 2001; Yeo et al., 2013). Anthocyanins are secondary compounds of the phenylpropanoid pathway and they are synthesized in or immediately below epidermal layers of leaves in response to various abiotic stresses such as high light or low temperature (Close and Beadle, 2003; Kovinich et al., 2015; Landi et al., 2015). The significance of anthocyanins in agricultural crop production and the relationship to plant nutrient status has been reviewed in chapter 5. Foliar anthocyanin synthesis is commonly, but often erroneously, associated with N or P deficiency. However, a multitude of nutrient imbalances – including Mg deficiency – causes leaf purpling. The genetic background of this nutrient stress-adaptation response remains largely unknown especially in crops. Detailed transcriptomic analysis has only been conducted for N and P deficiency in Arabidopsis thaliana but anthocyanin synthesis in response to Mg shortage has never been examined in detail. It has been suggested that foliar anthocyanins are accumulated when production of carbohydrates in source leaves exceeds their utilization in sink tissue. Since anthocyanins can be highly decorated with sugar moieties, they are supposed to function as carbon (C) sinks to sequester excess photoassimilates (Zhang et al., 2013). This hypothesis is supported by the finding that external sugar feeding (Kumar and Sharma, 1999; Rajendran et al., 1992) or artificial disruption of phloem loading in source leaves (Botha et al., 2000; Jeannette et al., 2000; Lloyd and Zakhleniuk, 2004; Russin et al., 1996) also results in anthocyanin accumulation and the major regulating transcription factors for anthocyanin synthesis respond positively to increased sucrose concentrations (Lloyd and Zakhleniuk, 2004; Solfanelli et al., 2006; Teng et al., 2005). This indicates a direct link between nutrient deficiency induced sugar accumulation and anthocyanin synthesis. As outlined in detail before, accumulation of soluble sugars in photosynthetic active leaves is a typical symptom in response to Mg starvation (Cai et al., 2012; Cakmak et al., 1994; Hermans et al., 2005; Hermans and Verbruggen, 2005) and immensely increased levels of soluble sucrose, glucose and fructose were found in Mgdeficient maize leaves in this work (Chapter 4, Fig. 7). Especially sucrose, the main circulating photoassimilate in maize (Hirel, 2005), was increased and reached up to 168 mg g<sup>-1</sup> DW, which was 33-times the concentration of plants grown under optimal nutrition (Chapter 4, Fig. 7C). Likewise, soluble glucose and fructose increased 8- and 3-fold, resulting in concentrations of 84 mg g<sup>-1</sup> DW and 45 mg g<sup>-1</sup> DW in the lowest analysed leaf (Chapter 4, Fig. 7A, B). However, anthocyanin concentrations in whole leaf extracts measured by HPLC analysis were in the range of few µg g<sup>-1</sup> DW or even below the detection limit (Chapter 4; data not shown). Since soluble sugar and anthocyanin concentrations were by far not in the same order of magnitude, anthocyanins cannot contribute significantly to C storage in Mg-deficient maize leaves. However, it has been suggested that sucrose is an important signalling element for the induction of anthocyanin synthesis-related genes (Solfanelli et al., 2006; Van den Ende and El-Esawe, 2014; Zhang et al., 2012), therefore a general causal connection between photoassimilate accumulation and anthocyanin synthesis is possible, but a direct functional relationship is unlikely. This assumption is supported by the finding that anthocyanins accumulate disproportionately to other phenylpropanoids in response to nutrient deficiency (Bongue-Bartelsman and Phillips, 1995; Peng et al., 2008). Channelling of the phenylpropanoid metabolic flux specifically towards anthocyanin synthesis indicates that they fulfil certain ecophysiological functions under limited nutrient supply, such as photoprotection via light attenuation and/or ROS scavenging. This would be of physiological advantage because Mg-deficient plants are highly sensitive to high light (Cakmak and Kirkby, 2008) and suffer from oxidative stress (Cakmak and Marschner, 1992; Hermans et al., 2010b; Tewari et al., 2006). The ability of anthocyanins to prevent photoinhibition and photodamage, as conferred by their chemical structure, has been explained in detail before (Chapter 5). Since the pattern of hydroxylation and glycosylation in anthocyanins modulates their antioxidant properties (Manganaris et al., 2014; Rice-Evans et al., 1996; Wang et al., 1997), it would be of future interest to analyse in detail the anthocyanin composition in response to Mg shortage and to compare "anthocyanin fingerprints" among diverse abiotic stresses. Differences in foliar anthocyanin accumulation between nutrient deficiencies were already shown in this work. Maize plants grown under controlled conditions synthesized anthocyanins in response to low Mg supply, whereas no leaf purpling was visible in N-deficient plants (Chapter 4, Fig. S3A). Furthermore, phosphorous deficiency was shown to result in purpling preferentially along the midrib of maize leaves, whereas anthocyanin accumulation was spread across the lamina under Mg deprivation (Chapter 5, Fig. 3, 4).

Since Mg deficiency is becoming an increasing agricultural problem, elicited by a variety of soil- and weather-born factors, early-stage diagnosis is of high interest in agriculture in order to immediately correct Mg deficiency by fertilization and consequently maintain crop yield and quality. In this work, the suitability of a handheld leaf-clip device for rapid, non-destructive measurement of epidermal flavonoids including anthocyanins in nutrient-deficient maize seedlings was tested. Next to assisting in genotype screening and fertilization management, monitoring the onset and progress of foliar anthocyanin synthesis could also provide valuable information in plant research. The examined "Dualex Scientific+TM" (Dualex) calculates an anthocyanin index by measuring chlorophyll (Chl) fluorescence excited with green and red light (Force-A, 2015). The former wavelength is strongly absorbed by anthocyanins and therefore the light amount exciting ChI fluorescence in mesophyll chloroplasts is assumed to be proportional to the epidermal anthocyanin concentration. The red light beam is supposed to penetrate into the mesophyll without being absorbed by epidermal anthocyanins and can therefore be used as a reference that accounts for anatomic leaf characteristics. However, the Dualex device did not prove to provide reliable information about epidermal anthocyanin levels in nutrient-deficient plants. The Anth index was measured in several leaves of N- and Mgdeficient maize seedlings and showed similar courses over time in leaf number 3 (Chapter 4. Fig. S2). However, anthocyanin accumulation, as indicated by leaf purpling, was only observed in plants with low Mg supply (Chapter 4, Fig. S3). Plotting of Anth index against the simultaneously measured Chl index revealed high correlations between these parameters in both N-deficient and control plants with optimal nutrient supply (Chapter 4, Fig. 4D; Table S1). Therefore, it is assumed, that the Anth index calculated by the Dualex is biased by a general methodical flaw, presumably caused by the wavelengths that are used for ChI fluorescence excitation, and which have distinct molar extinction coefficients for Chl (Nichelmann, 2014). This methodical drawback leads to dramatic overestimation of epidermal anthocyanins at low chlorophyll levels caused by Chl fluorescence reabsorption (Chapter 4; Fig. 1B). Since decreasing chlorophyll concentrations is a characteristic symptom of Mg deficiency (Chapter 3, Fig. 2; Chapter 4, Fig. 2, 5), the Dualex device did not prove to be suitable for measuring epidermal anthocyanins in Mg- or N-deprived plants. Furthermore, anthocyanin detection using the Dualex was additionally complicated by anatomical and physiological characteristics of maize leaves, namely high proportion of leaf veins and unequal distribution of anthocyanin synthesis across the leaf blade (Chapter 4, Fig. S1).

Bilger et al. (1997) previously developed a similar method for measuring epidermal UV-lightabsorbing compounds, using Xe-PAM or UV-A-PAM fluorometers to determine the UV/bluegreen-excited Chl fluorescence excitation ratio (FER), with the rationale that blue radiation can freely penetrate into the leaf without being absorbed by UV-absorbing compounds and, hence, blue-excited ChI fluorescence can serve as a reference signal to which UV-excited fluorescence can be related (Bilger et al., 2001; Bilger et al., 1997). Hagen et al. (2006) extended this method by combining UV-A-PAM and PAM-2000 fluorometer measurements, in order to determine not only epidermal UV-A-screening compounds, but also anthocyanins. In contrast to the Dualex device, which excites anthocyanin-dependent Chl fluorescence with green light, this approach uses blue light at 470 nm and red light at 655 nm as a reference beam. Since the absorptivity of Chl for these wavelengths are comparable (Goulas et al., 2004), the measurements are not biased by potential Chl fluorescence reabsorption effects. Consequently, this technique proved to accurately assess epidermal anthocyanins in fruits (Hagen et al., 2006), as verified by HPLC analysis, and can hence be used to monitor epidermal anthocyanin levels. This is of high importance in agricultural crop production, because it provides valuable information about both physiological plant state and crop quality, as discussed in detail before (Chapter 5).

# 6.4.2 Flavonol Synthesis

Next to calculating ChI and Anth indices from ChI transmittance and fluorescence measurements, the Dualex also assesses epidermal flavonol levels by exciting Chl fluorescence with UV-A light at wavelengths that are maximally absorbed by flavonols. The suitability of the Dualex device for measuring epidermal flavonol contents has been extensively tested and good correlations between the Flav index and extracted UV-absorbing phenolic compounds were found in diverse crops and under different N levels (Cartelat et al., 2005; Cerovic et al., 2012; Goulas et al., 2004; Pfundel et al., 2007). Flavonols are supposed to function in UV-light protection because of their high molar absorptivity in the UV-B band (Falcone Ferreyra et al., 2010). The preferential accumulation of specific flavonols with high antioxidant potential in response to UV-B and high-light indicates that they may also act as ROS scavengers under abiotic stress, including N deficiency (Agati et al., 2012; Bongue-Bartelsman and Phillips, 1995; Falcone Ferreyra et al., 2012; Pollastri and Tattini, 2011). In contrast to stress-induced anthocyanins, which are solely located in the vacuole and predominantly in those of epidermal cells, flavonols also occur in mesophyll cell vacuoles (Agati et al., 2011) and in chloroplasts (Saunders and McClure, 1976)(Saunders, 1976), and it is questionable, if flavonoids located in internal leaf tissue can be accurately assessed by the Dualex technique. However, little is known about tissue-specific occurrence of flavonols in response to nutrient stress.

Spatio-temporal monitoring of the Flav index in several leaves of Mg-deficient maize seedlings using the Dualex, revealed rising levels of epidermal UV-A light absorbing compounds - presumably flavonols – in lower leaves (Chapter 4, Fig. 3A, B, Fig. 6A, B). The Flav index gradually increased over time under Mg shortage beginning at the tip of leaves 3 and 4 (Chapter 4, Fig. 6C), and this was not observed in control plants with optimal nutrient supply, where the Flav index decreased over time (Chapter 4, Fig. 3, 6A). Effects of Mg deficiency on flavonol biosynthesis have hitherto not been reported and might be of interest in future agricultural and ecophysiological plant research.

Low correlation between Chl and Flav index was found in leaves of nutrient-deficient maize (Chapter 4, Fig. S4, Table S1), which might indicate that accumulation of UV-A light absorbing compounds in response to low N or Mg supply is not solely induced by light-stress. Sugar analysis of leaves did not provide conclusive information about the impact of photoassimilate accumulation on flavonol synthesis in response to nutrient shortage. Monitoring the nitrogen-balance-index (NBI) – which is defined as the ratio of Chl and Flav indices and is supposed to be a more accurate parameter displaying the crop N status (Cartelat *et al.*, 2005; Force-A, 2015) – in lower leaves was not discriminative enough for identification of N deficiency, because the mean NBI along the whole leaf blade also decreased significantly in Mg-deficient plants when compared to the control (Chapter 4, Fig. 4A, B). However, differences in the spatiotemporal distribution of epidermal flavonols were found between N and Mg deficiency. The Flav index gradually increased under Mg deficiency beginning at the tip of lower leaves, whereas the Flav index was also highly elevated in young leaves of N-deprived maize seedlings (Chapter 4, Fig. 6B, C).

#### 6.4.3 Conclusion

Secondary metabolites such as anthocyanins and flavonols are of major significance for plant stress resistance and quality of agricultural goods. Rapid, non-destructive monitoring of foliar flavonoid levels by chlorophyll fluorescence measurements can be a valuable phenotyping tool for genotype screening and fertilization management. However, leaves consisting of different cell-type layers with numerous boundaries and containing high amounts of pigments are very complicated from an optical point of view (Gitelson et al., 2003), and therefore, techniques for non-destructive assessment of leaf metabolites have to be carefully tested and their suitability for specific purposes has to be assessed on a case-by-case basis. The results of this study revealed difficulties in the utilization of the latest Dualex device for early-stage diagnosis of nutrient deficiency in maize in agriculture or plant research, namely measurement of only epidermal tissue, anatomical leaf features and unequal flavonoid distribution in maize, dramatic overestimation of epidermal anthocyanin levels in leaves with low chlorophyll content. and restricted discriminative power of the NBI for differentiation between N and Mg deficiency. Effects of Mg deficiency on flavonol synthesis in leaves of maize seedlings were shown for the first time and spatiotemporal monitoring of the Flav index revealed differences between N and Mg deficiency in youngest leaves. This knowledge might be useful in the future for optimizing early-stage diagnosis of nutrient deficiency in maize seedlings using ChI fluorescence-based detection methods.

## 6.5 Evaluation of Magnesium Sulphate Leaf Fertilization in Maize

Soil fertilizers with a gradual but strong release of Mg are most desirable to maximise root uptake and minimize losses in field-grown maize (Hardter *et al.*, 2004). However, these properties are not met by most commercially available soil fertilizers and are highly dependent on weather and soil conditions. Consequently, foliar nutrient application can be an ecologically and economically beneficial alternative to soil fertilization under certain conditions, such as restricted root Mg uptake or detection of acute Mg deficiency in crops. Magnesium deficiency in the field is caused by naturally low soil Mg concentrations or induced Mg deficiency resulting from antagonistic ions, poor soil structure, low soil moisture and transpiration, and decreased root activity during the development of reproductive organs. Direct Mg application to the plant tissue with the highest Mg demand is target-oriented and can lead to an immediate improvement of the nutritional status and the Mg concentration reaching the leaves is not dependent on transpiration-driven water flow. Furthermore, foliar fertilization allows better

timing of nutrient supply in order to meet the higher Mg demand during critical stages of plant growth or in the case of acute deficiency. In contrast, slow Mg releasing soil sources may lead to a temporal under-supply and fast releasing soil fertilizers are prone to dramatic Mg losses by leaching when applied to soils with high water conductivity under heavy rainfall conditions because of the nutrient's high soil mobility (Hardter *et al.*, 2004). Therefore, prudent foliar fertilization management can lower the quantitative fertilizer demand and avoid the uncontrolled release of nutrients into the environment.

Foliar fertilization is already of significant commercial importance worldwide (Fernández and Brown, 2013). However, there are several reports that Mg leaf-fertilization raises the foliar Mg concentration but without any considerable effects on crop yield and quality (Craighead, 2001). The current understanding of the factors that influence the ultimate efficacy of foliar nutrient applications is incomplete because nutrient uptake and plant response to the treatments is a complex scenario (Fernández and Brown, 2013) and only few studies have directly compared the efficiency of root and leaf application, respectively.

Therefore, maize was grown hydroponically under severe Mg deficiency in this work and resupplied with Mg via the roots or the leaves, respectively, in order to assess the effectiveness and efficiency of Mg leaf fertilization in this crop of global significance. In the following, the efficacy of MgSO<sub>4</sub> leaf-application in ameliorating metabolic processes in Mg-deficient maize will the assessed by the results of the conducted experiments. Particular considerations concerning the experimental setup will be discussed and possible physiological drawbacks of Mg leaf-fertilization in general will be addressed.

# 6.5.1 Experimental setup

A large variety of factors related to environmental conditions, the chemical formulation of the applied liquid fertilizer, anatomic and chemical properties of the leaves, and the developmental plant stage affect the uptake efficiency and effectiveness of foliar fertilization, some of which have been thoroughly described and reviewed elsewhere (Fernández and Brown, 2013; Fernández and Eichert, 2009; Fernández et al., 2013).

The intrinsic leaf permeability is one of the major parameters controlling the penetration of foliar-applied substances. Dissolved nutrient salts can be absorbed by the foliage via the cuticle, cuticular cracks and imperfections, stomata, and modified epidermal cells such as trichomes (Fernández and Eichert, 2009). The cuticle, synthesized by epidermal cells, is the outermost layer of leaves and serves as protection against non-stomatal water loss and pathogens. The cuticle membrane is composed of the monomer cutin - a network of interesterified hydroxyl and epoxy derivatives of C<sub>16</sub> and C<sub>18</sub> fatty acids - and overlaid with epicuticular waxes. Although the extra-cellular cuticle is a hydrophobic barrier between the inner leaf and the environment, it is not completely impermeable to liquids and applied nutrient solutions can enter the inner leaf for example through polar pores within the cutin meshwork of the cuticle. However, the mechanisms of penetration of hydrophilic, polar solutes through the cuticle are currently not fully understood (Fernández and Eichert, 2009). Applied nutrient solutions can also enter into the leaves through stomata and surfactant-assisted stomatal uptake of nutrient salt solutions was proven in both dicotyledonous and monocotyledonous species (Burkhardt et al., 2012; Kaiser, 2014). Presumably, hydrophilic pathways within the cuticle are increased in the close vicinity of guard cells and therefore, the solution uptake rate depends on the presence of stomata without the need of a direct involvement of the stomatal pore (Fernández and Eichert, 2009).

The uptake rate into the inner leaf is affected by the specific chemistry and physical structure of every particular leaf surface, which varies with plant development and physiological plant status. Morphological traits of maize leaves promote penetration of foliar fertilizer into the leaves and may therefore also favour the effectiveness and efficiency of leaf fertilization in this crop. Owing to their relatively flat topography, maize leaves show a high wettability when compared with other crops, meaning that water contact angles of droplets on the leaf surface are below 90°, which reduces solute leaching (Urrego-Pereira et al., 2013). Furthermore, maize leaves are amphistomatic and hence applied solution can enter through stomata on both the adaxial and abaxial side. The maize leaf is also covered with trichomes, which contribute to its conductivity for nutrient solutions (Fernández et al., 2013). The cuticle thickness and composition differs among maize lines (Ristic and Jenks, 2002). Moreover, the composition of the outermost wax layer changes during leaf maturation in maize, which gives juvenile maize leaves a glaucous surface appearance, whereas later appearing leaves have a glossy surface (Sturaro et al., 2005). Both ontogenetic differences in wax composition and variety-inherent cuticle properties can potentially affect leaf permeability for applied foliar fertilizer.

Next to biochemical characteristics of the leaf surface, the leaf-fertilizer formulation highly impacts the fertilizer's penetration rate and plant responses. In this work, 200 mM magnesium sulphate (MgSO<sub>4</sub>) solution including 0.1% Silwet was applied to both leaf sides of 4-5 weeks-old Mg-deficient plants for four times within ten days. In order to exactly quantify how much Mg was given to the plants, the solution was applied by brushing the leaves instead of spraying, because the latter is associated with higher losses of the liquid.

The mobility of the applied nutrient solution is influenced by the relative humidity (RH). Nutrient salts can only be taken up into the leaves in the liquid phase and a rapid uptake of the applied liquid fertilizer is favourable in order to not only avoid leaching loss but also to lower the rate of crystallization and immobilization of the dissolved nutrient salt on the leaf surface. The utilized nutrient salt MgSO<sub>4</sub> is known to be least mobile over a range of RH when compared to magnesium chloride (MgCl<sub>2</sub>) and magnesium nitrate (Mg(NO<sub>3</sub>)<sub>2</sub>). Consequently, the MgSO<sub>4</sub> uptake rate is relatively low. However, Mg(NO<sub>3</sub>)<sub>2</sub> and MgCl<sub>2</sub> both cause severe leaf damage when applied in high concentrations (Fernández and Eichert, 2009). Since the nutrient concentration of the foliar fertilizer must be high enough to meet the plant's high demand under the artificial conditions of severe Mg deficiency, application of 200 mM MgSO<sub>4</sub> with lower uptake efficiency was chosen in this work and only minimal leaf damages of short duration were observed in the single leaves (data not shown). Magnesium was applied with SO<sub>4</sub><sup>2</sup>- as counterion and a potential influence of sulphate on the analysed physiological plant responses was minimized by optimal sulphate supply with the nutrient solution. In order to increase the flux of dissolved nutrients over the cuticle barrier, the applied liquid fertilizer contained 0.1% of the surface-active agent Silwet, which lowers the surface tension of the solution, thereby maximizing the leaf coverage and enhancing the leaf penetration. Addition of surfactants such as Silwet to liquid spraying solutions of e.g. plant growth regulators, herbicides, and fungicides is a common procedure in agriculture.

#### 6.5.2 Effectiveness and Efficiency

According to expectations, the Mg concentration in Mg-deficient plants was raised by foliar fertilization in the treated leaves (Chapter 3, Fig. 5A) and rapid physiological responses to the treatment were observed in maize plants grown under severe Mg deficiency. Both the chlorophyll content as estimated by SPAD measurements and the net CO<sub>2</sub> assimilation rate

were significantly raised already within two days after the first MgSO<sub>4</sub> application and the increases were not different from plants that were resupplied with Mg via the roots (Chapter 3, Fig. 2, 3A). A re-greening was also visibly detected in the treated leaves (Chapter 3, Fig. 6). After nine days and three treatments with MgSO<sub>4</sub>, the CO<sub>2</sub>-assimilation rate was as high as in the positive control, which grew under optimal Mg nutrition throughout the whole experiment (Chapter 3, Fig. 3A). The same trend was recorded in the transpiration rate, which increased to levels of the positive control after six days and two application treatments (Chapter 3, Fig. 3B). Concentrations of K, total free amino acids, and the organic N transport forms Asn and Gln, which were highly increased in maize grown under severe Mg deficiency, were not different from the positive control at harvest (Chapter 3, Fig. 5B; Chapter 2, Table 2), indicating that Mg leaf-application successfully restored the phloem loading capacity and the distribution of metabolites and nutrients within the plant. Magnesium concentrations in the roots and in the youngest leaves, which emerged after leaf-fertilization treatments and were therefore not directly supplied with Mg solution, did not increase but remained at levels of the Mg-deficient plants (Chapter 3, Fig. 5A), indicating that applied Mg is not readily transported into untreated plant parts. This finding is astonishing, because Mg is known to be readily phloem-mobile and basipetal transport of leaf-applied Mg inside the phloem was demonstrated with <sup>28</sup>Mg isotopes (Steucek and Koontz, 1970). However, phloem transport of nutrients depends on the Mg demand in sink tissues and therefore, these results may display low Mg demand in roots and youngest leaves. In fact, the Mg demand of roots is in general relatively low, given its primary role in chloroplasts (Maathuis, 2009) and the Mg concentration in youngest leaves were obviously high enough to significantly increase the chlorophyll content in youngest leaves (Chapter 3, Table 1). In fact, increased SPAD values in youngest leaves prove for the systemic physiological effect of Mg leaf-application in distant, untreated plant parts.

Manganese concentrations in the roots were lowered to levels of the positive control by foliar Mg fertilization (Chapter 3, Fig. 5C), thereby lowering the risk of Mn toxicity in the roots, which supposedly occurred in Mg-deficient plants as indicated by shedding of lateral roots (Chapter 3, data not shown). Low Mn concentrations in roots of plants with Mg resupply to the nutrient solution can result from decreased Mn uptake caused by antagonistic effects of Mg. Since this cannot be the case for leaf-fertilized plants, where the nutrient solution Mg concentration was marginal, the low Mn concentrations in the roots are attributed to enhanced root growth (Chapter 3, data not shown), presumably resulting from increased phloem loading in source leaves. Surprisingly, Mn concentrations in leaves were not lowered to levels of the positive control by Mg leaf-application in mature leaves (Chapter 3, Fig. 5C). However, elevated Mn concentrations did not negatively impact physiological processes such as photosynthesis and transpiration in these plants (Chapter 3, Fig. 3).

The ameliorative effect of Mg leaf-fertilization on yield-relevant metabolic processes was also manifested in the fresh weight of whole plants and single leaves at harvest (Chapter 3, Fig. 4). No significant differences were measured between root and leaf resupply of Mg, respectively. The results of this work demonstrate that foliar-applied Mg enters the symplast and becomes biologically active. The majority of analysed parameters did not differ quantitatively and temporally from the Mg-deficient group, which was resupplied with Mg via the roots. This proves for both the effectiveness and the efficiency of Mg leaf-fertilization under severe Mg deficiency in maize.

# 6.5.3 Adverse Effects of Magnesium Leaf-Fertilization

As already outlined above, the chemical composition of liquid fertilizer must be chosen deliberately, because high Mg concentrations in the applied solution can not only induce leaf

burn but elevated Mg content in leaves can be critical under certain circumstances. Magnesium toxicity has rarely been described, presumably because of the high vacuolar Mg storage capacity in most plants (Gao et al., 2015; Verbruggen and Hermans, 2013). However, high Mg content in the leaves might become detrimental under drought stress (Shaul, 2002). The Mg concentrations in leaves including the Mg concentration in the chloroplast rises with increasing water-loss and stromal concentrations of free Mg above 5 mM around the thylakoid membranes were shown to repress photophosphorylation (Rao et al., 1987). In order to maintain a high stromal pH in the light, which is essential for the activity of photosynthesis enzymes, H<sup>+</sup> are pumped from the chloroplast stroma into the cytosol. This induces K channelmediated K influx into the stroma to counterbalance the changing electrochemical gradient. However, Wu et al. (1991) revealed that chloroplast envelope-associated Mg decreases the K conductance across the chloroplast membrane and hence inhibits K trafficking into the chloroplast. This consequently prevents stromal alkalization and decreases the photosynthetic capacity (Wu et al., 1991). Hence, the repressive effect of high chloroplast Mg concentrations is not caused by direct inhibition of chloroplast enzymes by free stromal Mg (Kaiser et al., 1986) but by Mg bound to the chloroplast membrane. However, the inhibitory role of Mg in photosynthesis was only shown in vitro in isolated chloroplasts and therefore, the potential risk of an adverse effect of elevated leaf Mg concentrations on photosynthesis remains speculative. It has also to be considered, that foliar applied Mg can displace calcium (Ca) at negatively charged sites in the apoplast, as observed in Vicia faba in response to application of 200 mM MgSO<sub>4</sub> (Geilfus et al., 2016). Since Ca is important for cell wall stability by bridging matrix pectin chains, and membrane stabilization (Kirkby and Pilbeam, 1984), Geilfus et al. (2016) emphasise that Mg foliar application could have negative effects on cell wall structure and membrane permeability.

#### 6.5.4 Conclusion

The results of the presented experiments in hydroponically-grown maize clearly indicate that Mg leaf-fertilization is as effective as Mg root supply in restoring basic physiological activities, such as photosynthesis, which is primarily impaired under Mg deficiency. Morphological traits of maize such as high foliar wettability and amphistomatic leaves may promote the effectiveness and efficiency of leaf fertilization, and therefore favour this alternative means of nutrition application in this crop. However, unexpected differences between plants with Mg supply via leaves or roots, respectively, were also revealed. For example, the low Mg translocation into untreated plant parts in foliar-fertilized maize needs further investigation. Nevertheless, the striking effects of Mg leaf-application on growth performance of maize justify testing the applicability of Mg foliar fertilization in the field, in order to examine the effects on yield and crop quality under non-artificial and adverse environmental conditions. The results of this work can contribute to the development of new fertilization strategies to instantaneously ameliorate acute deficiencies in the field with environmental and economic benefits.

#### 6.6 Final Conclusions

Detrimental effects on photosynthetic capacity, sugar allocation, homeostasis of potassium and manganese, and overall growth of maize were demonstrated in Mg-deficient maize in this work. Furthermore, accumulation of epidermal UV-A light absorbing compounds – presumably flavonols – were found in Mg-deficient maize seedlings. Unexpectedly, the spatiotemporal flavonol synthesis was found to be different from the flavonol patterns in N-deprived plants. Up-regulation of the flavonol biosynthesis pathway in response to Mg deficiency has hitherto not been demonstrated in crops and is of interest for future research on the relationship between the nutritional state and the secondary metabolism. Moreover, the results of this work are of value for the development of tools for early-stage diagnosis of nutrient deficiency in the field.

In contrast to previous findings in rice and spinach (Ding *et al.*, 2006; Yin *et al.*, 2009) and against expectations, the results of this work in maize show that even severe Mg deficiency is not generally accompanied by decreased NH<sub>4</sub><sup>+</sup> assimilation capacity and impaired leaf protein biosynthesis. Hence, these basic metabolic processes are not growth limiting steps under Mg deficiency. These findings might be related to species-specific efficient N metabolism of maize throughout the whole plant life cycle (Hirel *et al.*, 2005).

Moreover, it was proven in this work that MgSO<sub>4</sub> leaf application constitutes an effective and efficient alternative to Mg root supply for ameliorating acute Mg deficiency symptoms under controlled conditions. Therefore, the results of this work justify testing the applicability of Mg foliar fertilization in the field, in order to develop new economically and ecologically beneficial fertilization strategies.

# 7. Summary

The essential plant nutrient magnesium (Mg) is of fundamental importance for optimal plant growth and development. Magnesium fulfils pivotal roles in various metabolic processes including photosynthesis, gene transcription, protein biosynthesis, energy transfer, and internal distribution of photoassimilates and nutrients. However, Mg nutrition of crops is frequently overlooked and Mg deficiency is increasingly becoming an important limiting production factor in both industrialized intensive crop production (Cakmak and Yazici, 2010) and rural subsistence agriculture (Krishna, 2014). Yet, Mg is declared as the 'forgotten nutrient' in plant nutrition research (McHoul, 2006), and various aspects of the physiological responses of crops to Mg deficiency have hitherto not been conclusively clarified.

This work aimed at providing new insight into the metabolic adaptation of maize (*Zea mays* L.) to Mg undersupply, because of the major significance of this crop for global agriculture. Moreover, the suitability of a chlorophyll fluorescence-based method for Mg deficiency detection was tested and the effectiveness and efficiency of foliar Mg fertilization for ameliorating Mg deficiency were evaluated in maize.

It was shown that neither the glutamine synthetase-mediated ammonium assimilation nor the protein biosynthesis in maize leaves were reduced under Mg deficiency, and hence are not growth limiting steps in response to low Mg supply in this crop. These findings are in contrast to previous reports in other crops (Ding *et al.*, 2006; Gerendas and Fuehrs, 2013; Peng *et al.*, 2015; Yin *et al.*, 2009) and contradict the assumption that disturbed polypeptide synthesis is the primary effect of Mg shortage and the concomitant growth inhibition (Mengel and Kirkby, 2001).

Furthermore, Mg deficiency-induced accumulation of foliar flavonols was shown for the first time and nutrient stress-specific differences in the spatiotemporal synthesis of flavonoids were revealed in this work. This knowledge can contribute to future development of devices for rapid, non-destructive early-stage diagnosis of nutrient deficiency in agriculture and plant research. Such an instrument was tested in this work and was found to be not suitable for the detection of epidermal anthocyanins in nutrient-deficient leaves. Additionally, the results of the conducted sugar analysis argue against the prevalent hypothesis that anthocyanins synthesized in response to abiotic stress act as carbon storage compounds in maize leaves (Bergmann, 1993; Coley *et al.*, 1985; Herms and Mattson, 1992).

Foliar MgSO<sub>4</sub> application proved to be effective and efficient in ameliorating physiological constraints caused by severe Mg deficiency such as decreased photosynthesis and transpiration rate, disturbed homeostasis of potassium and manganese, and reduced overall growth. These striking effects of foliar Mg supply on growth performance of maize under controlled conditions justify testing the applicability of Mg foliar fertilization in the field, and can contribute to the development of new, environmentally and economically beneficial fertilization strategies for instantaneous amelioration of an acute Mg deficiency.

# 8. Zusammenfassung

Der Pflanzennährstoff Magnesium (Mg) ist von essentieller Bedeutung für optimales Pflanzenwachstum. Magnesium erfüllt elementare Funktionen in diversen physiologischen Prozessen einschließlich Fotosynthese, Proteinbiosynthese, Energietransfer und interner Verteilung von Fotoassimilaten und Nährstoffen. Dennoch wird die Magnesiumversorgung von Nutzpflanzen vielfach unzureichend beachtet und Mg-Mangel wird zunehmend zum Wachstum-limitierenden Faktor sowohl in industrialisierter Intensivlandwirtschaft (Cakmak und Yazici, 2010) als auch in ländlicher Selbstversorgerwirtschaft (Krishna, 2014). Nichtsdestotrotz wird Mg als "vergessener Nährstoff" der Pflanzenernährungswissenschaft bezeichnet (McHoul, 2006) und eine Vielzahl an physiologischen Folgen von Mg-Mangel wurde bislang nicht vollständig ergründet.

Diese Arbeit zielte darauf ab, neue Einblicke in die physiologischen Anpassungen von Mais (*Zea* mays L.) an Mg-Mangel zu gewähren, da diese Nutzpflanze auf globaler Ebene von höchster Bedeutung in der Landwirtschaft ist. Darüber hinaus wurden die Eignung einer Chlorophyll-Fluoreszenz-basierten Messmethodik zur Diagnose von Mg-Mangel getestet und die Effektivität und Effizienz von Mg-Blattdüngung zur Abmilderung von starkem Mg-Mangel in Mais bewertet.

Es wurde gezeigt, dass weder die Glutaminsynthetase-vermittelte Assimilation von Ammonium noch die Proteinbiosynthese in Mg-defizienten Maisblättern reduziert waren und folglich keine Wachstum-limitierenden Schritte in dieser Nutzpflanze unter Mg-Mangel darstellen. Diese Ergebnisse stehen im Widerspruch zu vorherigen Berichten in anderen Nutzpflanzen (Ding *et al.*, 2006; Gerendas und Fuehrs, 2013; Peng *et al.*, 2015; Yin *et al.*, 2009) und widerlegen die Annahme, dass eine gestörte Polypeptidsynthese der primäre Effekt von Mg-Mangel und dem damit einhergehenden eingeschränkten Pflanzenwachstum ist (Mengel und Kirkby, 2001).

Des Weiteren wurde in dieser Arbeit erstmalig eine Mg-Mangel-induzierte Akkumulation von Flavonolen im Blatt gezeigt und Nährstoffstress-spezifische Unterschiede in der spatiotemporalen Synthese von Flavonoiden dargestellt. Dieses Wissen kann zukünftig zur Entwicklung von Messgeräten zur schnellen, nicht-invasiven Früherkennung von Nährstoffmangel für landwirtschaftliche und wissenschaftliche Zwecke genutzt werden. Eine solche Chlorophyll-Fluoreszenz-basierte Messmethode wurde in dieser Arbeit getestet und als ungeeignet befunden, um Anthocyane in der Blattepidermis von Nährstoff-defizienten Pflanzen zu detektieren. Darüber hinaus widersprachen die Ergebnisse der durchgeführten Zuckeranalysen der Hypothese, dass infolge von abiotischem Stress akkumulierte Anthocyane zum Zwecke der Kohlenstoffspeicherung synthetisiert werden (Bergmann, 1993; Coley *et al.*, 1985; Hermans und Mattson, 1992).

Blattapplikation von MgSO<sub>4</sub> erwies sich als effektive und effiziente Methode, um mit Mg-Mangel einhergehende Stoffwechseleinschränkungen wie verminderte Fotosynthese- und Transpirationsrate, gestörte Homöostase von Kalium und Mangan sowie reduziertes Gesamtwachstum von Mais zu beheben. Die eindrucksvollen Effekte von Mg-Blattapplikation auf die Wachstumsleistung von Mais unter kontrollierten Bedingungen rechtfertigen die Überprüfung der Anwendbarkeit von Mg-Blattdüngung unter Feldbedingungen und können zur Entwicklung ökologisch und ökonomisch vorteilhafter Düngestrategien zur sofortigen Behebung von akutem Mg-Mangel im Feld beitragen.

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# **Curriculum vitae**

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2016 - Present	Research Assistant Institute of Molecular, Cell Glasgow University	and Systems Biology		
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2012 – 2016	PhD position Institute of Plant Nutrition and Soil Science, Faculty of Agricultural and Nutritional Sciences, Kiel University			
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2011 – 2012	MSc Agricultural Sciences (final grade: 1.0) Faculty of Agricultural and Nutritional Sciences, Kiel University			
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# Additional Information

2013 – 2016 Holder of a PhD scholarship of the Heinrich-Böll Foundation

#### **Publications**

Jezek, Mareike; Geilfus, Christoph-Martin; Bayer, Anne; Mühling, Karl-Hermann.

Photosynthetic capacity, nutrient status and growth of maize (*Zea mays* L.) as affected by MgSO<sub>4</sub> leaf-application.

Frontiers in Plant Science 5 Published: 2015

doi: 10.3389/fpls.2014.00781

Jezek, Mareike; Geilfus, Christoph-Martin; Mühling, Karl-Hermann.

Glutamine synthetase activity in leaves of *Zea mays* L. as influenced by magnesium status.

Planta 242:1309-1319 Published: 2015

doi: 10.1007/s00425-015-2371-8

Geilfus, Christoph-Martin Geilfus; Niehaus, Karsten; Gödde, Victoria; Hasler, Mario; Zörb, Christian; Gorzolka, Karin; Jezek, Mareike; Senbayram, Mehmet; Ludwig-Müller, Jutta; Mühling, Karl-Hermann.

Fast responses of metabolites in Vicia faba L. to moderate NaCl stress.

Plant Physiology and Biochemistry 92: 19-29 Published: 2015

doi: 10.1016/j.plaphy.2015.04.008

# Public presentations

Jezek, Mareike; Geilfus, Christoph-Martin; Bayer, Anne; Mühling, Karl-Hermann. Effects of a Mg leaf-application on K- and Mn-concentrations as well as N-assimilation in maize.

- 21. Conference Workgroup leaf-fertilization basics, consulting and praxis
- 29. September 2014, Frankfurt, Germany.

#### Poster presentations

Jezek, Mareike; Geilfus, Christoph-Martin; Bayer, Anne; Mühling, Karl-Hermann.

Photosynthetic capacity, nutrient status and growth of maize (*Zea mays* L.) as affected by MgSO<sub>4</sub> leaf-application.

International Conference of the German Society of Plant Nutrition 10. – 12. September 2014, Halle, Germany.

Neuhaus, Christina; Jezek, Mareike; Geilfus, Christoph-Martin; Mühling, Karl-Hermann. Increasing vegetative growth and yield in Mg-deficient faba beans (*Vicia faba* L.) by MgSO<sub>4</sub> foliar application.

Annual Conference of the German Society of Plant Nutrition 9. – 10. May 2013, München-Freising, Germany.