2 Introduction

2.1 Micronutrients are essential for plants and humans

Plant growth and development is highly dependent on the uptake of nutrients. According to the amount required by plants, nutrients can be divided into macronutrients (N, P, Ca, P, Mg and S) and micronutrients (Fe, Cl, Mn, Zn, B, Cu, Mo and Ni) (Marschner, 2012). The amount of nutrients present in plant tissues is mainly dependent on the plant demand and soil availability for each particular nutrient. Similar to plants, also humans require most of the same nutrients, which are obtained from the daily diet. Unfortunately, not all plant-derived food contains the necessary amounts of nutrients to meet the dietary requirements of humans. This becomes even more critical when the diet is based on a poor variety of plant derived food, and the majority of the plant derived food contains low amounts of bioavailable nutrients. For instance, a diet based only on staple cereals (e.g. maize, rice, wheat) is not able to cover the demand of many nutrients, since cereals are low in protein and micronutrients, such as Fe and Zn (White and Broadley, 2005; Cakmak, 2008; Newell-McGloughlin, 2008). As a consequence of insufficient nutrient intake, several health issues can develop. In fact, almost half of the world's population suffers from Fe and/or Zn deficiencies (WHO, 2002; Nestel et al., 2006).

In an attempt to reverse this scenario, research has been carried out to improve nutrient concentrations in edible crops, what is generally known as biofortification (White and Broadley, 2005; Nestel et al., 2006; Mayer et al., 2008; Bouis et al., 2011; Murgia et al., 2012). Biofortification can be achieved by combining breeding strategies with improved fertilization management (White and Broadley, 2005; Pfeiffer and McClafferty, 2007; Cakmak et al., 2010; Bouis et al., 2011).

The availability of biofortified food appears as a cost-effective strategy to reduce the incidence of micronutrient malnutrition and the health disorders associated therewith, especially in case of poor populations (Nestel et al., 2006; Mayer et al., 2008; Zhao and McGrath, 2009; Bouis et al., 2011).

In recent years, the main efforts have been concentrated towards the enrichment of micronutrients in edible parts of plants (e.g. grains). As important cereals, wheat, rice and barley are the most targeted in nutritional programs in order to increase micronutrient contents in the human diet (Borg et al., 2009). Perhaps the most straightforward alternative for biofortification is to supply crops with micronutrients in highly soluble forms, such as Fe(III)-chelates. This approach is known as agronomic biofortification. However, under certain circumstances this strategy is costly and its effectiveness is highly dependent on the soil and plant species (White and Broadley, 2009).

In addition to mineral fertilization, micronutrient-enriched crops can be obtained by conventional breeding or by biotechnological approaches (Goto et al., 1999; Brinch-Pedersen et al., 2007; Mayer et al., 2008). However, the success of conventional breeding heavily relies on the presence of sufficient genotypical variation for micronutrient contents in grains in the available germplasm (White and Broadley, 2005; Ortiz-Monasterio et al., 2007; White and Broadley, 2009). In wheat, Fe and Zn concentrations are lower and the genetic variability is narrower among cultivated tetraploid (*Triticum aestivum* ssp. *durum*) and hexaploid (*Triticum aestivum* ssp. *aestivum*) varieties as compared to wild wheat varieties (Balint et al., 2001; Cakmak et al., 2004; White and Broadley, 2005). One important aspect to consider is that

the total amount of a specific nutrient is not directly related with its bioavailability, since nutrients may interact with other compounds present in the food.

Thus, besides enriching edible organs with micronutrients, an additional approach to boost micronutrient bioavailability is to increase the concentrations of pronutrients (e.g. ascorbate, ß-carotene and citrate) and/or decrease the levels of antinutrients (e.g. oxalates, tannins and phytates) (White and Broadley, 2009).

In the following section the various aspects related to micronutrient uptake and distribution in plants will be introduced, with a major emphasis on Fe and Zn.

2.2 Micronutrient uptake and distribution within the plant

2.2.1 Micronutrient uptake

The first step of nutrient accumulation in plants is the nutrient uptake by roots. This process depends on the plant species and on soil availability of each nutrient. For micronutrients such as Fe, Zn and Cu, their availability relies mainly on chemical and physical properties of the soil (Marschner, 2012). For example, although Fe is the second most abundant metal in the earth's crust, its availability to plants is very low under well-aerated calcareous or alkaline soils, since it can be precipitated in the form of Fe hydroxides, oxyhydroxides or oxides (Lemanceau et al., 2009; Marschner, 2012). Thus, plants have evolved two major strategies to take up Fe. Plants using the strategy I, common in all non-graminaceous species, increase Fe availability by actively extruding protons in the rhizosphere and by enhancing both Fe(III) reduction and Fe^{2+} uptake (Romheld and Marschner, 1986; Eide et al., 1996; Robinson et al., 1999; Kim and Guerinot, 2007). Graminaceous plants, such as barley, maize and wheat, use the strategy II, in which Fe(III) is mobilized by the release of

phytosiderophores (PS), low molecular weight molecules that derive from mugineic acid (Takagi et al., 1984; Romheld and Marschner, 1986; Kim and Guerinot, 2007).

The Fe(III)-PS complexes are then taken up by the plasma membrane-bound transporter YS1 (Curie et al., 2001; Schaaf et al., 2004).

As for Fe, Zn availability is also significantly reduced in calcareous or alkaline soils, especially under arid or semi-arid conditions (Broadley et al., 2007; Marschner, 2012). Plants can take up Zn as the free ionic form Zn^{2+} or as Zn-PS complex (von Wirén et al., 1996; Suzuki et al., 2006; Broadley et al., 2007). The ionic form is mainly taken up via transporters from the zinc-regulated transporter, iron-regulated transporter-like protein (ZIP) family (Pence et al., 2000; Lopez-Millan et al., 2004; Colangelo and Guerinot, 2006), whereas in strategy II plants YS1 or YS1-like (YSL) transporters are thought to mediate Zn-PS uptake (Schaaf et al., 2004).

2.2.2 Micronutrient forms and Phytosiderophore Biosynthesis

The amount and types of PS synthesized differs among different graminaceous species. In fact, barley plants secrete higher amounts of PS in the rhizosphere than wheat plants (Tagaki, 1993). In addition, whereas barley plants secrete more types of PS at larger amounts, wheat plants synthesize and release mainly 2'-deoxymugineic acid (Kawai et al., 1988).

The non-proteinogenic amino acid nicotianamine (NA) serves as precursor for the synthesis of all types of mugineic acids. Although NA is ubiquitous to all plant species, only strategy II plants possess nicotianamine aminotransferases (NAAT) that convert NA to 3'- keto DMA (Kanazawa et al., 1994). This molecule, in turn, is

further converted into DMA, from which all other types of PS can be synthesized (Bashir et al., 2006). In plants experiencing Fe deficiency, both the biosynthesis and the release of PS are enhanced (Römheld and Marschner, 1990; Gries et al., 1995; Nagasaka et al., 2009). Recently, the transporter that mediates PS secretion into the rhizosphere in rice and barley was identified (Nozoye et al., 2011). This transporter, named as TOM1 in rice and HvTOM1 in barley, belong to the major facilitator superfamily (MFS). The expression of both TOM1 and HvTOM1 in *Xenopus laevis* oocytes leads to the efflux of DMA (Nozoye et al., 2011). In addition, the authors also demonstrate that *TOM1* expression is upregulated by Fe deficiency in rice.

PS facilitate also the mobilization of Zn. Besides Fe, it has been shown that DMA can bind Zn (Murakami et al., 1989). In barley, the biosynthesis and the release of PS are not only induced upon Fe deficiency, but also upon Zn deficiency (Cakmak et al., 1994; Suzuki et al., 2006). As a consequence, in these plants the uptake of Zn(II)-DMA was higher than that of Zn^{2+} (Suzuki et al., 2006). The uptake of Zn(II)-PS is likely mediated by Fe(III)-PS transporters, since the maize YS1 transporter can also transport Zn(II)-DMA (Schaaf et al., 2004). Interestingly, the release of PS in Zn-deficient durum wheat is smaller as compared to Zn-deficient bread wheat, indicating that this might explain at least part of why durum wheat plants are more susceptible to Zn deficiency (Cakmak et al., 1996).

2.3 Micronutrient translocation within plants

Although considerable progress has been made in understanding the micronutrient uptake mechanisms in roots, very little is known on how micronutrients are translocated after being taken up in roots. In order to be translocated from and to

different plant tissues and organs, micronutrients must be loaded in and unloaded from the xylem or phloem, the long-distance routes for nutrient transport in plants (Marschner, 2012). Recent reports have shed light on these processes.

2.3.1 Translocation in the xylem

By employing positron-emitting tracer imaging system (PETIS), it has been shown that ^{52}Fe(III)-deoxymugineic acid moves to the basal part of the shoot (Tsukamoto et al., 2009). From there, Fe can take two distinct routes: it can be either translocated to older leaves via the xylem or to the youngest leaf via the phloem. Since the free ionic forms of Zn and Fe are highly reactive and tend to precipitate, it is assumed that these micronutrients are transported in the xylem and phloem as chelated forms. In strategy I plants, the main Fe chelates during xylem transport are thought to be organic acids, especially citrate (Cataldo et al., 1988; von Wirén et al., 1999; Rellan-Alvarez et al., 2010). Evidence pointing to citrate as a major Fe-binding form in the xylem has been provided by the analyses of mutants defective in loading citrate in the xylem. The loss of the citrate transporter *FRD3* in Arabidopsis or *OsFRDL1* in rice resulted in impaired root-to-shoot Fe translocation and increased Fe-deficiency chlorosis symptoms (Durrett et al., 2007; Yokosho et al., 2009). More recently, it has been shown by both HPLC coupled to electrospray time-of-flight mass spectrometry (HPLC-ESI-TOFMS) and inductively-coupled plasma mass spectrometry (HPLC-ICP-MS) that Fe is transported in the xylem sap of tomato plants mainly in the form of a tri-Fe(III)-tri-citrate (Fe_3Cit_3) complex (Rellan-Alvarez et al., 2010). Differently from Fe, Cu is translocated in the xylem sap as a complex with NA in Cu-deficient plants, and chelated with histidine and proline under excessive Cu supply (Irtelli et al., 2009).

The long-distance of Fe and Zn in the xylem of strategy II plants is less much less characterized. However, it is thought that a great proportion of these micronutrients are transported in the xylem complexed with PS.

In this regard, it has been shown by PETIS experiments that Zn is preferentially translocated as Zn(II)-DMA in Zn-deficient rice plants (Suzuki et al., 2008).

2.3.2 Translocation in the phloem

The phloem represents the main route by which nutrients are retranslocated from old leaves into young leaves and, in the case of wheat, from flag leaves into grains. As for the xylem transport, it is also assumed that micronutrients are retranslocated via the phloem in complexed forms. In the case of Fe and Zn, it is assumed that complexation avoids precipitation due to the slightly alkaline pH of and the relatively high P concentration in the phloem sap (Briat et al., 2007; Curie et al., 2009). The most important Fe ligands during phloem transport have been proposed to be NA (von Wirén et al., 1999), PS (Inoue et al., 2008), and small peptides (Kruger et al., 2002). In the phloem of *Ricinus communis*, Fe was detected mainly bound to the protein fraction, and was found particularly complexed to an iron transport protein (ITP;(Kruger et al., 2002)). However, until now no IPT orthologs have been described in other plant species.

In many plant species, NA appears to be the major candidate for Fe complexation for subsequent phloem loading. This compound is a hexadentate Fe chelator synthesized from S-adenosyl-methionine by the activity of nicotianamine synthase (Mori, 1999). Many observations have indicated that NA plays a role during Fe translocation in the phloem. Firstly, at neutral pHs, as those measured in the phloem

sap, NA efficiently complexes both Fe(II) and Fe(III) thereby preventing the participation of Fe(II) in Fenton reactions (von Wirén et al., 1999). Secondly, when NA levels were decreased in transgenic tobacco plants, Fe loading into seeds was significantly impaired (Takahashi et al., 2003). Thirdly, the loss of all four *NICOTIANAMINE SYNTHASE* (*NAS*) genes in *Arabidopsis* resulted in low NA levels in leaves and reduced Fe concentrations in flowers and seeds (Klatte et al., 2009). This effect was mainly restricted to Fe, since Zn levels in seeds were only slightly reduced and those of Cu unaltered in *nas4x-1* mutant plants. Fourthly, many studies with members of the transporter family YSL (YELLOW STRIPE-LIKE), which are the predicted to transport Fe-NA complexes, indicate that NA is required for Fe loading in seeds. In fact, reduced concentrations of NA and Fe were detected in the *ysl1* mutant (Le Jean et al., 2005). In addition, the seed concentrations of Fe, Zn and Cu were significantly reduced in *ysl1ysl3* double mutants (Waters et al., 2006).

In strategy II plants, Fe and Zn are presumably also translocated in the phloem as complexes with PS (Curie et al., 2009). In agreement with this assumption, DMA has been detected in the phloem sap of barley plants (Mori et al., 1991). In addition, recent ESI-TOFMS analysis of the phloem sap of rice has revealed that Fe is mainly complexed with DMA (Nishiyama et al., 2012). In the same study, it was observed that most Zn is translocated in the phloem as Zn(II)-NA.

2.4 Micronutrient remobilization and retranslocation

The micronutrient contents in seeds (grains) depends on the amount taken up by roots during the stage of grain filling and the amount that is retranslocated from the vegetative tissue via the phloem (Garnett and Graham, 2005). The proportion of

nutrients retranslocated via the phloem is highly dependent on the micronutrient's mobility in the phloem. For instance, Fe shows an intermediate mobility in the phloem (Kochian, 1991), whereas Zn is relatively phloem-mobile (Marschner, 1995). In fact, in wheat, up to 70% of the Zn in the vegetative parts of plants can be remobilized in the grains (Grusak et al., 1999).

The extent of Fe retranslocation rates reported in the literature is largely different. Whereas in *Arabidopsis thaliana* it has been estimated that only 10% of the shoot Fe is retranslocated to seeds (Waters and Grusak, 2008), in wheat the reported values range from less than 30% (Hocking, 1994) to about 75% (Garnett and Graham, 2005), depending on the genotype and growing conditions. Besides species-dependent differences, also the Fe status of plants appears to determine the extent of Fe which is remobilized. In fact, high Fe remobilization rates of up to 66% were recorded in hydroponically-grown wheat plants subjected to Fe starvation from the anthesis onwards (Waters et al., 2009). In addition, Zn retranslocation was higher in Zn-deficient than in Zn-sufficient rice plants (Hajiboland et al., 2002).

2.4.1 Leaf senescence and micronutrient retranslocation

During leaf senescence, nutrients accumulated in the vegetative tissue are exported to growing leaves or to developing seeds. This process allows plants to re-utilize nutrients that are stored in leaves during the photosynthetically active phase. Leaf senescence is a highly regulated process which is associated with dramatic biochemical and ultrastructural changes (Lim et al., 2007; Gregersen et al., 2008). In cereals, senescence is regulated in individual leaves and proceeds from the oldest to

the youngest leaves (Gregersen et al., 2008). In these plants, nutrients are eventually remobilized from the flag leaf into the seeds (Wiedemuth et al., 2005).

Many genes are up-regulated in flag leaves of wheat plants during senescence (Gregersen and Holm, 2007). Importantly, the import of nutrients in seeds (grains) is synchronized with leaf senescence, thus increasing the sink strength of developing seeds (Waters and Sankaran, 2011). For some nutrients, the remobilization from the vegetative tissues might represent the majority of the nutrient that ends up in the grains. In small-grain cereals like barley, wheat and rice, it is estimated that up to 90% of the N can be remobilized from the vegetative tissues to the grains (Gregersen et al., 2008). This remobilized N represents a major source for the final protein contents in the grains of these species (Barneix, 2007; Heidlebaugh et al., 2008; Masclaux-Daubresse et al., 2008). Unfortunately, less is known about the senescence-associated remobilization of other nutrients, particularly of micronutrients.

The nutrients that end up the grains during the grain developmental stage originate from the continuous uptake in roots and especially from the restranslocation from leaves. Micronutrient contents in seeds are positively correlated with the rates of retranslocation from the source tissues (e.g. flag leaves) to the sink (in this case the seeds). Prior to retranslocation, nutrients must be remobilized and it has been shown that senescence in source tissues induces nutrient remobilization (Marschner, 1995; Gregersen et al., 2008). In this regard, it was shown that whereas only 20% of the ^{59}Fe applied to bean leaves was exported to sink leaves, this amount was increased to 34% when senescence was induced by shading in ^{59}Fe-treated leaves (Zhang et al., 1995). In addition, it has recently been shown that leaf senescence enhances Fe

remobilization in old leaves and favors Fe retranslocation to sink leaves (Shi et al., 2012). Thus, Fe retranslocation can be significantly increased by leaf senescence.

One major breakthrough in understanding the correlation between senescence and micronutrient contents was provided by the study of the wheat *Gpc-B1* locus. This locus is associated with increased contents of protein (Joppa et al., 1997; Olmos et al., 2003) and of N, Fe and Zn in wheat grains (Cakmak et al., 2004; Distelfeld et al., 2007). Interestingly, this locus is also related with earlier senescence in flag leaves and with a reduced grain-filling period (Uauy et al., 2006). These observations indicated that the earlier senescence conferred by the *Gpc-B1* locus improved the remobilization of N, Zn, Fe from leaves to the grains. *Gpc-B1* was cloned by positional cloning and found to encode *NAM-B1*, a member of the NAM (NO APICAL MERISTEM) subfamily of NAC transcription factors (Uauy et al., 2006). When the expression of *NAM-B1* in wheat was down-regulated by RNA interference, leaf senescence is delayed and, as a consequence, the grain concentrations of protein, Fe and Zn are significantly reduced (Uauy et al., 2006; Waters et al., 2009). Altogether these observations provided strong indications that leaf senescence has a great impact on the remobilization of N, Fe and Zn. Thus, conditions that affect the onset and the duration of leaf senescence might significantly affect the amount of micronutrients, such as Fe and Zn that accumulate in grains.

2.4.2 Influence of the nitrogen nutritional status on micronutrient retranslocation

Recent reports highlighted a significant impact of N nutrition on the retranslocation of Fe and Zn in cereals. Since the biosynthesis of important Fe/Zn chelators or

transport peptides such as NA or the Fe transport peptide ITP requires N (von Wirén et al., 1999; Kruger et al., 2002), a high N nutritional status may potentially promote Fe and Zn retranslocation. In addition, grain protein appears to act as a sink for Zn and Fe (Persson et al., 2009; Kutman et al., 2010). Indeed, seed concentrations of protein, Fe and Zn have been reported to show significant positive correlations (Peleg et al., 2008; Zhao et al., 2009). It is assumed that because high N supplies increase grain protein concentration, the sink strength of the grain for Fe and Zn is enhanced, leading to increased accumulation of these micronutrients in the grains.

The accumulation of Fe and Zn in wheat grains is enhanced by improving the N nutritional status of the plants (Kutman et al., 2010; Shi et al., 2010; Erenoglu et al., 2011; Kutman et al., 2011). In barley, whereas N sufficiency inhibits, N deficiency stimulates Fe export out of source leaves, indicating that the N status has contrasting effects on Fe pools in source leaves (Shi et al., 2012). However, when high N was supplied to durum wheat, almost 60% of Zn stored in the vegetative tissue was retranslocated to grains, whereas for Fe this was limited to 40% (Kutman et al., 2011). In the case of Zn, increasing N supplies not only significantly enhanced Zn uptake and root-to-shoot Zn translocation in wheat, but also increased Zn retranslocation from flag leaves into grains (Erenoglu et al., 2011). Thus, these studies indicate that N management represents a promising agronomic strategy to improve micronutrient contents in wheat grains. As discussed before, it has been shown that the wheat locus *Gpc-B1* controls amino acid remobilization from the flag leaf thereby increasing grain protein contents (Joppa et al., 1997; Olmos et al., 2003) and is associated with higher Fe, Zn and Mn concentrations in grains (Cakmak et al., 2004; Distelfeld et al., 2007). In addition, transgenic wheat *TaNAM* RNAi plants

accumulated lower grain concentrations of N, Fe and Zn (Waters et al., 2009). Thus, factors that can interfere with N retranslocation can also potentially improve the partitioning of micronutrients to grains.

2.4.3 Influence of citrate on Fe retranslocation

As presented in section 2.3.1 the organic acid citrate is an important Fe chelator during the long-distance transport of Fe in the xylem and Fe-citrate complexes have been detected in xylem exudates (Rellan-Alvarez et al., 2010). In Fe-deficient plants, citrate concentrations increase in the xylem sap (López-Millán et al, 2000). In Arabidopsis, citrate loading in the xylem vessels is mediated by a member of the multidrug and toxin efflux (MATE) transporter family, named FRD3 (Durrett et al., 2007). In *frd3-1* mutants, although total Fe concentrations in roots and shoots are markedly increased, Fe concentrations in leaf cells are constitutively low (Green and Rogers, 2004). Thus, these observations indicate that citrate is required for the proper translocation of Fe inside the plant. In rice, it has been shown that the MATE transporter OsFRDL1 moves citrate into the xylem and thereby affects root to shoot Fe translocation (Yokosho et al., 2009). More recently, evidence has been reported that citrate is important to move Fe between symplastically disconnected tissues, because citrate can solubilize Fe present outside cells (Roschzttardtz et al., 2011). This process seems to be also important in the seeds, since *FRD3* is also expressed in the embryo (Roschzttardtz et al., 2011).

It has been assumed that, similarly to what happens in the rhizosphere, when the apoplastic pH is high, Fe can be precipitated in the leaf mesophyll (Mengel et al., 1994; Kosegarten et al., 2001). Thus, under some circumstances the leaf apoplastic

Fe pool may be considerably large. This Fe pool could also contribute significantly to the Fe which is remobilized from leaves. Interestingly, some attempts have been made to increase the availability of the leaf apoplastic Fe pool via the foliar application of diluted acids, such as sulphuric or citric acids (Dungarwall et al., 1974; Kosegarten et al., 2001; Alvarez-Fernandez et al., 2004). In these studies, when diluted acids were sprayed on Fe-deficient plants, leaf chlorophyll levels were significantly increased. Thus, citrate might not only improve Fe movement inside plants because it serves as a Fe chelator during the long-distance transport of Fe, but also improves solubilization of Fe outside cells. However, the efficiency of foliar citric acid spraying in improving micronutrient accumulation in grains still remains to be addressed.

2.5 Aims of the thesis

The mechanisms that underlie the uptake of micronutrients from soil have been very well documented, however less is known on how they are distributed inside the plants. Of particular interest is to improve the accumulation of micronutrients such as Fe and Zn in the grains of crops, such as wheat, that inherently exhibit low concentrations of these micronutrients in grains. Thus, in the present thesis the main goal was to investigate fertilization practices that could be applied to improve the accumulation of micronutrients in the grains of bread wheat (*Triticum aestivum*). In addition, this work also aimed at characterizing the effect of the different fertilization regimes on the remobilization of micronutrients from flag leaves into the grains. All experiments were carried out in the field to better assess the impact of the treatments when plants are grown in agronomically relevant settings.

The first part of this thesis presents the effect of the supply of different N forms on the onset of leaf senescence and on the accumulation of micronutrients in wheat grains In addition, the effect of these treatments on the concentration of N-containing Fe and Zn chelators is reported. In the second part of this thesis, besides N forms also the effect of an additional foliar supply of citric acid was assessed. The main goal here was not only to validate the experiments from the first field trial, but also to investigate the feasibility of improving with the leaf remobilization of micronutrients, particularly Fe, by the supply of a diluted acid that can also chelate Fe, in this case citric acid.

In order to investigate the effects of the different treatments on micronutrient accumulation in grains, various analyses were carried out, such as mineral analyses via ICP-OES or ICP-MS, the concentration of chelators via HPLC, and the expression of relevant genes via real time quantitative RT-PCR.

The last section of this thesis summarizes the results and discusses the role of leaf senescence and N nutrition on the accumulation of micronutrients, especially Fe and Zn, in grains. In addition, the future challenges that hamper the biofortification of wheat grains with micronutrients are also discussed.

3 Materials and Methods

3.1 Plants and growth conditions

The field experiments were conducted on an experimental field of the IPK Gatersleben. The soil is characterized as clay with pH at around 7.6 and 22% organic matter (data base, IPK Gaterseleben). Winter wheat (*Triticum aestivum* cv. Akteur) was grown using common agriculture practice and the experiments were carried out on two subsequent years, namely 2009 and 2010. The experimental design was randomized blocks, in which each plot was 3 x 9 m. Each treatment had 4 replicates (blocks) from which ten plants were sampled. The wheat plants were supplied by different nitrogen forms: nitrate, ammonium or urea. These N forms were used in order to manipulate the onset of senescence in plants. Nitrogen (80 kg N ha^{-1}) was supplied at different stages of plant development either before anthesis (EC49/51) or after anthesis (EC65). In order to prevent the nitrification of ammonium, the application of this N form was accompanied by the supply of the nitrification inhibitor DCD (dicyandiamide). In the case of the urea treatment, the urease inhibitor nBTPT (N-(n-butyl) thiophosphoric triamide) was supplied together with the urea fertilizer. Flag leaf samples were collected at two times: either when they were still fully green (EC75) or when they started to senesce (EC85). However, in addition to the N treatments, in the second field trial also the effect of a foliar supply of citric acid was assessed. Citric acid application was carried out when plants were at the developmental stage EC85. The samples were then taken two days after spraying (EC87). The citric acid concentration was 1 g L^{-1} and 300 L ha^{-1} were supply by foliar spray. The fully mature grains were harvested at EC104 when the plants were completely dried.