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Iron and zinc translocation from senescent leaves to grains of wheat (*Triticum aestivum* cv. Akteur) in response to nitrogen fertilization and citric acid application

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Table of contents

1 Summary/Zusammenfassung

1.1 Summary

The micronutrients Fe and Zn are required by seeds to sustain the development of seedlings. Cereal grains contain low contents of Fe and Zn, which makes them poor sources of these micronutrients for human nutrition. To accumulate in grains, micronutrients have to be taken up, translocated from roots to leaves or stems and retranslocated from senescing leaves or stems to the developing grains. Nitrogen nutrition has been reported to play an important role in micronutrient retranslocation to grains by stimulating the synthesis of N-containing Fe chelators like nicotianamine and phytosiderophores, which may facilitate the long distance-translocation of these metals. On the other hand, N fertilization may be counteractive by delaying senescence and retaining micronutrients in vegetative organs.

Since so far most studies on micronutrient retranslocation during leaf senescence were carried out in hydroponics or pot experiments, the present thesis assessed the effect of different N fertilizer forms on leaf senescence and on micronutrient accumulation in wheat grains (*Triticum aestivum* cv. Akteur) under field conditions in two consecutive years. It was observed that flag leaf senescence was delayed by N fertilization, especially when N was supplied in the form of nitrate or ammonium while urea was less effective. In both field experiments the supply of ammonium after anthesis resulted in higher Fe and Zn contents in grains, suggesting that this treatment is a promising management strategy to improve the accumulation of these micronutrients in wheat grains. Nitrogen supply also enhanced the levels of nicotianamine in leaves and leaf exudates, whereas phytosiderophore concentrations were less responsive to N treatments.

In an attempt to improve Fe and Zn mobilization, citric acid was sprayed onto senescing leaves of field-grown wheat and micronutrient accumulation in grains was assessed. In N-fertilized plants citric acid application did not increase citrate exudation from senescing leaves and Fe and Zn accumulation in grains remained affected. In unfertilized plants, however, citric acid application increased citrate levels in exudates collected from senescent leaves and led to significantly elevated Fe contents in grains. In this case, the positive effect of citrate was likely related to an improved Fe solubilization by citrate in flag leaves, since this treatment had no effect on the levels of nicotianamine and phytosiderophores. The results of the present thesis indicate that N fertilization can improve the fortification of wheat grains with Fe and Zn, in particular when the different effects of fertilizer N forms are respected, while citric acid application could not be proven efficient to increase Fe and Zn accumulation in grains.

1.2 Zusammenfassung

Die Mikronährstoffe Fe und Zn werden von Samen für die Entwicklung des Keimlings benötigt. Getreidekörner besitzen geringe Fe und Zn Gehalte und sind deshalb eine schlechte Quelle dieser Mikronährstoffe für die menschliche Ernährung. Um eine Anreicherung von Mikronährstoffen in Getreidekörnern zu erreichen, müssen jene aufgenommen, von der Wurzel zu Blättern oder Stängeln transloziert und von seneszenten Blättern oder Stängeln in die sich entwickelnden Körner retransloziert werden. Es ist bekannt, dass Stickstoffernährung eine wichtige Rolle bei der Retranslokation von Mikronährstoffen spielt, da die Anregung der Synthese von N-haltigen Fe Chelatoren wie Nikotianamin und Phytosiderophoren zu einer erleichterten Langstreckentranslokation dieser Metalle führen kann. Andererseits könnte eine N Düngung auch entgegengerichtet wirken, da dadurch Seneszenz verzögert wird und Mikronährstoffe in vegetativen Organen festgehalten werden.

Bisher wurden die meisten Studien zu Mirkronährstoffretranslokation während der Blattseneszenz in hydroponischer Kultur oder in Topfexperimenten durchgeführt. Die hier vorliegende Arbeit untersuchte deshalb den Effekt unterschiedlicher N Düngungsformen auf Blattseneszenz und Mikronährstoffanreicherung in Weizenkörnern (*Triticum aestivum* cv. Akteur) unter Feldbedingungen in zwei aufeinanderfolgenden Jahren. Es wurde beobachtet, dass die Seneszenz des Fahnenblattes durch N Düngung verzögert war und zwar besonders dann, wenn N in Form von Nitrat oder Ammonium angeboten wurde, wohingegen Harnstoff weniger effektvoll war. In beiden Feldexperimenten führte die Zugabe von Ammonium nach der Blüte zu einem erhöhten Fe und Zn Gehalt in den Körnern. Dies lässt darauf schließen, dass diese Behandlung eine vielversprechende Strategie zur

verbesserten Anreicherung dieser Mikronährstoffen in Weizenkörnern darstellt. Die Stickstoffversorgung führte auch zu erhöhten Nikotianamingehalten in Blättern und Blattexudaten, wohingegen die Phytosiderophorkonzentrationen weit weniger von den N Behandlungen beeinflusst waren.

In einem Versuch die Fe und Zn Mobilisierung zu verbessern wurde Zitrat auf seneszierende Blätter von Freilandweizen gesprüht und die Mikronährstoffanreicherung in den Körnern gemessen. In N-gedüngten Pflanzen erhöhte die Zitratapplikation die Zitratexudation von seneszierenden Blättern nicht und die Fe und Zn Anreicherung in Körnern blieb unverändert. In ungedüngten Pflanzen hingegen erhöhte die Zitratapplikation die Zitratgehalte in den Exudaten seneszenter Blätter und führte zu signifikant erhöhten Fe Gehalten in den Körnern. In diesem Fall ist der positive Effekt der Zitratapplikation höchstwahrscheinlich auf eine verbesserte Fe Solubilisierung durch Zitrat in den Fahnenblättern zurück zu führen, da diese Behandlung keinen Effekt auf die Nikotianamin- und Phytosiderophorgehalte hatte.

Die Ergebnisse dieser Arbeit zeigen, dass N Düngung die Fe und Zn Anreicherung von Weizenkörnern verbessern kann und zwar besonders dann, wenn die unterschiedlichen Effekte der N Düngerform berücksichtigt werden, während die Zitratapplikation zu keiner erhöhten Fe und Zn Anreicherung in den Körnern geführt hat.

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.

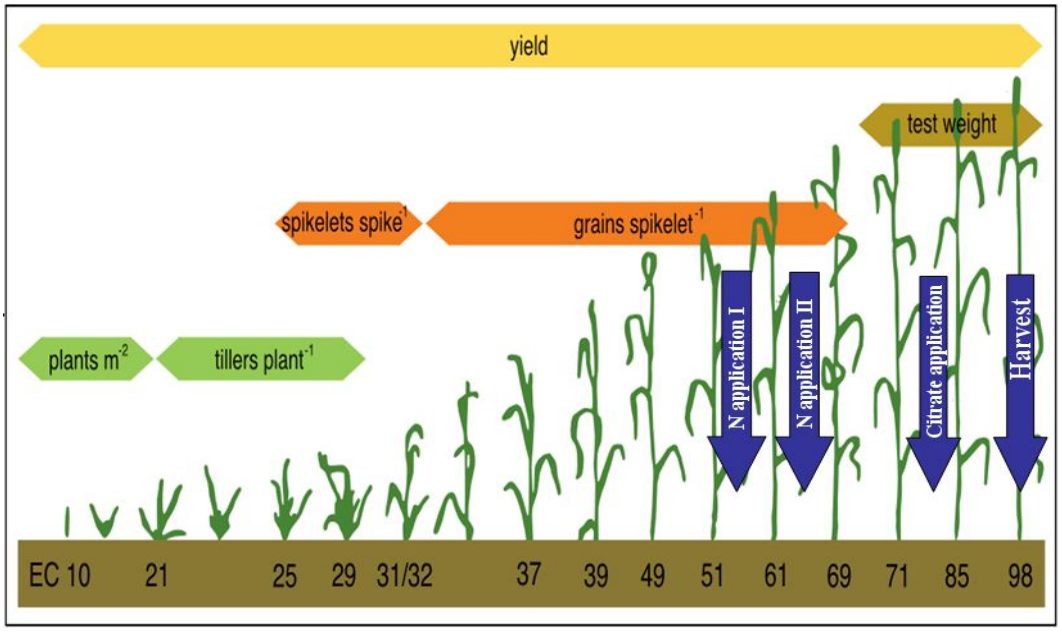

Figure 1. The developmental stages of winter wheat plants (*Triticum aestivum* cv. Akteur).
In the present study, stages EC49 to EC51 and EC61 to EC69 were named as "before anthesis" and "after anthesis" respectively. The supply of the different N forms (80 kg N ha^{-1}) was carried out before or after anthesis (N1 or N2). Leaf samples were taken at EC75 (as green leaves) or EC85 (as senescent leaves). In addition, the foliar supply of citric acid (300 L ha^{-1}) was carried out at EC85 when the leaves starting to senesce. In this case, the second harvest was at EC87, i.e. two days after citrate application. The spikes were harvested at EC75 (immature grains) or at EC104 (when all the grains were fully mature).

3.2 Determination of chlorophyll concentrations in flag leaves

Total chlorophyll were assessed in flag leaves by following the protocol described in Lichtenthaler (1987). In brief, about 20 mg of homogenized leaf samples were weighed. Then 80% ethanol was added to the samples and they were incubated at 80°C for 60 minutes. Samples were allowed to cool down for 15 minutes and then centrifuged under room temperature. Double-distilled water was added to the supernatant and homogenized. The chlorophyll concentrations were measured by

spectrophotometry (Guebel Instrumentelle Analytik, UVikon XL Bio-tek Instrument, Germany) at 652 nm.

3.3 Collection of leaf exudates

In order to collect the exudates of flag leaves, single flag leaves were cut at their basis and incubated immediately in an EDTA buffering solution. This solution contained 15 mM EDTA and the pH was adjusted to 7.5 (Weibull et al, 1990; Valle, E.M, 1998; King and Zeevaart, 1974; Crafts-Brandner, 2002). The leaf samples were incubated in the EDTA buffering solution for 3 hours. Leaf exudates were collected under control conditions at 21°C, 98 % humidity and normal light condition in the climate chamber Type HPS 1500/S (Heraeus GmBH, Germany).

3.4 Measurements of NA and DMA by HPLC

The deep-frozen flag leaves were homogenized and extracted in ultra pure water. After centrifugation at 3500 g for 30 min at 4°C, the supernatant was collected as the water-soluble fraction. The pellet, containing the water-insoluble fraction, was dried at 65°C for 72 h and wet-digested in the microwave. For this purpose, the fluorescing reagent 9-fluorenyl methoxycarboxyl chloride (FMOC) was used for a stable derivatization procedure. One miligram of FMOC was dissolved in 1 ml of acetonitrile and incubated for 10 min at 55°C. This solution was kept on ice and used for derivatization of the samples by adding 80 µl of 1 M sodium borate (pH 8.0) to 10 µl of the sample followed by the addition of 10 µl of FMOC reagent. The mixture was incubated at 55°C for 10 min and separated immediately by HPLC using a SynergyHydro C18 column (4 µm, 4.6 x 50 mm) for DMA or a Luna C18 column

(5 µm, 4.6 x 250 mm) for NA (Phenomenex, Aschaffenburg, Germany). The HPLC system consisted of a gradient pump, a degassing module, an autosampler and a fluorescence detector (Waters GmbH, Eschborn, Germany). Chromatograms were recorded using the software program MILLENNIUM 32 (Waters GmbH, Eschborn, Germany). For the determination of DMA, samples were separated for 15 min using an eluent consisting of 63.5% acetonitrile, 36% HPLC water and 0.5% formic acid. The gradient for NA measurements, in turn, was formed using eluent A containing 0.5% formic acid in HPLC grade water and eluent B consisting of 0.5% formic acid in pure acetonitrile. The HPLC gradient was produced by the following concentration changes: 80% A and 20% B was used at the beginning and the ratio of A to B was set to 60 : 40 within the first 10 min and changed to 40 : 60 for the following 5 min to purify the column from contamination. The A to B ratio was set back to the initial ratio of 80% A to 20% B for another 5 min to recondition the column for the next sample. The column was equilibrated at a flow rate of 1 ml per min and tempered at 30°C.

3.5 Determination of citrate from flag leaves

Citrate concentration in leaves was determined by Dionex HPLC system (Dionex, Idstein, Germany), which includes a gradient pump (GP50), an autosampler (A50), and a conductivity detector and suppressor ASRS Ultra II 2-mm. Frozen flag leaves were grinded and homogenized and 50-100 mg was used for extraction without derivatization. Extraction was made by adding 0.5 ml 80% ethanol. Samples were then incubated at 80°C for 60 minutes and cooled down for about 15 minutes. After centrifugation, the supernatant was removed and evaporated in speed vacuum at 55°C. Samples were resuspended with 250 µl ultrapure H_2O.

The separation of organic acids was carried out using an AS11-HC column (2 x 250 mm) that was connected to an AG11-HC (2 x 50 mm) column and an ATC anion trap column. The gradient was accomplished with ultrapure H_2O and increasing concentrations of potassium hydroxide from a concentrated EluGen Catride EGC-KOH (Dionex, Idstein, Germany) and Eluent Generator EG40. The column was equilibrated at a flow rate of 0.25 ml per minute with 4% KOH. The duration of one measurement was 40 minutes and the quantitative calculation of organic acids was carried out using the Chromeleon software.

3.6 Measurements of amino acids from flag leaves and leaf exudates

For amino acid determinations, samples were prepared and concentrated by speed vacuum as described in 3.5. Then, 250 µl of ultrapure H_2O was used to resuspend the samples. The ACQ reagent (aminoquinolyl-N-hydroxysuccimidyl carbamate) was used for derivatization at 55°C for both leaves and leaf exudates. The amino acids were determined by HPLC mounted to an ALT2, Waters 2795 separation module and a 2475 Multi Fluorescence detector (Waters GmbH, Eschborn, Germany). The reagents reacted with primary and secondary amino acids to yield highly stable urea that fluoresce strongly at 400 nm after passing through a xBridg™ C18 3 µm column. The buffer had 140 mM sodium acetate, pH 5.8 adjusted by acetic acid, Suprapur (Merck, Germany) and 7 mM triethanolamine (Sigma, Germany), acetonitrile (Roti C Solv HPLC, Roth) and ultrapure H_2O. Data collection and calculations were carried out by the Empower software (Waters GmbH, Eschborn, Germany).

3.7 Determination of nitrogen and carbon content from flag leaves and grains

Nitrogen and carbon concentrations were determined in dried and grinded samples from flag leaves and grains using ES-MS (EA; Elemental Analyzer, Mass Spectrometry, Hekatech Germany). The EA3000 series instrument was based on the well established Dumas principle of Dynamic Flash Combustion followed by gas chromatography separation of the resultant gaseous species (N_2, CO_2). In order to calculate the N and C content, the results have been multiplied by dry weight. The Horizon Stable Gas Control version 1.063 software was used for data collection and calculation of the results.

3.8 Micronutrient measurements

The concentrations of the micronutrients Fe, Zn, Mn and Cu in flag leaves and in grains were measured by inductively-coupled plasma optic emission spectrometry (ICP-OES; iCAP, Thermo Scientific). About 1.5 mg dried grinded samples from flag leaves and grains were weighed. Then, 2.0 ml ultrapure HNO_3 65% (Merck, Germany) was added to the samples. Samples were wet-digested in a high-pressure digestion apparatus (UltraClave III, MLS, Leutkirch, Germany). Micronutrient determination in leaf exudates was carried out by high resolution inductively-coupled plasma mass spectrometry (HR-ICP-MS, Element 2, Thermo Scientific). For quality control, the certified reference from International multielement standard (CPI GmBH) was used. The recovery rate was >95%. The software used to analyze the data was iTEVA version 9.1.

3.9 Determination of citrate and malate in leaf exudates

The relative concentration of citrate and malate were measured by Günther Weber (ISAS, Dortmund). Samples from leaf exudates were separated by hydrophilic interaction chromatography (HILIC), using a ZIC®-HILIC stationary phase (i.e. zwitterionic functional groups) and an acetonitrile/acetate gradient. The pH of the sample was adjusted to 6.5 and mass spectrometric detection was done using FT-ICR MS (nano-electrospray ionization Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS) in negative ESI mode ('negative ionization') and in the mass range m/z 50-1000. Phe-D5 (a modified phenylalanine in which five protons are replaced by five deuterons) was used as internal standard.

3.10 Gene expression analyses

Total RNA was extracted from frozen flag leaves using Trizol reagent. One microgram of total RNA was treated with DNAase (Invitrogen, Germany) and used for reverse transcription (Superscript™II reverse transcriptase, Invitrogen). GAPDH rRNA and the primers GAPDH-for (TTAGACTTG CGAAGCCAGCA); GAPDH-rev AATGCCCTTGAGGTTTCCC) were used as control for cDNA synthesis and amplification. Real-time quantitative PCR analysis was performed using a commercial PCR kit containing fluorescent dyes (QuantiTectTM SYBR® Green; Qiagen, Valencia, CA, USA) in the presence of 1 µg of gene-specific primers. After enzyme activation at 95°C for 15 min, amplification was carried out in a two-step PCR procedure. Dissociation curves for each amplicon were analyzed to verify the specificity of each amplification reaction. The dissociation curve was obtained by heating the amplicon from 60 to 95°C. Relative gene expression was determined

using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). The levels of mRNA for each sample were normalized with respect to GAPDH rRNA. Quantifications were expressed relative to control plants grown in complete nutrient solution. The following primers were used: TaSAG12for (GCACGACTACATCATCGTGAAG) and TaSAG12rev (CTCTCAAGGATGGT TCAGTGC) designed to specifically amplify *TaSAG12* (GenBank accession number: AK335587); TaNAS1for (CATGGATGGATGTGGCTACT) and TaNAS1rev (CTCGACTCGATCTCACCACA) designed to amplify *TaNAS1* (clone: WT004_I13); TaDMAS1for (GCCTCATCGTCAAGAGCTTC) and TaDMAS1rev(GAGCTCCTCGAGGGACTTGT) designed to amplify *TaDMAS1* (GenBank accession number AB269908.1); and TaYSL15for (GAGGATCCACACAACGTCAG) and TaYSL15rev (CTGTAGGCGACAAC AAGCAA) design to amplify a sequence that is highly similar to the rice gene *OsYSL15*, named herein as *TaYSL15* (GenBank accession number AK334282.1).

4 Results

I Influence of N forms on metal retranslocation in senescing wheat (*Triticum aestivum* cv. Akteur)

4.1 Effect of different N forms on chlorophyll levels and dry matter of flag leaves

The first experiment was designed to manipulate Fe retranslocation by the onset of flag leaf senescence. In order to determine whether the different N forms supplied to wheat plants affected leaf senescence, chlorophyll levels were determined (Figure 2). Compared to first harvest when flag leaves were still green, leaves harvested during senescence, showed significantly lower chlorophyll concentrations.

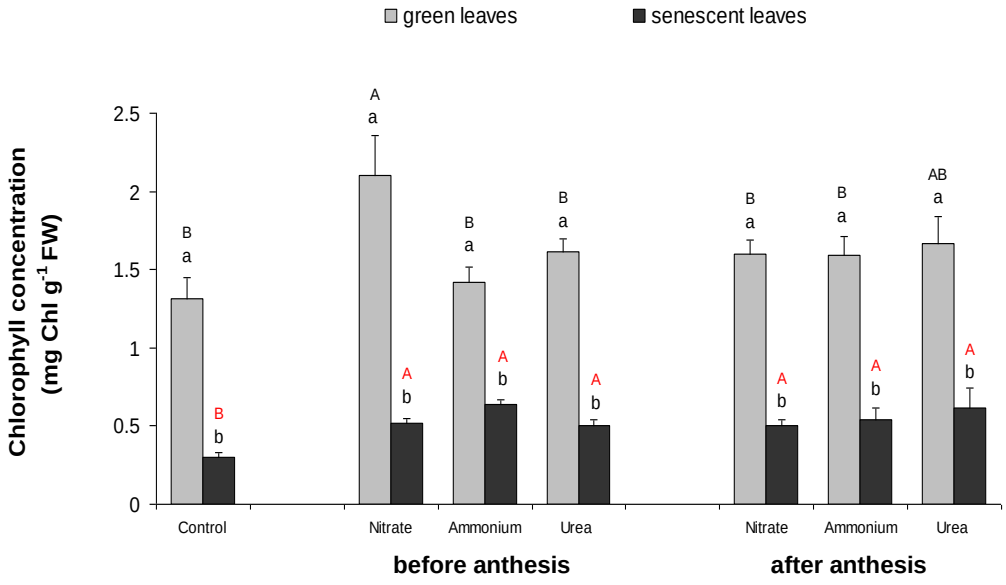

Figure 2. Effect of N forms on chlorophyll concentrations in flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (green leaves) or 85 days (senescent leaves), flag leaves were harvested and the chlorophyll concentrations were determined. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$.

The reduced chlorophyll levels measured at the second harvest reflected advanced senescence (Figure 2). Thus, flag leaves from the early and late harvest were described as green and senescent leaves, respectively. Nitrate application led to higher levels of chlorophyll in green leaves (Figure 2). In senescent leaves, chlorophyll concentrations were significantly lower in unfertilized plants than in N-fertilized plants. To assess whether the N forms affected the dry matter of leaves, the dry weight of flag leaves was determined (Figure 3). Unlike the expectation, there was no significant effect of the N form on the dry matter of flag leaves either when measured in green leaves or senescent leaves. Although the absolute dry weights of leaves harvested at the late date were lower, these differences were not statistically significant (Figure 3).

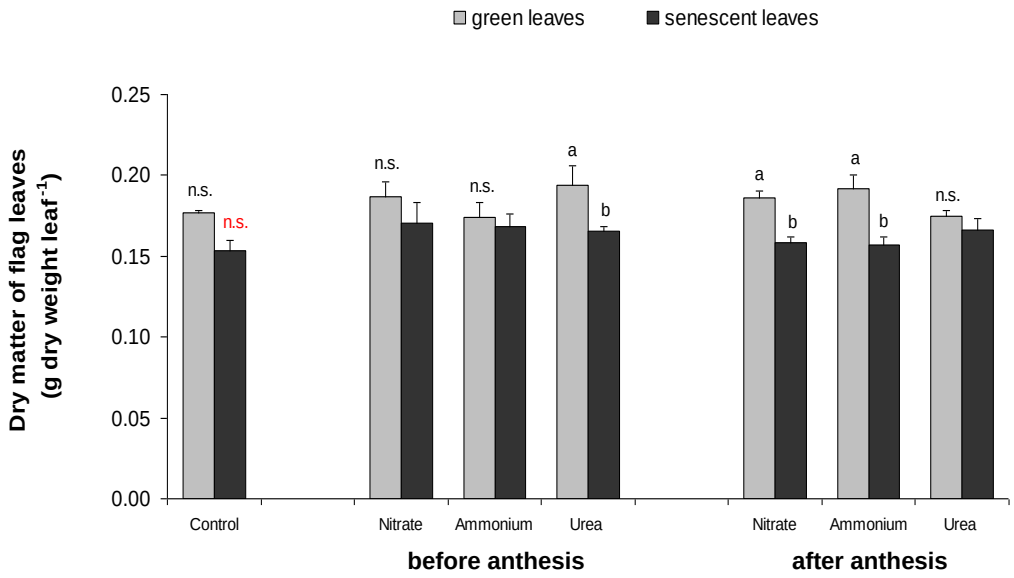

Figure 3. Effect of N forms on dry weight of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (green leaves) or 85 days (senescent leaves), flag leaves were harvested and the dry weight of leaves was determined. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

In order to assess micronutrient contents in grains, plants were grown until grain maturity. Whole spikes were harvested 75 and 104 days after sowing, and grains were collected from each spike, being considered in the present work as immature and mature grains, respectively. As expected, the weight of mature grains was greater than that of grains harvested at an earlier developmental stage (Figure 4). The treatments tested did not significantly affect the weight of immature grains. However, in the case of mature grains, it was observed that compared to control and nitrate, ammonium fertilization increased grain weight. The difference was more prominent when ammonium was supplied after anthesis.

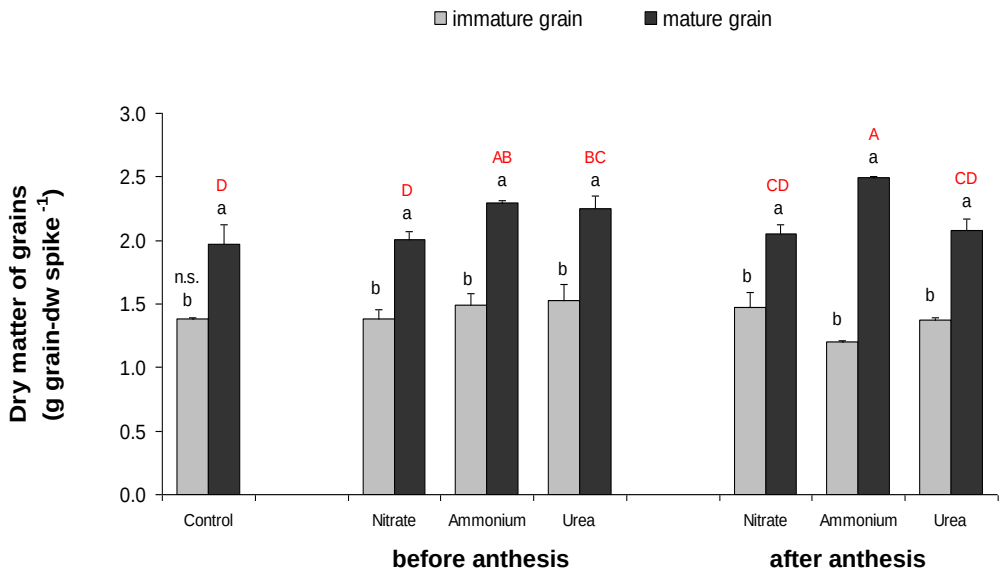

Figure 4. Effect of N forms on dry weight of grains.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (immature grain) or 104 days (mature grain), spikes were harvested, then grains were collected from each spike, and the dry weight of grains was determined. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for immature grain and red letters for mature grain), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

4.2 Effect of different N forms on accumulation of N and micronutrients

Several reports indicated that the remobilization of micronutrients is associated with the remobilization of N (Kutman et al., 2010; Shi et al., 2010; Erenoglu et al., 2011; Kutman et al., 2011). Thus, the effect of the different N treatments on N contents in leaves and grains was assessed. Compared to control plants, the application of N efficiently increased N contents in both green and senescent leaves, except for the supply of urea after anthesis (Figure 5). It was observed that leaf senescence was accompanied by a significant decrease in the total N contents.

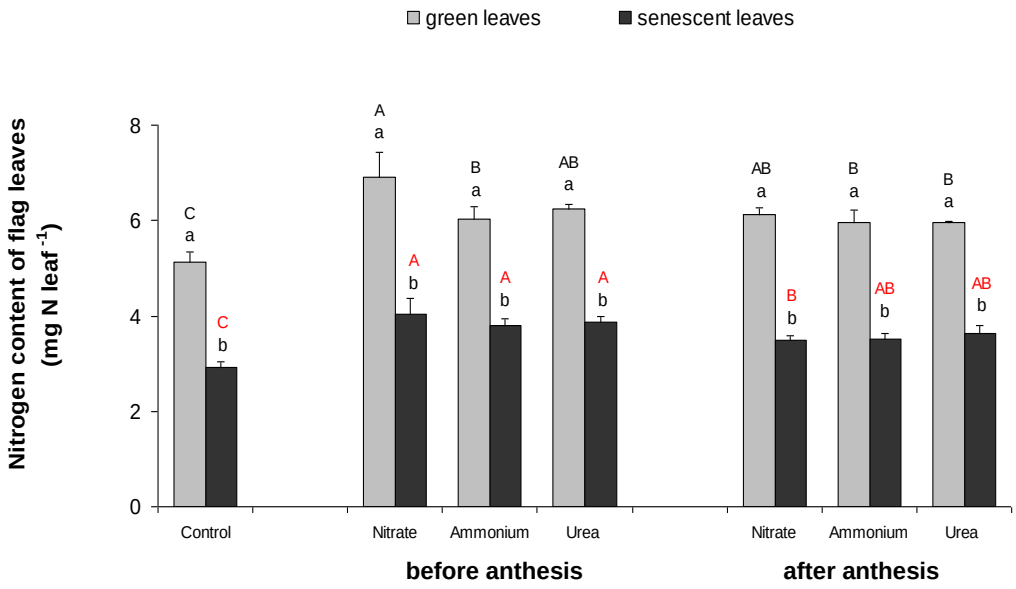

Figure 5. Effect of N forms on N contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (green leaves) or 85 days (senescent leaves), flag leaves were harvested and the N contents of leaves were determined by Elemental Analysis-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$.

Importantly, the supply of N, irrespective of the form, efficiently maintained higher N levels in senescing leaves (Figure 5). This effect was more prominent when N forms were supplied before anthesis.

In grains, total N contents increased as grains matured, except when nitrate or ammonium was supplied before anthesis (Figure 6). This observation was mainly due to the fact that these two treatments were the most efficient ones in inducing a significant accumulation of N already in immature grains. Nitrogen application resulted in a significant increase in grain N contents, particularly when ammonium was supplied to plants after anthesis (Figure 6).

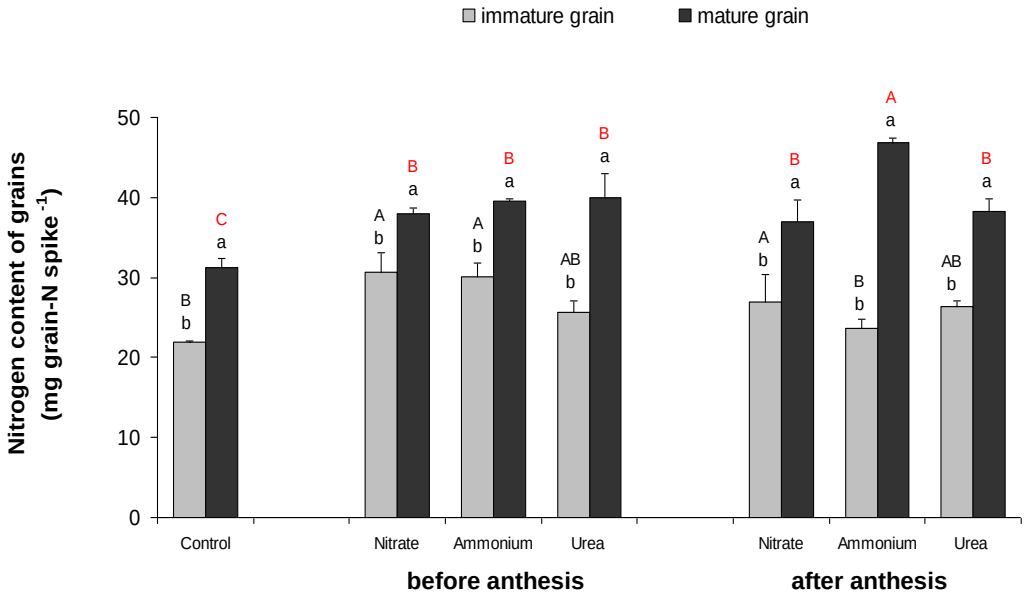

Figure 6. Effect of N forms on N contents of grains.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (immature grain) or 104 days (mature grain), spikes were harvested and grains were collected from single spike then the N content was determined by Elemental Analysis-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for immature grain and red letters for mature grain), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$.

Since the main aim of this study was to determine the effect of N forms on Fe accumulation in grains, Fe contents were determined in flag leaves and grains. Iron

contents decreased significantly in senescent leaves, except when nitrate was supplied to plants after anthesis (Figure 7). This reduction could be indicative of enhanced Fe retranslocation in senescent leaves.

In green leaves, Fe contents were higher when nitrate was fertilized before anthesis. In senescent leaves, however, Fe contents were lowest in control plants and in plants fertilized with urea after anthesis (Figure 7).

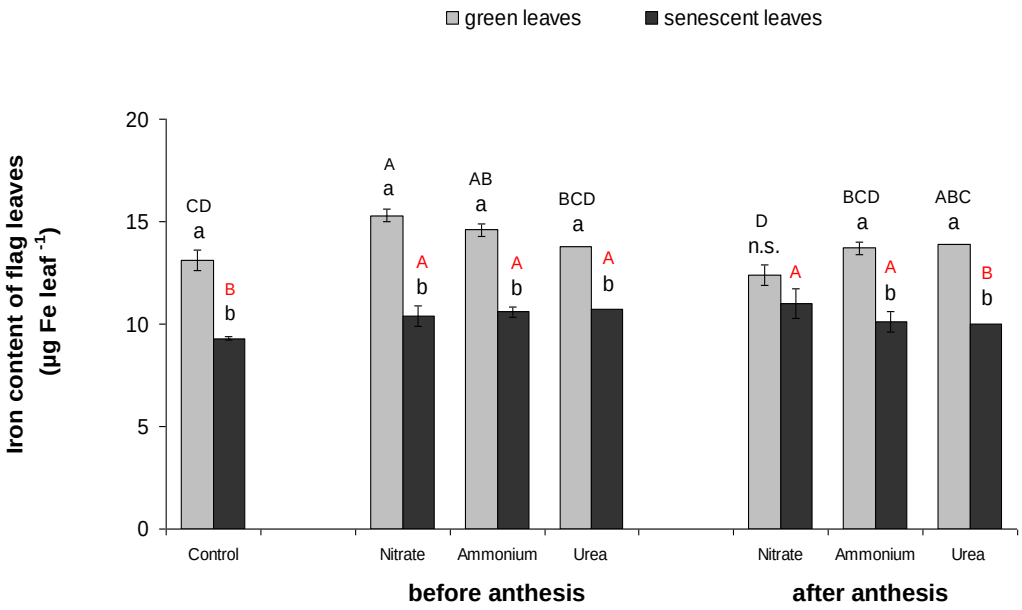

Figure 7. Effect of N forms on Fe contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (green leaves) or 85 days (senescent leaves), flag leaves were harvested and the Fe contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

In order to assess more directly Fe retranslocation, Fe concentrations were measured in exudates collected from flag leaves. Contrary to what was observed in flag leaves, Fe concentrations did not change significantly in exudates as flag leaves became senescent (Figure 9). However, the supply of ammonium before anthesis or

the late supply of N (after anthesis) significantly decreased the Fe levels in the flag leaf exudates. In grains, Fe contents increased as grain matured (Figure 8). Irrespective of the N form in immature grains the supply of N before anthesis

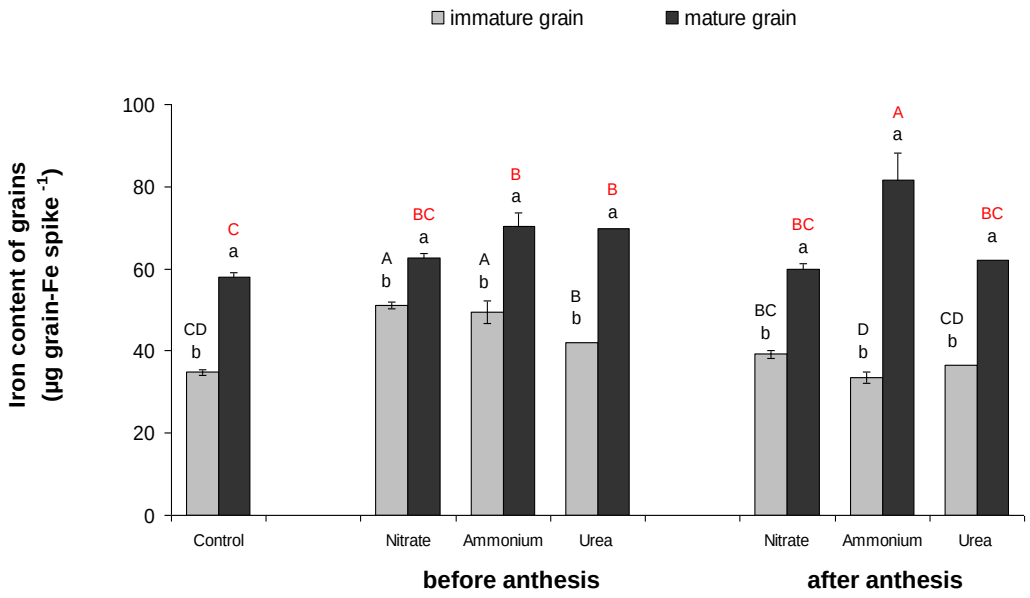

Figure 8. Effect of N forms on Fe contents of grains.
Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (immature grain) or 104 days (mature grain), spikes were harvested and grains were collected from single spike, then the Fe contents were determined with ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for immature grain and red letters for mature grain), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$.

increased Fe contents when compared to the control treatment or N application after anthesis. In green leaves, only the late urea supply resulted in significantly increased Zn contents (Figure 10), whereas in senescent leaves none of the treatments did significantly alter Zn accumulation. Opposite to what was observed in flag leaves, Zn contents increased significantly as plants progressed in their development. Thus, Zn contents increased in mature grains. Importantly, the supply of ammonium either

before or after anthesis or the supply urea before anthesis resulted in higher Zn concentrations in mature spikes (Figure 11).

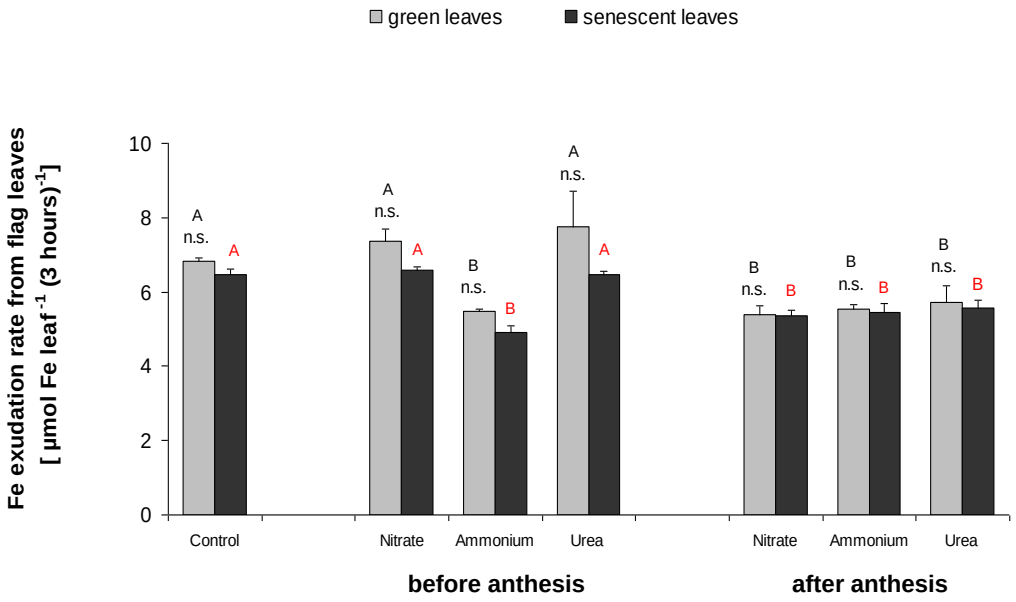

Figure 9. Effect of N forms on Fe exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). The leaf exudates were collected into 15 mM EDTA solution pH 7.5 from green leaves after 75 days and from senescent leaves after 85 days. The Fe concentrations of leaf exudates were determined by ICP-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

The Zn exudation rates from flag leaves were also assessed (Figure 12). It was observed that all N treatments reduced significantly Zn contents in the exudates collected from either green or senescent leaves. In addition, although the developmental stage of leaves had no effect on the Zn exudation rates in control plants, the supply of nitrate to plants significantly reduced Zn exudation in senescent leaves (Figure 12). By contrast, the supply of ammonium after anthesis increased Zn exudation in senescent leaves.

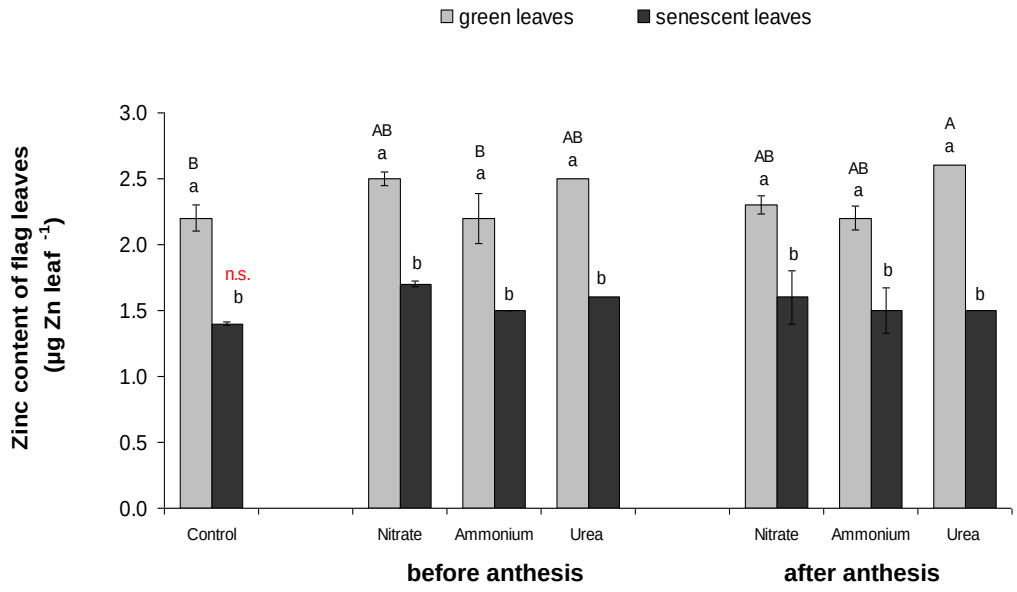

Figure 10. Effect of N forms on Zn contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (green leaves) or 85 days (senescent leaves), flag leaves were harvested and the Zn contens were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

Mn and Cu decreased in senescent leaves, although the N forms maintained the content of Mn compared to control plants (Appendix 3). The application of N forms increased the contents of Mn and Cu in mature grains (Appendix 6), particularly when ammonium and urea were supplied at a later stage. Interestingly, in leaf exudates it was found that Mn contents increased during senescence especially by nitrate being supplied before anthesis (Appendix 5). By contrast, Cu in leaf exudates decreased during leaf senescence irrespective of the N form supplied (Appendix 8).

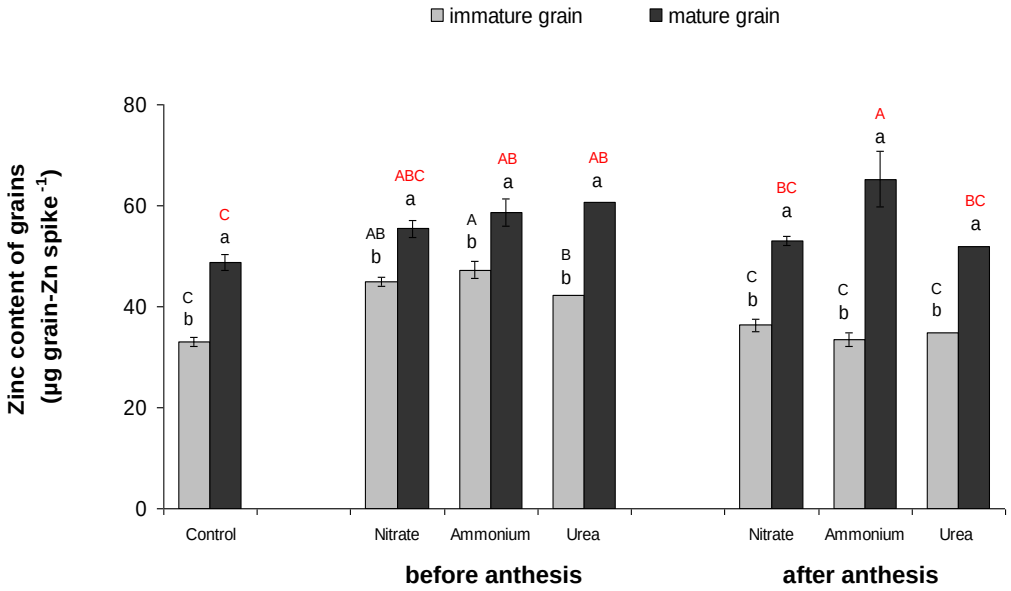

Figure 11. Effect of N forms on Zn contents of grains.
Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (immature grain) or 104 days (mature grain), spikes were harvested and grains were collected from single spike, and the Zn concentrations were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for immature grain and red letters for mature grain), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$.

The contents of Mn and Cu were also determined in grains observed to increase by ammonium when applied or after anthesis, or by urea when supplied before anthesis (Appendix 4 and 7) .

Altogether, these results indicate that N treatments increased N contents in flag leaves and delayed leaf senescence. In addition, N fertilization maintained higher Fe contents in senescent flag leaves, whereas the supply of N before anthesis increased Fe and Zn contents in grains as well as it was found in Mn and Cu grains contents.

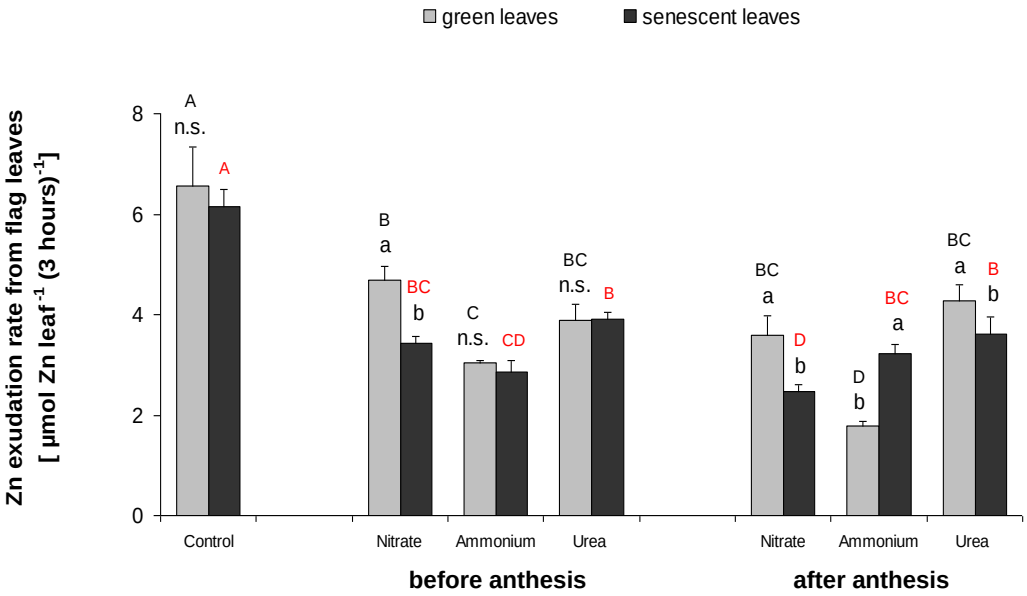

Figure 12. Effect of N forms on Zn exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). The leaf exudates were collected into 15 mM EDTA solution pH 7.5 from green leaves after 75 days and from senescent leaves after 85 days and the Zn concentrations were determined by ICP-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

4.3 Concentration of Fe chelators in the flag leaves and leaf exudates as affected by different N forms

The determination of Fe contents in flag leaves (Figure 7), flag leaf exudates (Figure 9) and in grains (Figure 8) indicated that Fe remobilization and retranslocation were likely affected by the N forms. Because it is assumed that during retranslocation via the phloem Fe is maintained in a complexed form (Briat et al., 2007; Curie et al., 2009), the concentrations of two major N-containing Fe chelators – NA and DMA – were assessed. It has been shown that the non-proteinogenic amino acid NA is able

to chelate Fe(II) at a high affinity under slightly alkaline pH (von Wirén et al., 1999) as usually found in the phloem sap (Hall and Baker, 1972). Thus, NA is thought to play a key role in the long-distance transport of Fe via the phloem (Scholz, 1989; von Wirén et al., 1999). In addition, NA was found as the main chelator of Zn in the phloem sap of rice plants (Nishiyama et al., 2012). In flag leaves, except for control plants, NA concentrations were remarkably lower in senescent leaves (Figure 13). Compared to unfertilized plants, N application resulted in significantly higher NA concentrations, especially in green leaves. This effect was very pronounced when N fertilization was carried out before anthesis (Figure 13). The effect of the different N forms on NA concentrations in flag leaves was less significant. Interestingly, except in control and when nitrate was applied before anthesis, NA concentrations were significantly higher in the exudates collected from senescent leaves (Figure 15). Since NA concentrations were reduced in senescent flag leaves (Figure 13), but increased in the exudates of these leaves, it appears that during leaf senescence NA is exported via the phloem (Figure 16). Furthermore, although all N forms had similar effect when applied after anthesis, only ammonium and urea increased NA levels in the leaf exudates when supplied before anthesis (Figure 14).

The concentrations of DMA in leaves and in leaf exudates were also assessed in response to the different N treatments. Different to NA, DMA concentrations were significantly higher in senescent leaves (Figure 14).

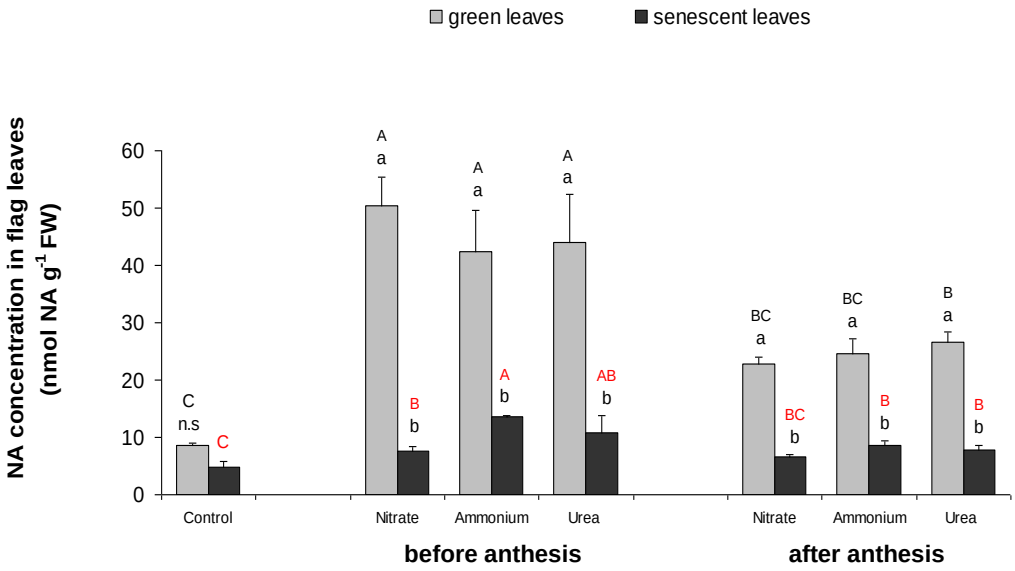

Figure 13. Effect of N forms on nicotianamine (NA) concentrations of flag leaves. Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (green leaves) and 85 days (senescent leaves), flag leaves were harvested and the NA concentrations were determined by HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

However, this effect was restricted to plants supplied with N before anthesis, not being observed in control treatment or under N application after anthesis. In addition, compared to the control treatment, only the supply of nitrate or urea before anthesis resulted in a significant increase of DMA levels in senescent leaves (Figure 15).

In addition to NA, in strategy II plants, like wheat, also the PS 2'-deoxymugineic acid (DMA) is assumed to play a key role in the movement of micronutrients in the phloem. In fact, in the phloem sap of rice plants it was found that most Fe was chelated to DMA (Nishiyama et al., 2012). In the present study with wheat, it was observed that contrary to NA in leaves (Figure 13), the levels of DMA were significantly higher in senescent leaves (Figure 14).

However, this effect was restricted to plants supplied with N before anthesis, not being observed in unfertilized plants or when N application was carried out after anthesis. In addition, compared to the control treatment, only the supply of nitrate or urea before anthesis resulted in a significant increase of DMA levels in senescent leaves (Figure 14).

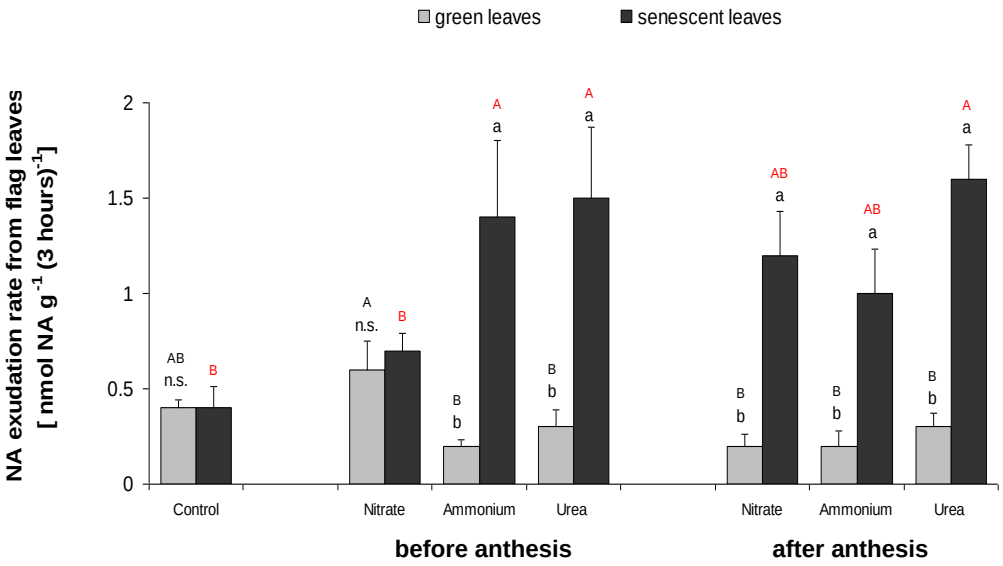

Figure 14. Effect of N forms on nicotianamine (NA) exudation rates from flag leaves. Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). The leaf exudates were collected into 15 mM EDTA solution pH 7.5 from green leaves after 75 days and from senescent leaves after 85 days and NA concentrations were determined by HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

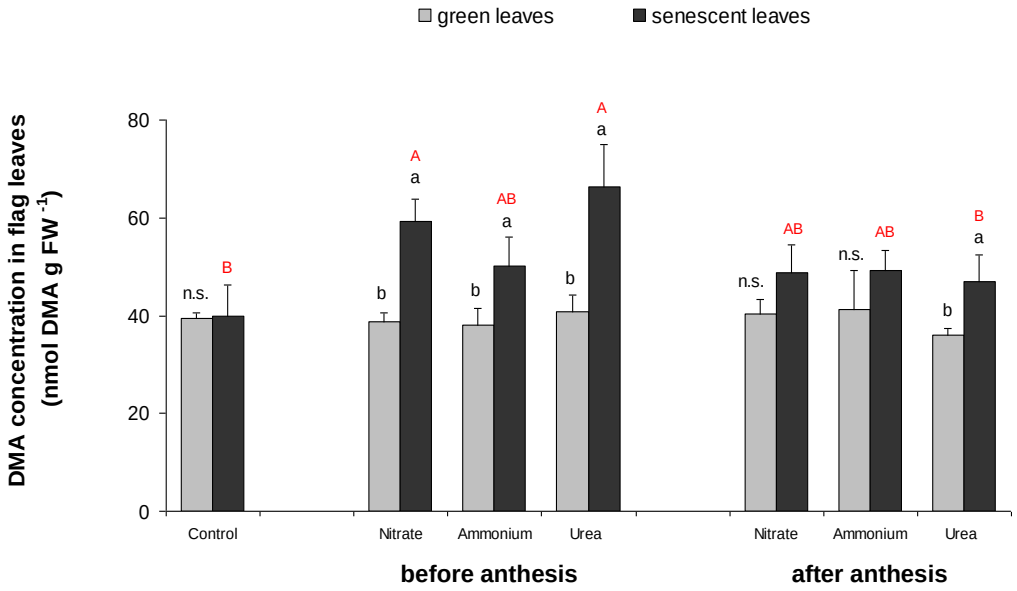

Figure 15. Effect of N forms on deoxy-mugineic acid (DMA) concentration in flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (green leaves) and 85 days (senescent leaves), flag leaves were harvested and the DMA concentrations were determined by HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

DMA levels were generally higher in exudates collected from green leaves (Figure 16). At this harvest time, N fertilization and N forms did not significantly affect DMA concentrations in leaf exudates. However, in the exudates of senescent leaves DMA concentrations were higher particularly when no N was supplied (control) or when nitrate was supplied before anthesis (Figure 16). The late N fertilization with urea also increased DMA concentrations in leaf exudates as compared with nitrate or ammonium. Besides NA and DMA, also the organic acid citrate is able to serve as a Fe chelator during long-distance transport. In the present work relatively high levels of citrate were detected in green leaves (Figure 17). The citrate concentrations decreased significantly in senescent leaves.

In these leaves, when compared to unfertilized plants, the early supply of nitrate or urea reduced significantly the citrate concentrations.

It has been shown that the foliar application of natural Fe-amino acid chelates like glutamate, glycine and arginine (Sanchez-Sanchez et al, 2002; Rodríguez-Lucena (2010) can reduce leaf chlorosis. In general, most amino acid concentration in green leves increased after supply of N form (Table 1). In particular glutamine and asparagines concentration were much higher when N was supplied after anthesis. In the leaf exudates again glutamine as asparagine levels were most elevated when N form was supplied after anthesis.

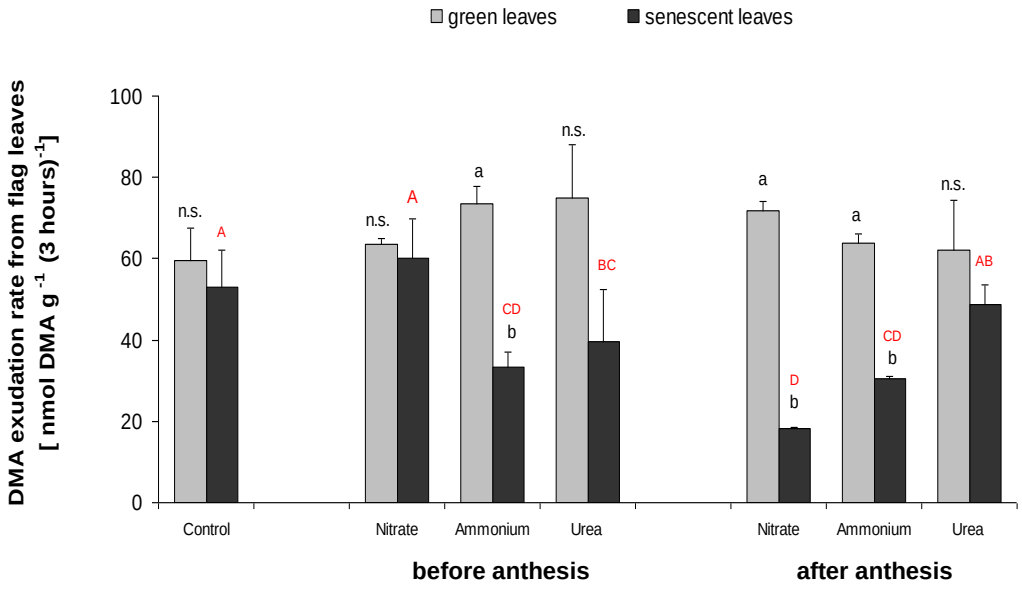

Figure 16. Effect of N forms on deoxy-mugineic acid (DMA) exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). The leaf exudates were collected into 15 mM EDTA solution pH 7.5 from green leaves after 75 days and from senescent leaves after 85 days and DMA concentrations were determined by HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

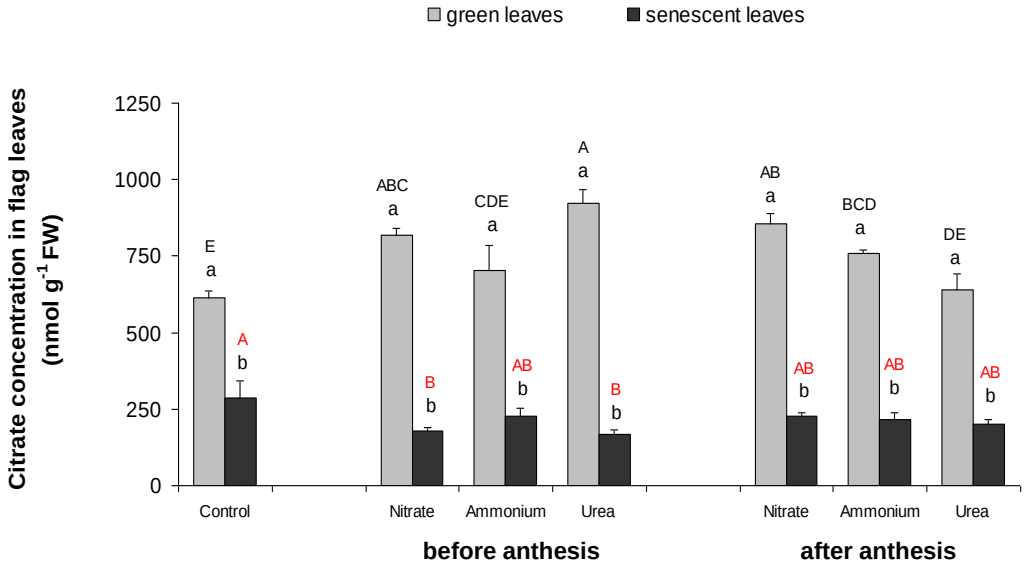

Figure 17. Effect of N forms on citrate concentration of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (green leaves) and 85 days (senescent leaves), flag leaves were harvested and the citrate concentrations were determined by Dionex HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

In addition, the concentration of other amino acids (e.g. glycine, histidine and alanine) also decreased sharply during senescence in flag leaves (data not shown). Furthermore, the supply of N did not significantly affect the contents of these amino acids in green and senescent leaves (data not shown).

Altogether, these results demonstrate that whereas in senescent leaves NA levels decreased significantly, the exudates of these leaves contained higher NA concentrations. The supply of N increased the NA concentrations in the exudates of senescent leaves. The treatments did not consistently affect DMA levels in flag leaves and in leaf exudates.

Table 1. Concentration of essential amino acids measured in flag leaves and their exudation rate in response to different developmental stages and forms of N application.

	Control	Application of nitrogen					
		Before anthesis (EC49)			After anthesis (EC65)		
		NO_3^-	NH_4^+	Urea	NO_3^-	NH_4^+	Urea
Flag leaves							
Green leaves				**(nmol g^{-1} fresh wt)**			
Glutamine	380a C	823a AB	541a BC	858a AB	1071a A	1035a A	955a A
Asparagine	164a C	1648a B	563a C	1756a B	1450a B	2995a A	2772a A
Glutamate	2132a C	2671a C	2367a C	3868a AB	2660a C	4114a A	3040a BC
Aspartate	940a C	1659a AB	1474a BC	2085a A	1734a AB	2100a A	1725a AB
Senescent leaves							
Glutamine	193b D	406b BC	280b CD	569b AB	205b CD	413b BC	618b A
Asparagine	29b B	92b B	76b B	132b AB	74b B	145b AB	213b A
Glutamate	747b B	1032b A	1001b A	1001b AB	695b B	113b A	1216b A
Aspartate	509b C	1159b AB	835b BC	894b BC	545b C	853b BC	1472a A
Leaf exudates				**[nmol $leaf^{-1}(3\ h)^{-1}$]**			
Green leaves							
Glutamine	105b BC	108b BC	99b C	77b D	98b C	117b AB	131b A
Asparagine	4n.s. D	15a A	12a ABC	11b BC	3n.s. AB	9n.s. C	12n.s. BC
Glutamate	147a BC	202a A	37n.s. BC	109b C	151a BC	159n.s. AB	150n.s BC
Aspartate	75n.s. BC	113a A	70n.s. BC	55b C	83a B	81b B	108a A
Senescent leaves							
Glutamine	449a DE	569a CD	378a E	644a BC	697a BC	1039a A	753a B
Asparagine	7n.s CD	7b CD	5b D	19a A	10n.s. BC	10n.s BC	13n.s. B
Glutamate	112b C	154b AB	123n.s. BC	184a A	107b C	183n.s A	183n.s A
Aspartate	60n.s B	60b B	59n.s. B	96a A	50b B	106a A	87b A

*** Small letter → comparison between harvest time. Capital letter → comparison on row**

II. Influence of foliar citric acid application on micronutrients retranslocation in senescing wheat (*Triticum aestivum* cv. Akteur).

4.4 Effects of N forms and citric acid on flag leaf senescence

Following the objective to investigate whether foliar application of the Fe chelator citric acid can improve Fe retranslocation from flag leaves to grains, wheat plants pre-treated with different N forms to alter senescence behaviour were then subjected to foliar application with citric acid.

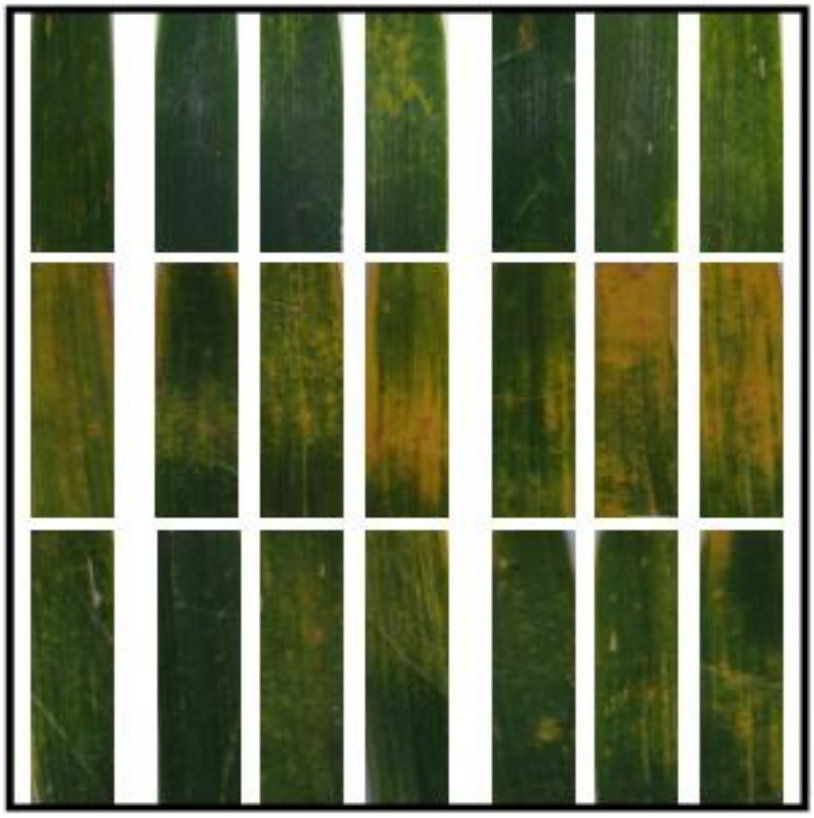

Figure 18. Effect of N supply and foliar citric acid application on the visual appearance of flag leaves
Nitrate, ammonium or urea (80 kg ha^{-1}) were supplied either before (EC49) or after the antheis (EC65) to wheat plants (*Triticum aestivum* cv. Akteur). The foliar application of citric acid (300 L ha^{-1} of 1.0 g citric acid L^{-1}) was performed to plants 87 days after sowing. Flag leaves were harvested when still green, or when starting to senesce.

As expected, with supply N forms tended to accelerate senescence at a stage when control plants leaves were still green (Figure 18). This trend continued when flag leaves were senescing, whereas nitrate supplied leaves tended to remain green for longer. This was also supported by chlorophyll concentration in senescent leaves, which were higher after N supply, in particular when citric acid was used (Figure 19). In addition, a foliar application of citric acid (300 L ha^{-1}) was carried out when plants were at the developmental stage EC85. Although citric acid application tended to increase chlorophyll concentration, these values significantly altered, suggesting that citric acid does not influence leaf senescence.

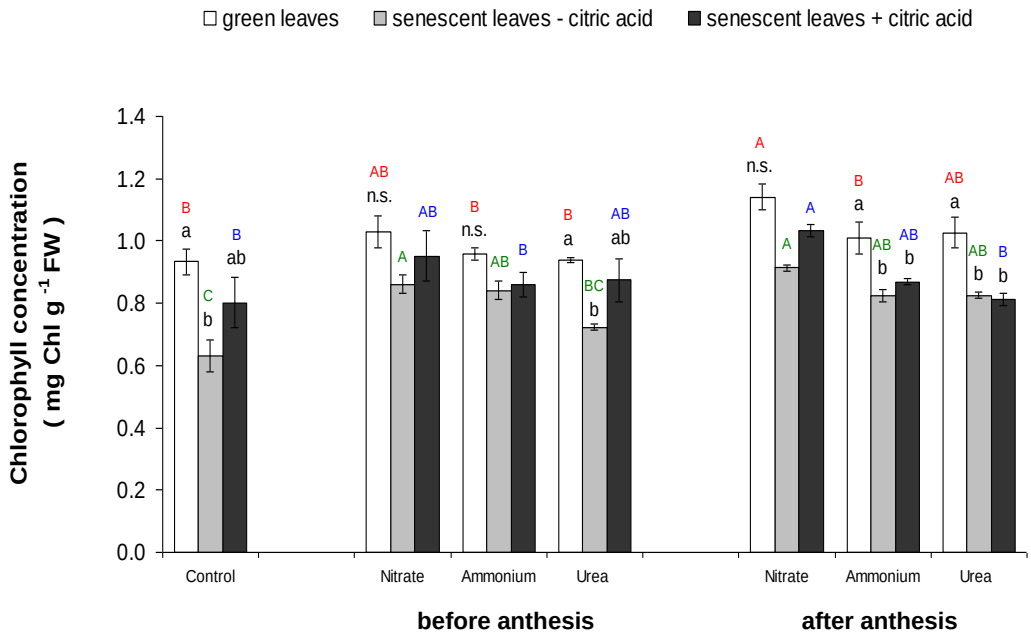

Figure 19. Effect of N supply and foliar citric acid application on chlorophyll levels of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

Moreover, the expression of the senescence-asosiated gene *TaSAG12* was determined as a molecular marker for leaf senescence. It has been demonstrated that *TaSAG12* transcript levels are upregulated during leaf senescence (Lohman et al, 1994; Sun-Noh et al., 1999). Unfortunately, in the present study, the expression of *TaSAG12* did not seem to correlate with chlorophyll levels. In fact, *TaSAG12* expression was only significantly increased in senescent leaves of ammonium-supplied plants, remaining largely unchanged in leaves of control and nitrate-treated plants (Figure 20). In the case of urea, the expression of this gene was unchanged with the late application of urea whereas *TaSAG12* transcription was actually downregulated in senescent leaves when urea was supplied before anthesis.

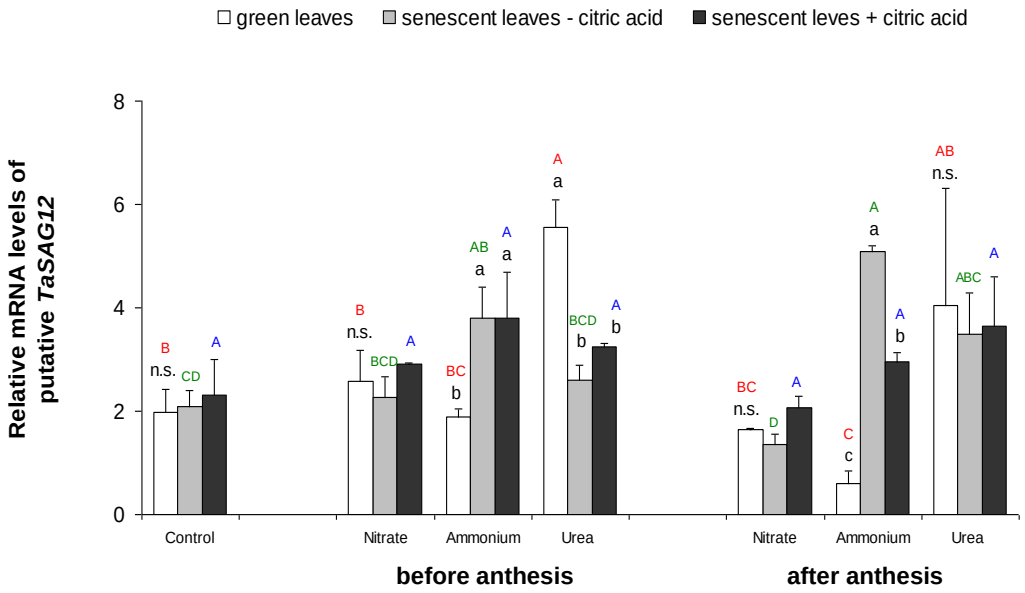

Figure 20. Effect of N supply and foliar citric acid application on the expression of *TaSAG12* in flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) or 87 days (senescent leaves). *TaSAG12* expression levels were determined by means of quantitative real time PCR. Values are means of 3 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

Except in the case, of late supply of ammonium, the application of citric acid had no significant effect on the expression of *TaSAG12* (Figure 20).

4.5 Effects of N forms and citric acid on dry matter of flag leaves and grains

The dry matter of green flag leaves was hardly affected by the supply of N (Figure 21). In general, the dry matter of senescent leaves decreased as compared to green leaves and, except for the late urea application, was not affected by spraying citric acid.

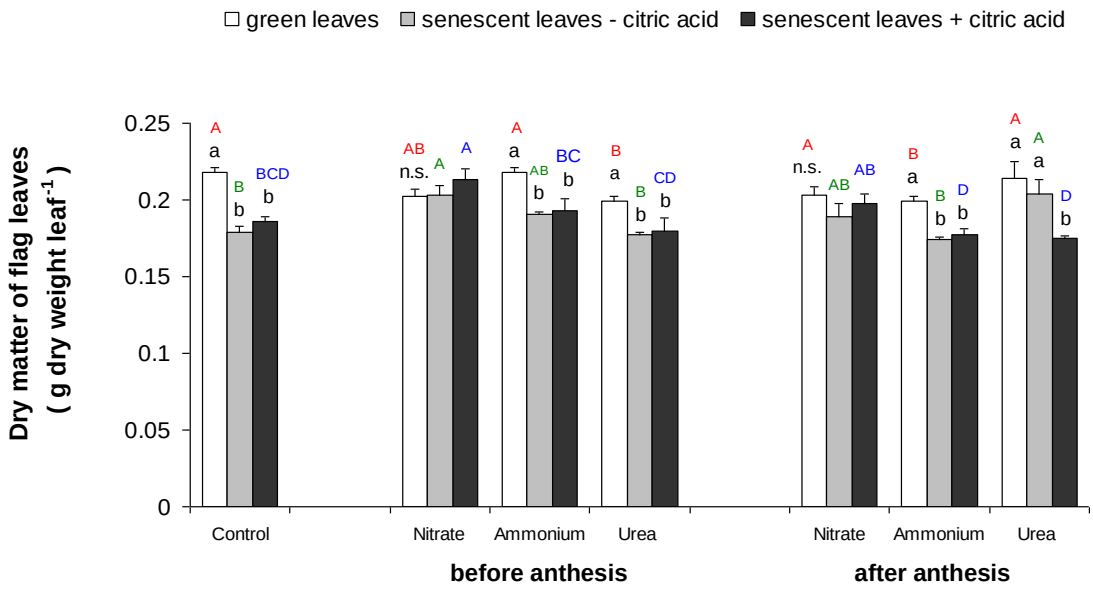

Figure 21. Effect of N supply and foliar citric acid application on flag leaf dry weight. Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying, and the dry weight was determined. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

As expected, the dry weight of grains increased significantly as they matured (Figure 22). The supply of N, irrespective of the form and the time, significantly increased the dry weight of mature grains. Interestingly, the application of citric acid was able to significantly increase grain biomass in control plants, whereas in N-fertilized plants this treatment resulted resulted in no obvious effects (Figure 22).

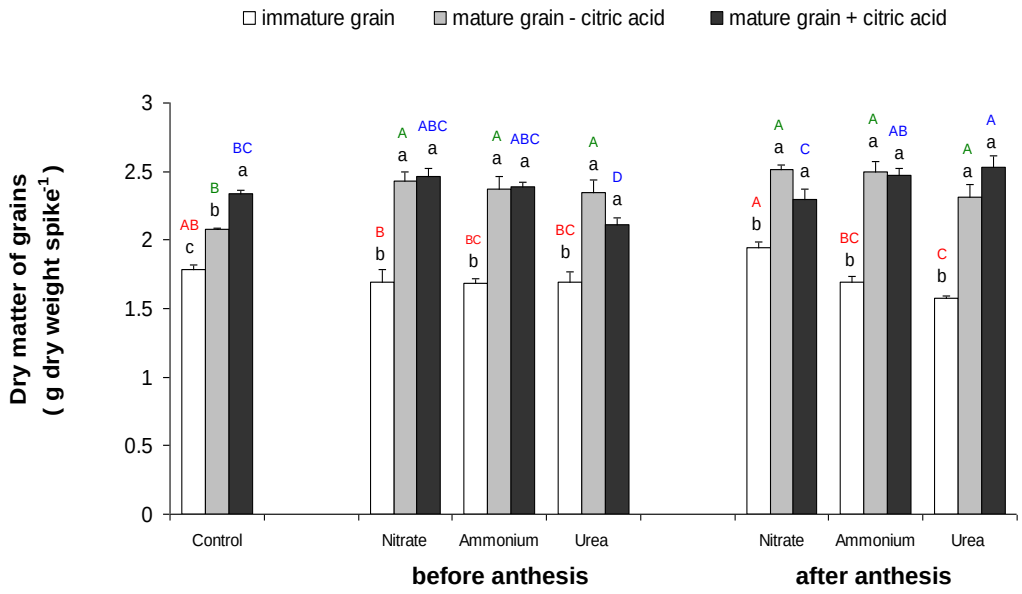

Figure 22. Effect of N supply and foliar citric acid application on grains dry weight. Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). In addition, foliar application of citric acid was carried out after 85 days and samples were harvested 2 days after spraying. Grains were collected from each spike and harvested after 75 days (immature grains), or 104 days (mature grains) and the dry weight was determined. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for immature grain, green letters for mature grain without citrate and blue letters for mature grain with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

4.6 Effects of N forms and citric acid on N content and micronutrients in flag leaves and grains

As for the first trial, the total N contents were measured in flag leaves and in grains. It was again observed that N contents decreased as flag leaves became senescent (Figure 23). The only exception for this was when urea was supplied to plants before anthesis, which resulted in lower N contents already in green leaves. In general nitrogen contents were higher in plants treated with N, as compared to non-fertilized plants (Figure 23). However, this increase was rather small. The supply of citric acid to plants, in turn, did not significantly affect N contents in leaves.

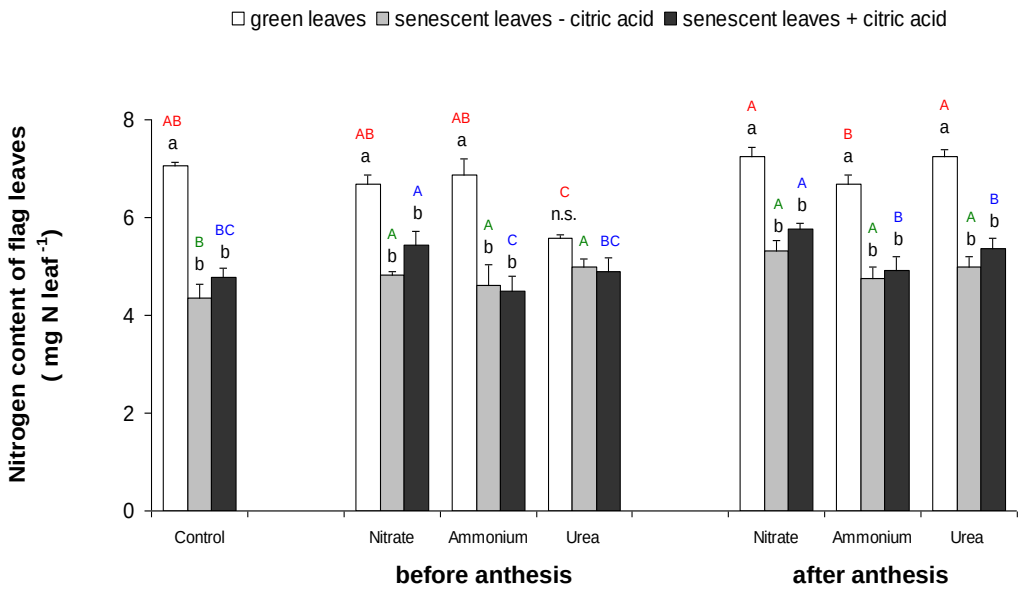

Figure 23. Effect of N supply and foliar citric acid application on N contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. The flag leaves were harvested and dried, then the N contents were determined by Elemental Analysis-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

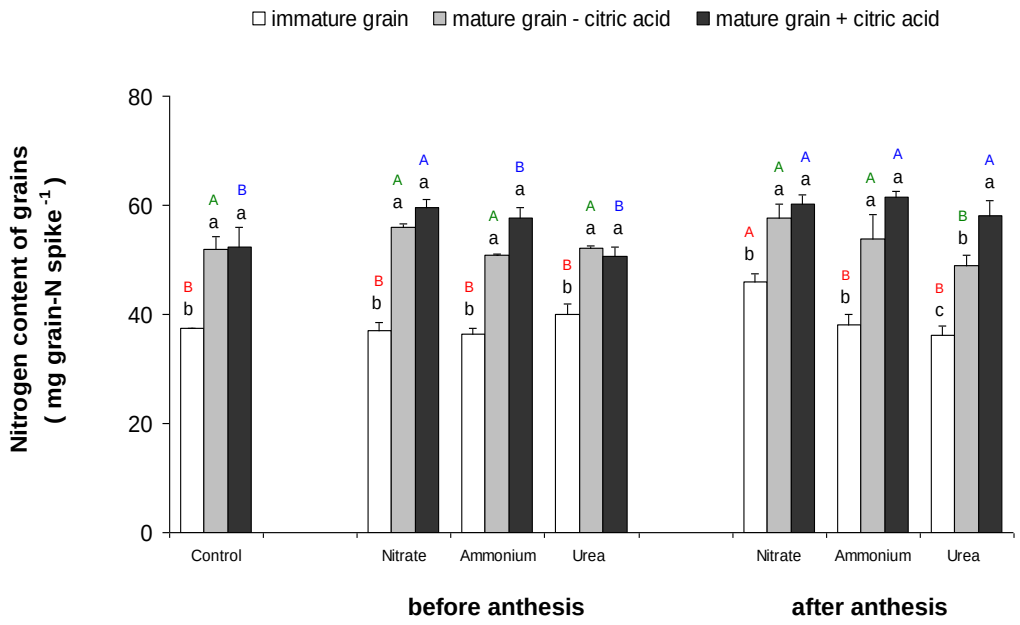

Figure 24. Effect of N supply and foliar citric acid application on N contents of grains. Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). In addition, foliar application of citric acid was carried out after 85 days and samples were harvested 2 days after spraying. The spikes were harvested after 75 days (immature grains), or 104 days (mature grains). Grains collected from each spike were dried and the N contents were determined by Elemental Analysis-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for immature grain, green letters for mature grain without citrate and blue letters for mature grain with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

In grains, N contents increased consistently in all treatments throughout grain maturation (Figure 24). This observation together with the reduction of N contents in flag leaves indicates that N was imported from leaves into grains. However, in mature grains the supply of N did not result in a significant change in N contents (Figure 24). One exception was the late supply of urea, which resulted in significantly lower N contents in mature grains. Interestingly, an additional supply of citrate was only able to elevate the N contents in mature grains when plants supplied with urea after anthesis (Figure 24).

As for the first trial, the Fe and Zn were measured in flag leaves as well as in grains, and in exudates collected from flag leaves. In addition, the contents of Cu and Mn were determined. Similarly to what was observed in the first field experiment, Fe contents in flag leaves were lower in senescent as compared to green leaves (Figure 25). In green leaves only the supply of urea before anthesis led to significantly lower leaf Fe contents. In general, Fe contents in senescent leaves not treated with citric acid were significantly lower when N was supplied to plants, independent of the form and the time of application (Figure 25). The supply of citric acid had a particular effect in control leaves, where Fe contents were markedly reduced.

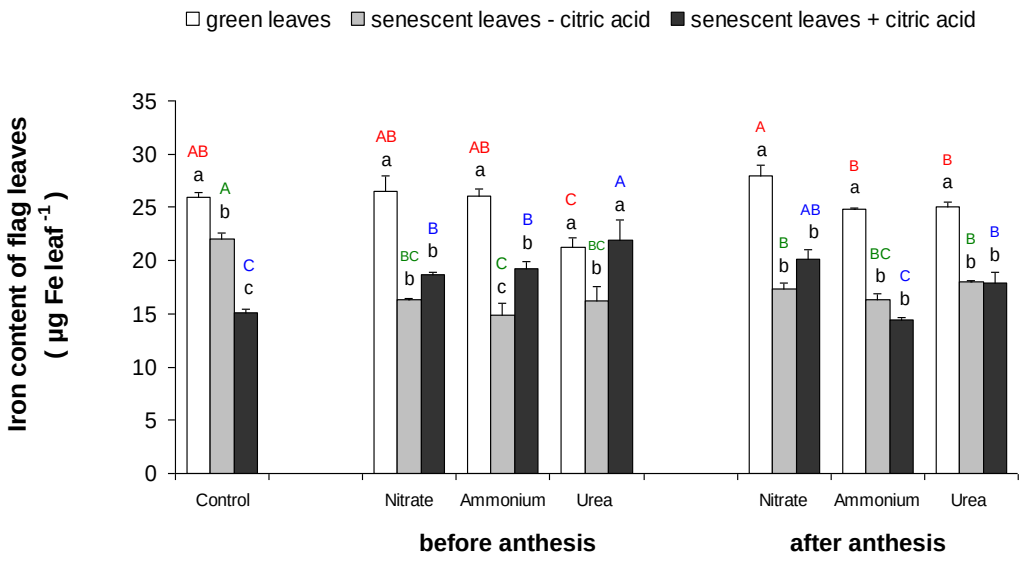

Figure 25. Effect of N supply and foliar citric acid application on Fe contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying and Fe contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

By contrast, leaf spray with citric acid led to higher Fe contents in leaves of plants fertilized with ammonium or urea before anthesis supply, whereas there was no significant effect on the remaining treatments (Figure 25).

Nitrogen application also affected Zn contents in flag leaves (Figure 26). Zinc contents were remarkably lower in senescent leaves when N was applied. Like for Fe, the lowest contents of Zn were observed when urea was supplied to plants either before or after anthesis (Figure 26). Citric acid application to plants reduced Zn contents only in control leaves, showing no significant effect on leaves from other treatments.

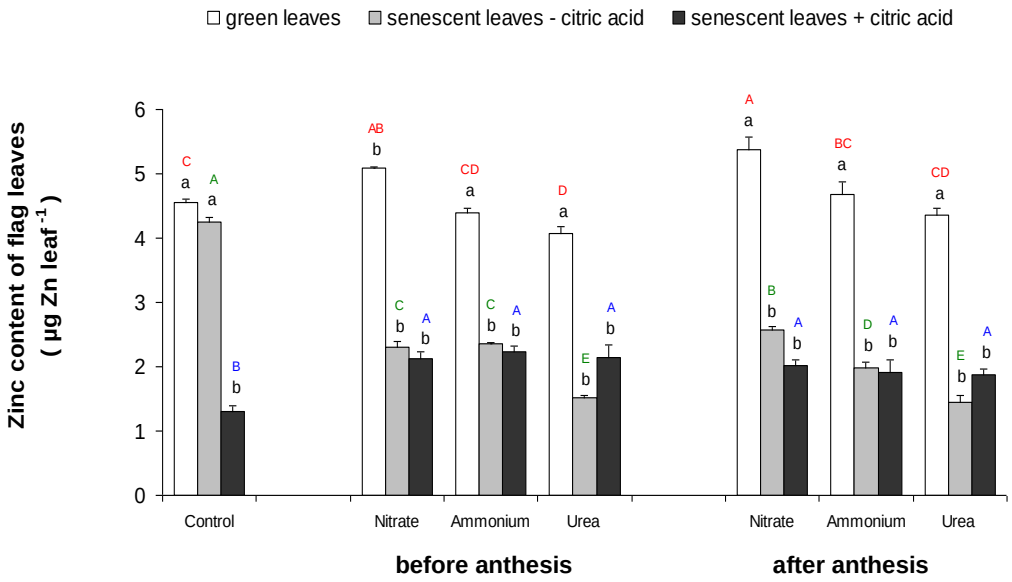

Figure 26. Effect of N supply and foliar citric acid application on Zn contents of flag leaves.
Nitrate, ammonium or urea (80kg N ha^{-1}) were supplied to wheat plants (*Triticum aestivum* cv. Akteur) either before (EC49) or after the anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid was carried out after 85 days and samples were harvested 2 days after spraying. The dried flag leaves were grinded, and the Zn content were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

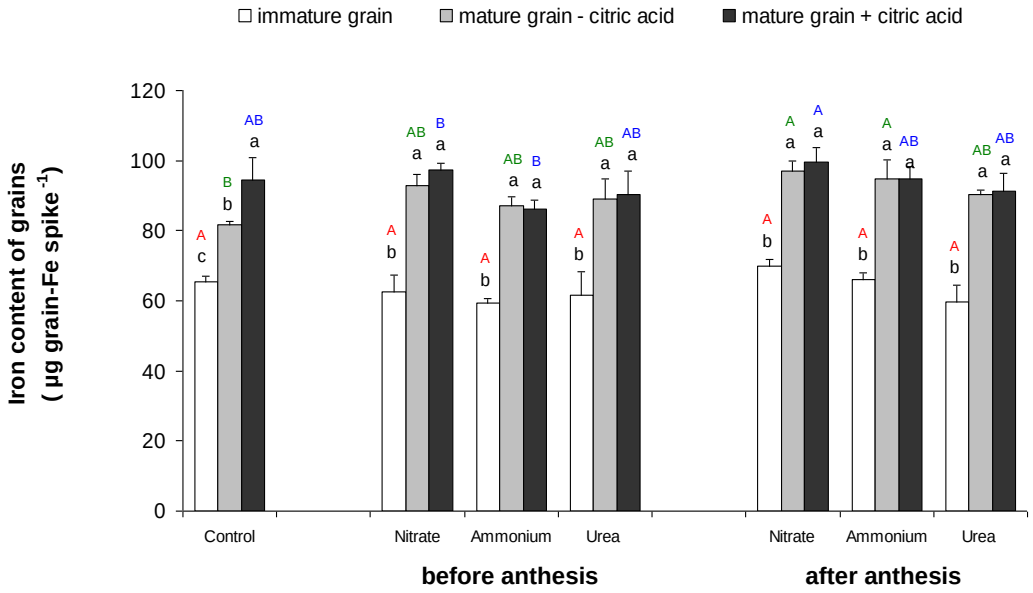

Figure 27. Effect of N supply and foliar citric acid application on Fe contents of grains. Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (immature grains) or 104 days (mature grains), spikes were harvested and grains from each spike collected, then the Fe concentration were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for immature grain, green letters for mature grain without citrate and blue letters for mature grain with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

The contents of two other metal micronutrients, Mn and Cu, were also assessed. Manganese contents decreased in senescent leaves of plants supplied with ammonium or urea before anthesis as well as with nitrate or urea after anthesis, not being significantly changed by the other treatments (Appendix 11 and 14). In senescent leaves, the supply of ammonium or urea before anthesis resulted in decreased Mn contents in flag leaves, but only urea showed a similar effect when supplied after anthesis. Citric acid application to control plants also reduced Mn contents in flag leaves (Appendix 11). However, specifically when N was applied to plants before anthesis, an additional supply of citric acid actually increased Mn contents in flag leaves.

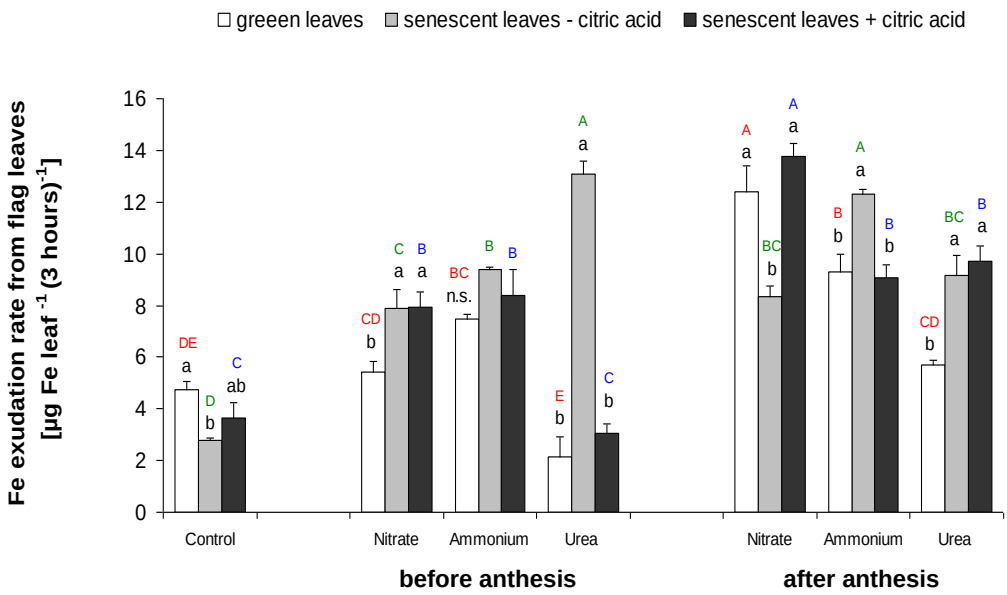

Figure 28. Effect of N supply and foliar citric acid application on Fe exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. Leaf exudates were collected from single flag leaf into EDTA solution after 75 days (green leaves) or 87 days (senescent leaves) and Fe contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

Similarly to what was observed for Fe and Zn, Cu contents in senescent leaves decreased significantly as compared to green leaves, except those from the control treatment (Appendix 14). There was no significant difference in Mn contents among N forms or due to the time of N application. Citric acid application caused Cu contents to decrease in control leaves. However, when urea was supplied before anthesis, citric acid supply increased Cu contents in leaves, whereas in the other treatments citric acid did not affect the levels of this micronutrient in leaves (Appendix 14). In general, these results indicate that when plants were supplied with N, the

contents of Fe, Zn and Cu become lower during flag leaf senescence. This suggests that a considerable amount of these micronutrients were re-out of flag leaves and then this process is stimulated by N supply. By contrast, the application of citric acid to (N fertilized) wheat plants slightly increased the flag leaf contents of Fe in plants which obtained early N application (Figure 25). This may reflect to enhanced Fe decreased in citrate–supplied leaves.

To gain additional insights on whether N treatments induced leaf-to-grain retranslocation of micronutrients, the levels of Fe, Zn, Mn and Cu were measured in the exudates collected from flag leaves. In control leaves, Fe exudation rates from senescent leaves were significantly lower (Figure 28). The application of N led to a significant increase in the Fe levels in the exudates, particularly in senescing leaves. The most remarkable effects were observed when urea was supplied to plants before anthesis or ammonium after anthesis (Figure 28). Also noteworthy, the late nitrate fertilization reduced Fe levels in the exudates, but an additional supply of citrate to these plants compensated for the negative effect of nitrate (Figure 27). However, when plants received a late supply of ammonium or particularly an earlier application of urea, the addition of citrate reduced Fe concentrations in leaves exudates.

When compared to control, the supply of N independently if before or after anthesis increased remarkably Zn levels in leaf exudates collected from senescent leaves (Figure 30). One exception for this was the supply of urea before anthesis, which resulted in Zn levels similar as in control samples. In addition, the supply of N increased Zn exudation rates at senescent as compared to green leaves (Figure 30). Probably because the supply of nitrate in the later time point resulted in the highest Zn levels in the exudates of green leaves, this treatment did not further increase the

concentrations of this micronutrient in the exudates of senescing leaves (Figure 28). The citric acid application was only able to increase Zn concentrations in exudates of control leaves. Particularly when N was supplied as ammonium or urea after anthesis, the additional application of citrate resulted in a remarkable reduction of Zn levels in leaf exudates (Figure 30).

Manganese levels were comparatively little altered in the exudates collected either from green or senescent leaves (Appendix 13). As an exception to this, the supply of nitrate or urea before anthesis increased Mn exudation rates. In addition, compared to control, N fertilization resulted in higher levels of Mn in the exudates of senescing leaves (Appendix 13). In most treatments the supply of citric acid tended to decrease Mn concentrations in leaf exudates.

The concentrations of Cu in leaf exudates were only affected by the leaf age when urea was supplied or when ammonium was applied after athesis (Appendix 16). In the exudates collected from senescent leaves, highest Cu concentrations were detected when either nitrate or urea were applied after anthesis. When urea was the N form, the application of citrate decreased Cu levels in leaf exudates (Appendix 16). In the other treatments, citric acid application did not affect significantly the concentration of this micronutrient.

The Fe contents in immature grains were not significantly affected by any of the treatments (Figure 26). However, in mature grains the supply of nitrate or ammonium after anthesis increased significantly Fe contents as compared to non-fertilized plants. In N-supplied plants, the additional supply of citric acid did not significantly affect grain Fe contents, although citrate enhanced grain Fe in control plants (Figure 26).

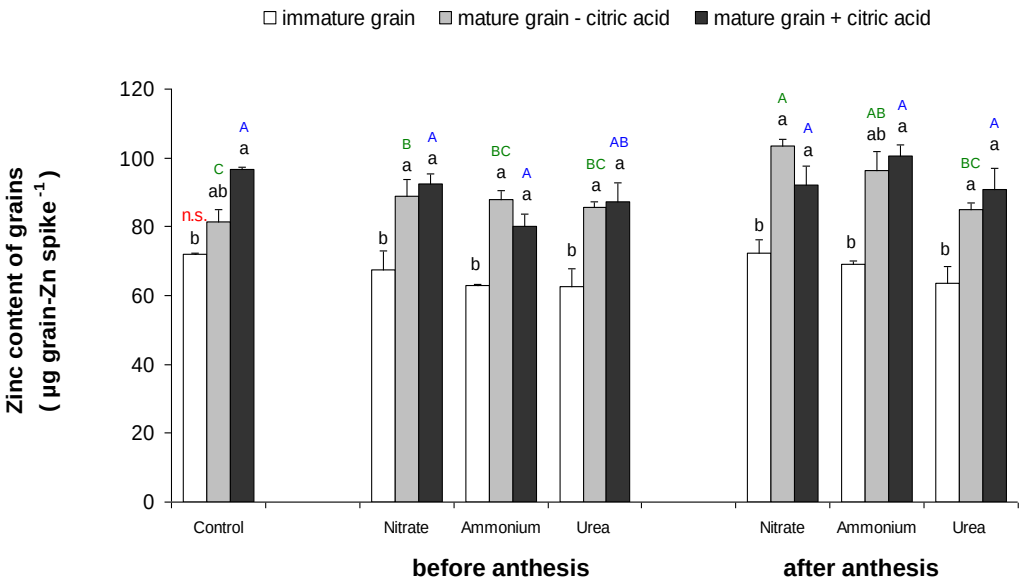

Figure 29. Effect of N supply and foliar citric acid application on Zn contents of grains. Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). In addition, foliar application of citric acid was carried out after 85 days and samples were harvested 2 days after spraying. Spikes were harvested after 75 days (immature grains) or 104 days (mature grains) and grains were collected from single spikes and the Zn concentration was determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for immature grain, green letters for mature grain without citrate and blue letters for mature grain with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

Noteworthy, these results indicate that the significantly higher levels of Fe detected in the leaf exudates from all N-treated plants (Figure 27) did not always result in higher Fe contents in grains (Figure 26). As for Fe, Zn contents did also increase in mature grains (Figure 29). In relative to control plants, the supply of nitrate either before or after anthesis as well as ammonium application after anthesis resulted in increased grain Zn contents. The application of citric acid had no considerable effect on Zn in grains accumulation (Figure 29). The Mn contents in grains were not significantly affected any of the treatments and remained largely unaffected as grains maturated (Appendix 12).

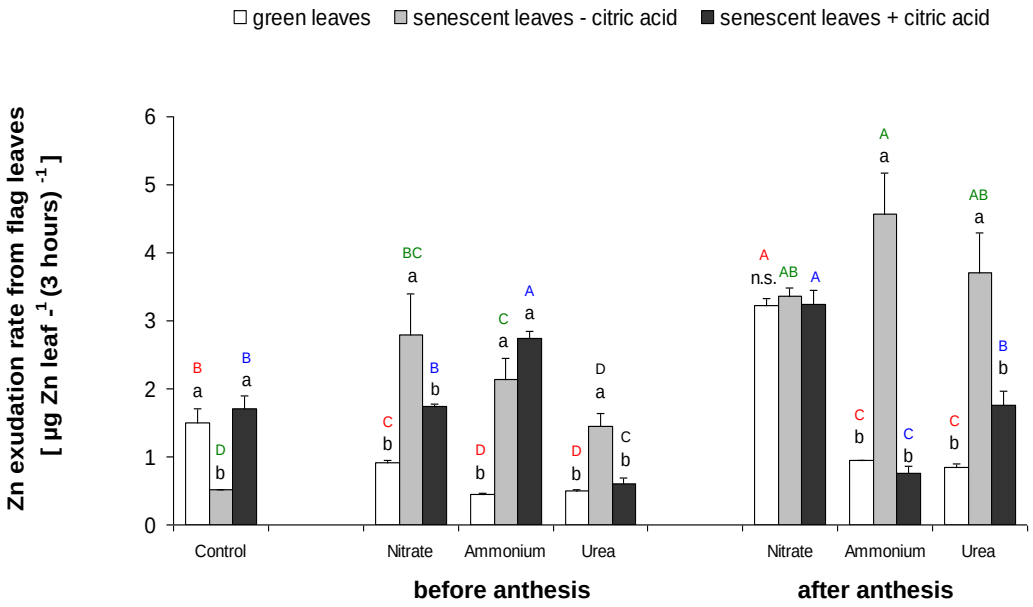

Figure 30. Effect of N supply and foliar citric acid application on Zn exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. The flag leaves exudates were collected into 15 mM EDTA solution pH 7.5 after 75 days (green leaves) or 87 days (senescent leaves) and the Zn concentration was determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.

However, when nitrate was supplied to plants, Mn contents increased in mature grains as compared to immature ones. A similar response was also observed when urea was supplied to plants after anthesis (Appendix 12). In turn, copper contents increased significantly in mature compared to immature grains, except in control plants (Appendix 15). However, most treatments had no significant effect on the Cu accumulation in grains, just the supply of nitrate after anthesis raised Cu grain contents above control level (Appendix 15).

Altogether, these results indicate that N fertilization enhanced Fe and Zn export out of flag leaves, since N-treated plants exhibited reduced contents of these micronutrients in senescent flag leaves. The supply of nitrate or ammonium after anthesis increased the contents of Fe and Zn in grains. The supply of citric acid was able to affect micronutrient contents mainly in control plants. In these plants, citric acid decreased Fe and Zn contents in flag leaves and increased the contents in grains.

4.7 Concentration of NA and PS in flag leaves and leaf exudates

Since significant changes in the contents of micronutrients were detected in flag leaves, leaf exudates and in grains at various degrees in response to the N and citrate treatments, the concentrations of two important metal chelators were also assessed. In flag leaves, NA leaves levels were reduced during senescence only when urea was supplied before or nitrate after anthesis, remaining unchanged under the other treatments (Figure 31). Compared to control plants, the fertilization of ammonium either before or after anthesis or the application of nitrate after anthesis resulted in higher NA levels in senescent leaves. The supply of citric acid, in turn, did not significantly change the concentration of NA in leaves (Figure 31).

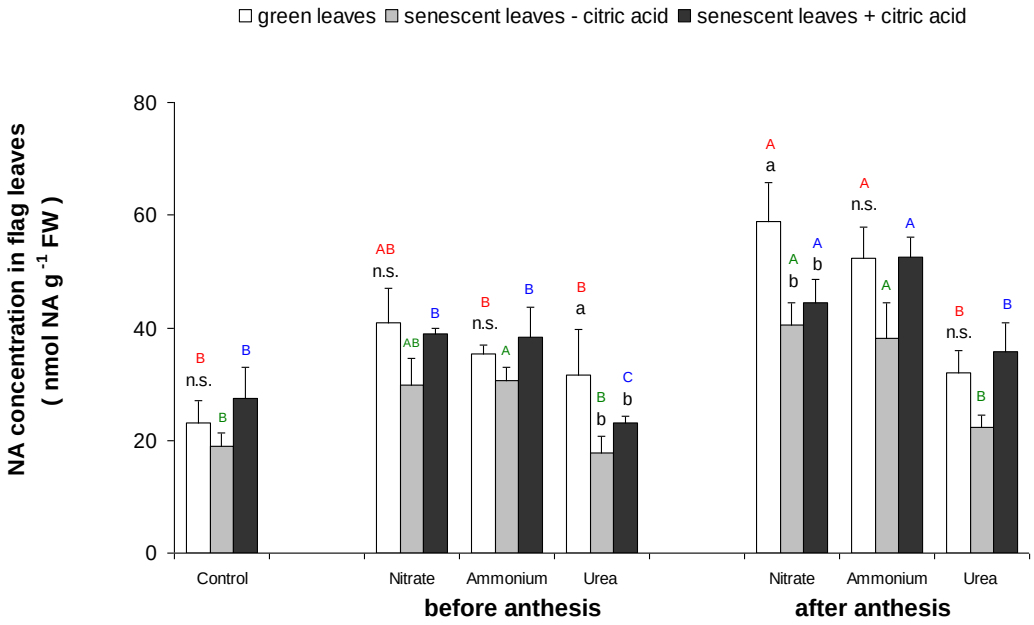

Figure 31. Effect of N supply and foliar citric acid application on nicotianamine (NA) concentrations of flag leaves.

Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. Flag leaves were grinded and the NA concentration was determined by HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

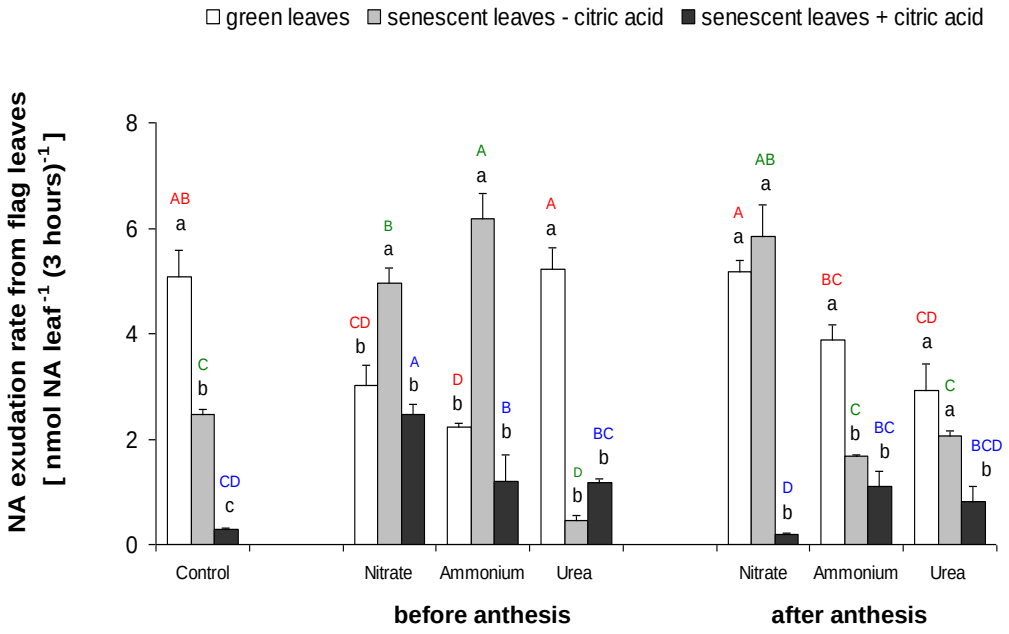

Figure 32. Effect of N supply and foliar citric acid application on nicotianamine (NA) exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. The exudates were collected into 15 mM EDTA solution pH 7.5 and the NA concentration was determined by HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

The measurement of NA in leaf exudates revealed that in control plants NA levels decreased significantly in senescent leaves (Figure 32). However, relative to unfertilized plants, the supply of nitrate to wheat plants maintained higher NA levels in the exudates of senescent leaves. A similar effect was observed when ammonium was supplied to plants before anthesis (Figure 32). The supply of urea before anthesis resulted in the lowest NA concentrations in the exudates of senescent leaves. Although citric acid application had no effect on NA levels in leaves (Figure

31), it caused a marked decrease in NA concentrations measured in leaf exudates of control plants (Figure 32).

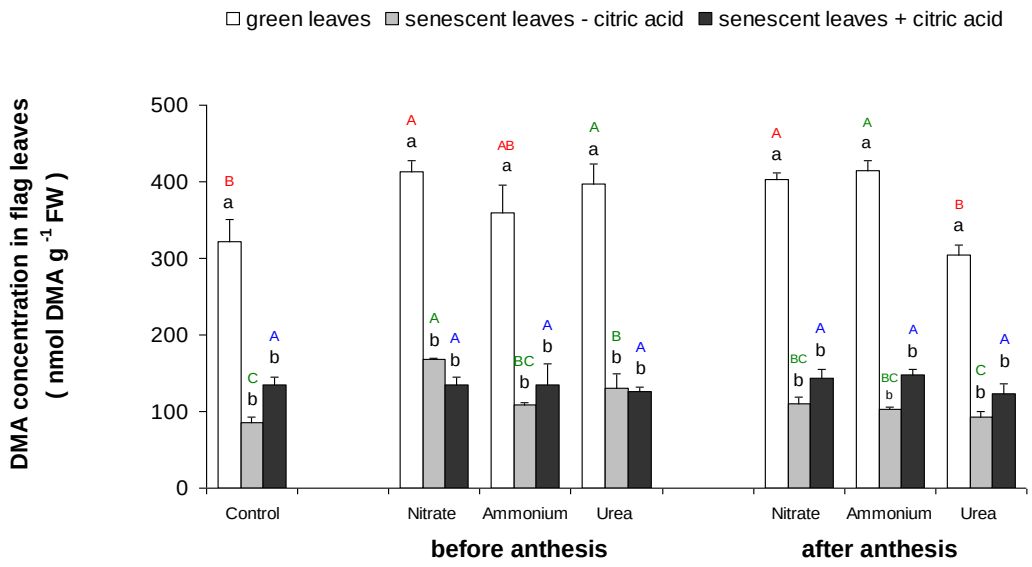

Figure 33. Effect of N supply and foliar citric acid application on deoxy-mugineic acid (DMA) concentrations of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. The flag leaves were extracted and the DMA concentration was determined by HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

Irrespectively of N fertilize treatment DMA levels were markedly decreased in senescing flag leaves, as compared to green leaves (Figure 32).

The supply of nitrate before anthesis led to the highest DMA levels in senescent leaves. Citric acid application did not significantly affect DMA concentrations in flag leaves (Figure 32). DMA exudation rates were significantly affected by N treatments and by leaf age (Figure 33).

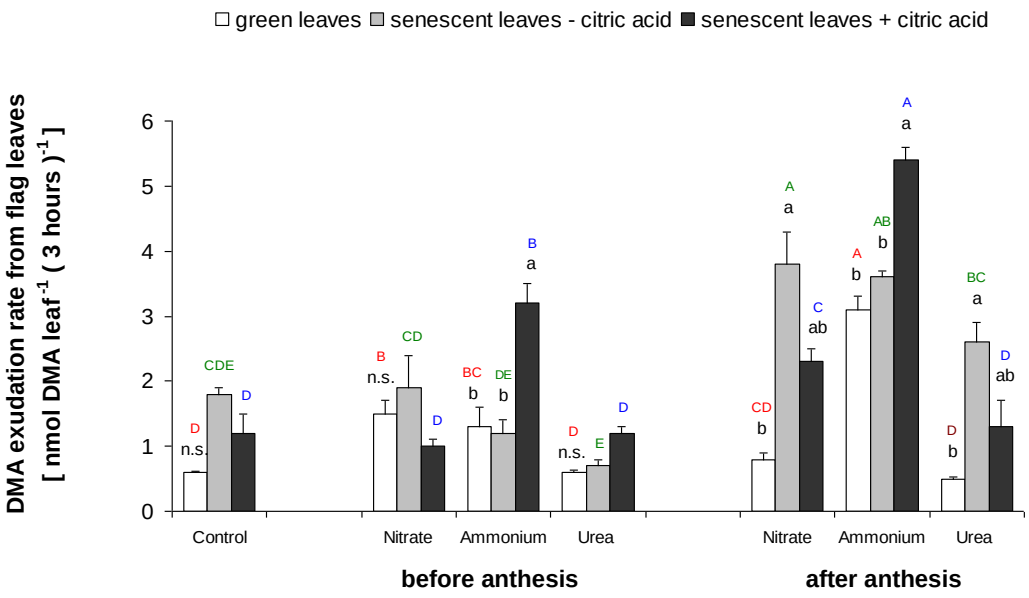

Figure 34. Effect of N supply and foliar citric acid application on deoxy-mugineic acid (DMA) exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. The exudates were collected from single flag leaf into 15 mM EDTA solution pH 7.5 and the DMA concentration was determined by HPLC. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

These changes in exudation rates, however, were not consistent with those detected in flag leaves. Only the application of nitrate or urea after anthesis led to a significant increase in DMA concentrations in the exudates of senescent as compare to green leaves (Figure 34). Interestingly, relative to control plants, the late fertilization with nitrate or ammonium was able to elevate remarkably DMA levels in leaf exudates.

However, when N forms were supplied before anthesis or when urea was fertilized after anthesis, DMA exudation rates were not significantly different from control plants (Figure 34). Citric acid did only significantly affect DMA levels when supplied to

ammonium-fertilized plants, causing an increase in the DMA concentrations detected in the leaf exudates.

Thus, these results demonstrate that, except for urea, the supply of N raised higher NA levels in senescent leaves as compared to control plants. In addition, the supply of nitrate resulted in more NA in the leaf exudates. DMA, in turn, sharply decreased during leaf senescence and was, in general, less responsive to the N treatments.

4.8 Gene expression analysis

Since NA levels, particularly in the leaf exudates, were significantly affected by the leaf developmental age and by some of the N treatments imposed to plants, the expression of *NICOTIANAMINE SYNTHASE1* (*TaNAS1*) in flag leaves was determined (Figures 35). *TaNAS1*, which has been characterized in barley encodes for the enzyme that converts S-adenosylmethionine (SAM) to nicotianamine (NA) (Higuchi et al., 1999).

It was observed that, except in control leaves, *TaNAS1* expression levels were significantly upregulated in senescent leaves (Figure 35). Interestingly, the highest *TaNAS1* expression was detected in senescent leaves harvested from plants fertilized with urea. However, the additional supply of citric acid to these plants prevented this increase in *TaNAS1* mRNA levels (Figure 35).

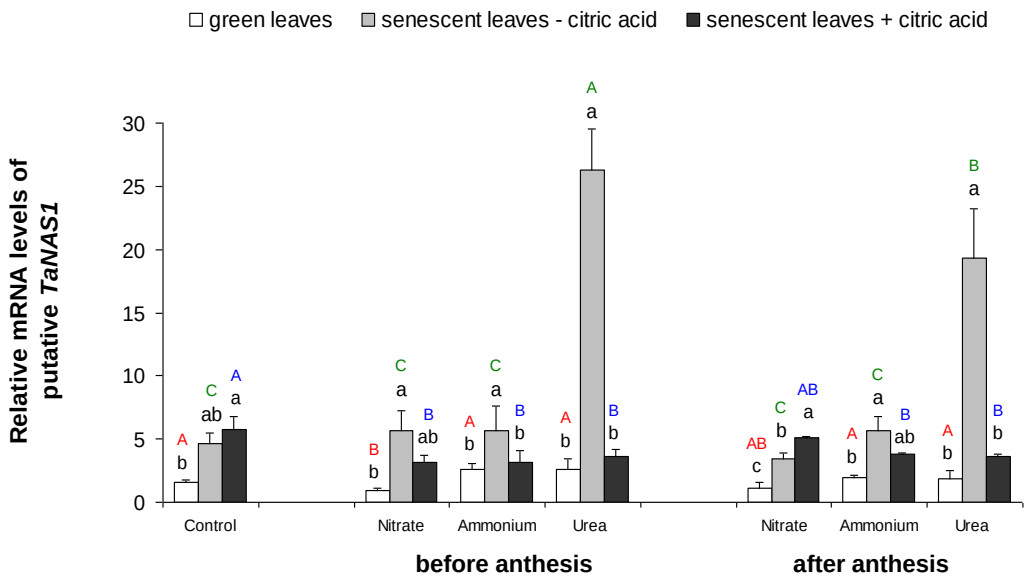

Figure 35. Effect of N supply and foliar citric acid application on the expression of the putative *TaNAS1* gene.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. RNA was isolated from flag leaves and the expression of *TaNAS1* was assessed by means of quantitative RT-PCR. Values are means of 3 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.

The transcript levels of the wheat homolog of the rice *OsYSL15 (YELLOW STRIPE-LIKE 15)* gene mirrored the expression of putative *TaNAS1* (Figures 37 and 35). In rice, it has been reported that *OsYSL15* is the major transporter responsible for Fe(III)-DMA uptake in roots and for Fe translocation in the phloem (Inoue et al., 2006; Lee et al., 2009). The expression of the *TaYSL15* was dramatically increased in plants supplied with urea, independently of the time of application (Figure 36). Similarly as for *TaNAS1*, the supply of citric acid to these plants prevented the upregulation of *TaYSL15*.

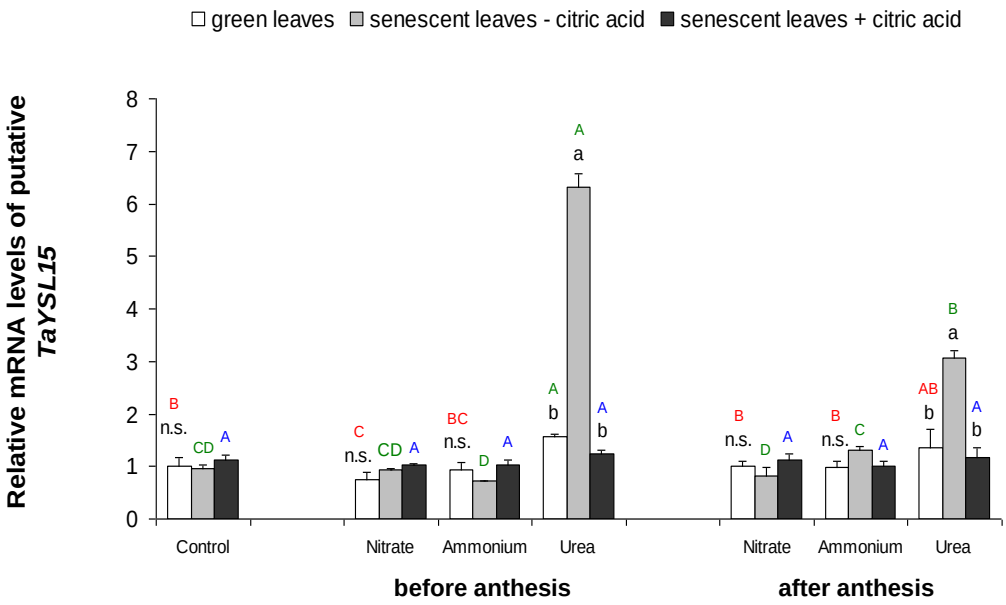

Figure 36. Effect of N supply and foliar citric acid application on the expression of the putative *TaYSL15* gene.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. RNA was isolated from flag leaves and the expression of the putative *TaYSL15* gene was assessed by means of quantitative RT-PCR. Values are means of 3 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

In addition to *TaNAS1*, also the expression of a putative *TaDMAS* homologue was assessed. DMA synthase (DMAS) converts NA to DMA in graminaceous plants (Bashir et al., 2006). The expression of the putative *TaDMAS* was also significantly upregulated in senescent leaves, except when plants were fertilized with urea (before or after anthesis) or with ammonium after anthesis (Figure 37). This expression pattern may be explained by the fact that in these plants the expression of *TaDMAS* was already higher in green leaves.

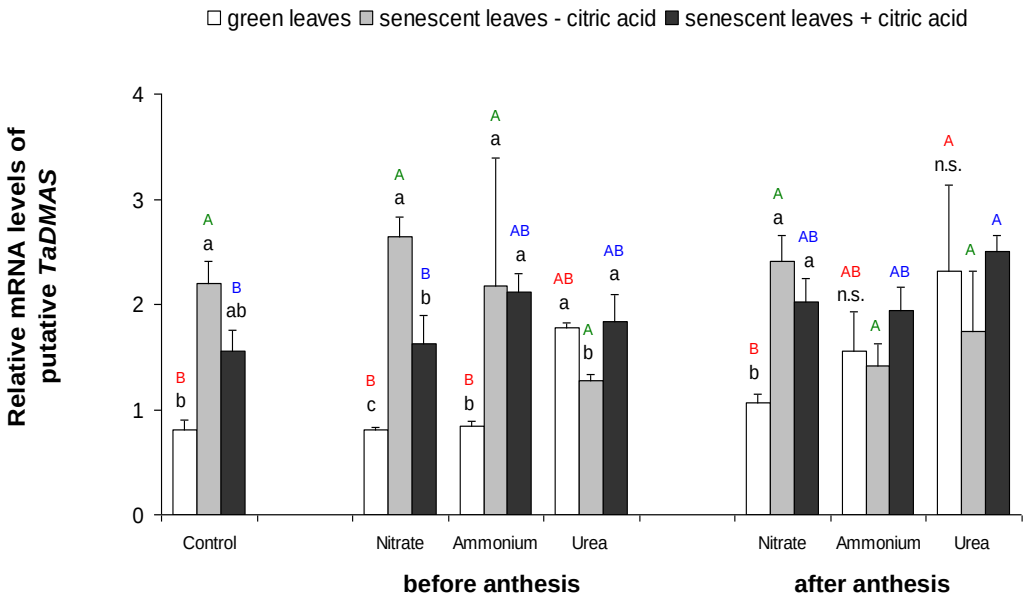

Figure 37. Effect of N supply and foliar citric acid application on the expression of the putative *TaDMAS* gene.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. RNA was isolated from flag leaves and the expression of *TaDMAS* was assessed by means of quantitative RT-PCR. Values are means of 3 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

Altogether, these results indicate that *TaNAS1* in flag leaves was induced by senescence, especially when urea was supplied to plants. The expression of *TaDMAS* was also induced by senescence in control and nitrate-treated plants. However, when urea was supplied *TaDMAS* expression was induced already in green leaves. In general, citric acid supply did not consistently affect the expression of the genes investigated in the present study.

4.9 The relative concentration of citrate and malate in leaf exudates

The concentration of citrate and malate in leaf exudates were detected by Fourier Transform Ion Cyclotron Resonance mass spectrometry. These analyses were carried out by Dr. Günter Weber (ISAS, Dortmund).

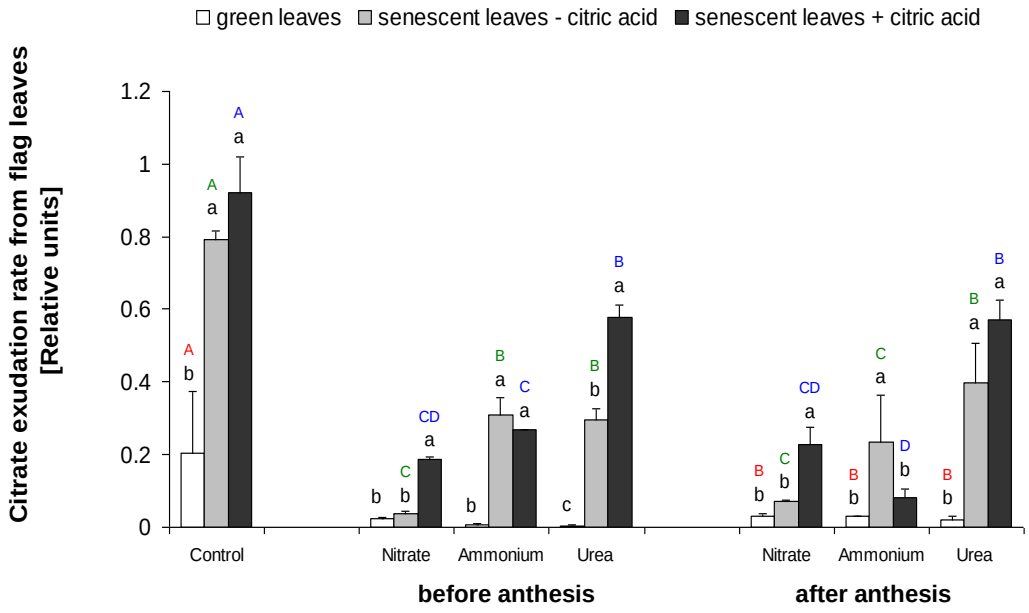

Figure 38. Effect of N supply and foliar citric acid application on the relative concentration of citrate in leaf exudates.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. The leaf exudates collected into 15 mM EDTA solution pH 7.5 were separated by hydrophilic interaction chromatography (HILIC), and the relative citrate concentrations were measured by Fourier Transform Ion Cyclotron Resonance mass spectrometry (FTICR-MS). Values are means of 3 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

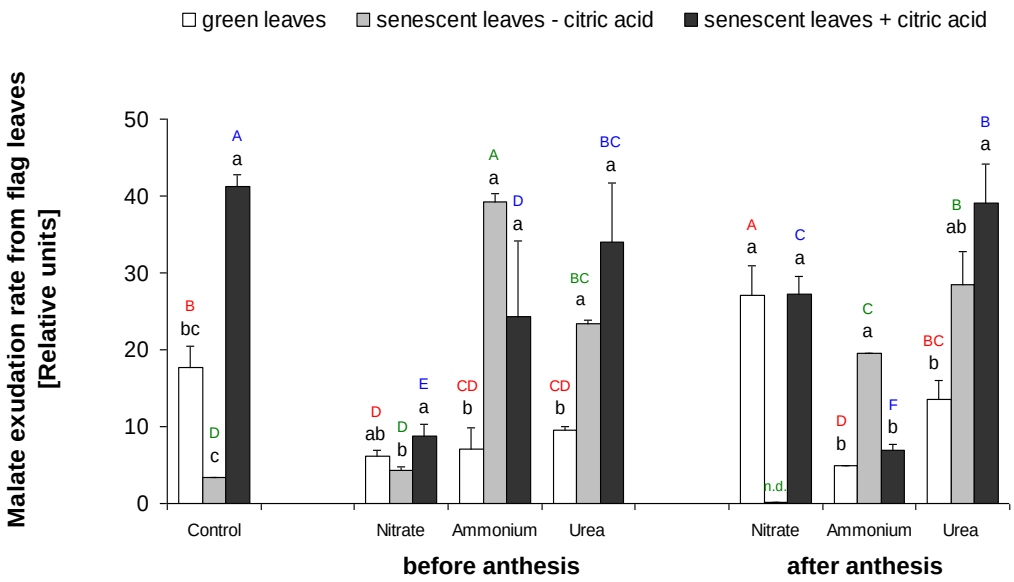

Figure 39. Effect of N supply and foliar citric acid application on the relative concentration of malate in leaf exudates.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. Leaf exudates collected into 15 mM EDTA solution pH 7.5 were separated by hydrophilic interaction chromatography (HILIC), and the relative malate concentrations were measured by Fourier Transform Ion Cyclotron Resonance mass spectrometry (FTICR-MS). Values are means of 3 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at $P<0.05$. ns denotes that there is no significant difference.

Regarding citrate concentration, one important observation was that, compared to unfertilized plants, the supply of N to plants reduced significantly the citrate concentration in exudates collected either from green, senescent or senescent leaves treated with citric acid (Figure 38). This reduction was stronger when either nitrate or ammonium was supplied. In addition, relative concentrations of citrate in leaf exudates were significantly higher in senescent leaves, except when plants were fertilized with nitrate.

Interestingly, an additional foliar treatment of citric acid to nitrate-treated plants was able to increase significantly citrate levels in leaf exudates (Figure 38). In fact, relative citrate concentrations were increased by foliar supply of citrate only when nitrate (before and after anthesis) or urea (before anthesis) was supplied to plants.

Compared to citrate, the relative concentrations of malate provide a less clear pattern (Figure 39). In control plants and in plants supplied with nitrate after anthesis, malate levels detected in exudates collected from senescent leaves decreased significantly as compared to green leaves. However, when plants were supplied with ammonium or urea, malate concentrations increased in leaf exudates (Figure 39). The foliar application of citric acid to unfertilized or nitrate-supplied plants resulted in a significant increase of malate levels (Figure 39).

Thus, these results indicate that, in general, senescence increased and N fertilization decreased citrate levels in the exudates collected from flag leaves. Malate exudation rates decreased in senescent leaves of control and nitrate-supplied and were enhanced by the supply ammonium and urea.

4.10 Effects of N forms on concentration of essential amino acids

Finally, an analysis of the quantitatively most abundant amino acids was conducted to determine the effects of citric acid and N supply on amino acid metabolism (Table 2). The concentrations of glutamine decreased significantly in senescent leaves of control plants (Table 2). This was similar in N-treated plants, except when they were supplied with nitrate before or after anthesis or with urea after anthesis. Contrary to

glutamine, the concentrations of asparagine increased significantly in senescent leaves and this increase was observed after application of any N forms (Table 2).

In control plants, glutamate concentrations in flag leaves decreased as leaves turned into senescence (Table 2). In plants treated with nitrate or ammonium, glutamate concentrations were not affected by leaf senescence. The concentration of another amino acid, aspartate, was not considerably altered when green and senescent leaves of control plants were compared (Table 2). Higher aspartate concentrations were observed in senescent leaves treated with N, especially when nitrate was the supplied N form.

Interestingly, the supply of citric acid reduced dramatically the concentrations of glutamine, asparagine, glutamate and aspartate in senescent leaves, independent of the N fertilization (Table 2). In addition, other amino acids decreased during leaf senescence even more by citrate application, and their concentrations tended to increase slightly in leaf exudates (data not shown).

Table 2. Concentration of essential amino acids measured in flag leaves in response to different developmental stages and forms of N application.

	Control	Application of nitrogen					
		Before anthesis (EC49)			After anthesis (EC65)		
		NO_3^-	NH_4^+	Urea	NO_3^-	NH_4^+	Urea
Flag leaves				**(nmol g^{-1} fresh wt)**			
Green leaves							
Glutamine	1387a C	1960a AB	1957a AB	1412a C	1382a C	2157a A	1644a BC
Asparagine	190b C	332b B	403b AB	565b A	577b A	516b A	381b B
Glutamate	6757a DE	6994a DE	7942a BC	7315a CD	6300a E	9485a A	142a B
Aspartate	2505a C	3120b C	4350a AB	4950a A	3120b C	4305a AB	4125a B
Senescent leaves (without citrate)							
Glutamine	922b B	2218a A	1046b B	841b B	1234a B	861b B	1838a A
Asparagine	346a C	147a B	2766a A	1299a B	1314a B	1389a B	1110a B
Glutamate	3397b D	6415a B	7678a A	3841b D	5310a BC	5083b C	5704b BC
Aspartate	1844a D	4232a A	4041a AB	3401b B	4669a A	2605b C	3124b BC
Senescent leaves (with citrate)							
Glutamine	166c B	228b B	501c A	260c B	301b B	205c B	219b B
Asparagine	20b D	147b AB	77c CD	159c A	140c AB	100c BC	120c BC
Glutamate	542c C	632b AB	603b BC	606c BC	705b A	590c BC	420c D
Aspartate	177b C	487c A	353b B	325c B	319c B	252c B	293c B

*** Small letter → comparison between harvest time. Capital letter → comparison on row.**

5 Discussion

One important challenge for modern agriculture is not only to produce enough food to a constantly growing population, but also to deliver highly nutritious food to consumers. In many countries, poor populations have access to only a limited variety of food and, in most cases, cereals represent the main staple food. Although cereal grains are excellent sources of calories, they are inherently poor in Fe and Zn (White and Broadley, 2005; Cakmak, 2008; Newell-McGloughlin, 2008). Thus, a diet which is mainly based on cereals can lead to micronutrient malnutrition. In this regard, almost half of the world's population suffers from diseases related to the deficiencies of Fe and/or Zn (WHO, 2002; Nestel et al., 2006). Against this background, the improvement of Fe and Zn contents in the edible parts of cereals, i.e. grains, has gained particular attention (White and Broadley, 2005; Bouis et al., 2009). In addition, it has been reported that biofortification strategies are economically efficient and can cause a significant impact on the reduction of micronutrient malnutrition in developing countries (Stein et al., 2007).

Earlier studies have indicated that the plant's N nutritional status can affect significantly the retranslocation of Fe and Zn in plants. The supply of high N to durum wheat enhanced the acquisition and the allocation of Fe and Zn in grains (Erenoglu et al., 2011; Kutman et al., 2011). Thus, these studies indicate that N management might represent a promising strategy to improve micronutrient contents in wheat grains. However, excessive N might also exert a negative effect on the retranslocation of Fe and Zn, since a significant proportion of Fe and Zn can be fixed to proteins under high N (Marschner, 1995), thereby limiting the retranslocation of these micronutrients to the grains. Thus, in order to obtain maximum gain in terms of

micronutrient accumulation in grains, N fertilization has to be managed in a rational manner.

One important drawback of the studies in which N nutrition and micronutrient accumulation in grains were assessed is the fact that most of these studies were not carried out in field conditions (Erenoglu et al., 2011; Kutman et al., 2011; Shi et al., 2012). Thus, it is not yet clear to which extent N fertilization practices can indeed affect micronutrient levels in grains. In the present study, two field trials were carried out with wheat plants (*Triticum aestivum* cv. Akteur) in two subsequent years to assess the effect of N nutrition on the accumulation of Fe and Zn in grains.

5.1 The effect of N forms on the senescence of flag leaves

Micronutrient accumulation in grains is highly dependent on their rate of retranslocation from leaves, a process that starts with the onset of leaf senescence. The remobilization and retranslocation of nutrients is significantly enhanced during senescence (Marschner, 1995). The importance of leaf senescence for the accumulation of Fe and Zn in wheat grains has been clearly demonstrated by the studies on the *Gpc-B1* locus. This locus has been associated with increased accumulation of Fe and Zn in grains (Cakmak et al., 2004; Distelfeld et al., 2007). In fact, it has been shown that the increased micronutrient accumulation related to the *Gpc-B1* locus is linked with an early onset of senescence in flag leaves (Uauy et al., 2006). These observations suggest that by manipulating senescence one could affect micronutrient retranslocation in plants.

In the present study, it was observed that as flag leaves started to senesce the contents of Fe and Zn in leaves decreased significantly (Figures 7, 10, 25 and 26)

whereas they started to increase in developing grains (Figures 8 and 11). This indicates that both micronutrients were exported out of flag leaves during senescence. It has been reported previously that in sand-grown wheat up to 77% of shoot Fe was retranslocated to grains (Garnett and Graham, 2005). However, in field-grown wheat poor Fe retranslocation during seed filling has been reported (Hocking, 1994). Unfortunately, in the present study the decrease in Fe and Zn contents during leaf senescence were not accompanied by increases in the exudation rates of these micronutrients (Figures 9, 12, 28 and 30).

The N nutritional status of plants can affect the development of senescence, since a high N nutrition delays the onset of senescence (Marschner, 1995). In contrast, N deficiency induces leaf senescence (Guitman et al., 1991; Crafts-Brandner et al., 1998; Aguera et al., 2010; Shi et al., 2012). In the present work, different N forms were used and are supplied to wheat plants at two distinct stages. The main goal was to assess whether senescence could be manipulated without affecting negatively the pool of N-dependent chelators. In fact, it was observed that compared to control plants, in particular the supply of nitrate retained higher chlorophyll levels in flag leaves, indicating that leaf senescence is sensitive to fertilizer N forms and that nitrate is most effective in delaying leaf senescence (Figures 2 and 19).

Unfortunately, the observations from the chlorophyll analysis were not reflected by the expression of *TaSAG12*, the wheat ortholog of *SENESCENCE ASSOCIATED GENE12* of *Arabidopsis thaliana* (Figure 20). However, the expression of *TaSAG12* was also not significantly different in control leaves harvested in the green or senescent stage, although leaves from the senescent stage were obviously more

chlorotic (Figure 18). Thus, although the expression of *AtSAG12* is a reliable indicatior for leaf senescence in *A. thaliana* (Lohman et al., 1994; Noh and Amasino, 1999), *TaSAG12* expression is not associated with flag leaf senescence in wheat.

In the present study, the timing of the N application had only a minor effect on the progression of leaf senescence, since flag leaves exhibited similar chlorophyll levels irrespective of whether they received the N fertilization before or after anthesis (Figures 2 and 19). Thus, the increased micronutrient accumulation during grain filling in wheat was associated with the reduction of micronutrients in flag leaves during senescence. The supply of N, particularly in the form of nitrate, can delay the onset of leaf senescence and can therefore be used to manipulate this developmental process in field-grown wheat.

5.2 The effect of N forms on the accumulation of N and micronutrients in grains

In wheat, N contents in grains correlate positively with the accumulation of micronutrient in grains (Cakmak et al., 2010; Kutman et al., 2010). Therefore, the effect of the different N treatments on grain N contents was assessed (Figures 6 and 24). In the first field trial, the application of N increased significantly N contents in grains, independently of the N form used (Figures 6). However, this effect was limited to the first year, since in the second field trial grains of control plants also exhibited relatively high N contents (> 50 mg N spike^{-1}) and no additional effect was observed with the supply of N (Figure 24).

It has been reported that most of the N that ends up in wheat grains originates from the retranslocation of amino acids out of the vegetative organs, especially from flag

leaves (Gregersen et al., 2008; Masclaux-Daubresse et al., 2008). In the present study, the supply of N increased significantly the concentration of glutamine, asparagine and aspartate in flag leaves (Table 1). In addition, the levels of glutamine were significantly increased in leaf exudates when N was supplied to plants. The form of N had no significant effect on this response, since N contents in flag leaves and grains were increased by N supply independently of the N form used (Figures 5 and 6). Previously, it has been reported that the supply of nitrate to durum wheat (*Triticum turgidum* ssp. *durum*) can enhance the rate of N remobilization to grains (Spano et al., 2003). More recent reports also indicate that the increase in grain N contents can stimulate the accumulation of Zn, and likely of Fe, in grains (Kutman et al., 2010).

Among the N forms tested in the present study, the supply of ammonium after anthesis increased most consistently the accumulation of Fe in grains, since a significant effect was observed in both field experiments (Figures 8 and 27). The effect of ammonium was not only restricted to Fe, since the application of this N form also enhanced the Zn contents in grains (Figures 11 and 29). The effect of ammonium on micronutrient accumulation was likely not related to senescence, since ammonium-treated plants did not show early leaf senescence (Figures 2 and 19). Recent studies indicated that N can stimulate the uptake and the root-to-shoot translocation of Fe and Zn in barley (Erenoglu et al., 2011; Shi et al., 2012).

Thus, it is more likely that ammonium affected the synthesis of N-containing chelators required to translocate micronutrients and/or stimulated the activity of transporters responsible for loading the phloem with Fe and Zn.

The transport of Fe and Zn in the phloem is facilitated by nitrogenous compounds, such as NA and DMA (Suzuki et al., 2008; Curie et al., 2009). In the first field trial, it was observed that the supply of N to wheat plants especially before anthesis increased strongly NA concentrations in flag leaves (Figure 13). In addition, N supply enhanced NA exudation rates from senescing flag leaves (Figure 14), suggesting that more NA was loaded into the phloem for long-distance transport. Regarding the effect of the N forms, there was no consistently more effective action of any N form on NA translocation in these two trials (Figures 14 and 32). An unexpected observation from this study was that N nutrition had only a minor effect on the DMA levels of flag leaves (Figures 15 and 33) and on DMA exudation rates (Figures 16 and 34). Thus, NA was the metal chelator that responded more sensitively to the N treatments. In agreement with this, it has been reported that NA concentrations were increased in old leaves of barley plants when sufficient N was supplied to plants, especially under Fe deficient conditions (Shi et al., 2012). By contrast, DMA levels were increased when plants experienced N deficiency.

Altogether, the present study indicates that the supply of N particularly ammonium to wheat plants after the anthesis is a promising agronomic strategy to increase micronutrient contents in grains, while the fertilizer N form appears to play a minor role.

5.3 The effect of citric acid on the micronutrient accumulation in grains

In addition to NA and DMA, also citrate plays a role during long-distance transport of Fe. However, because citrate exhibits a higher affinity to Fe under low pH conditions (von Wirén et al., 1999) it is more likely that citrate effectively contributes to Fe transport in the xylem rather than to Fe transport in the phloem (Rellan-Alvaréz et al., 2010). In addition to its function as a Fe-complexing molecule during transport, citrate appears to be important for the solubilization of the Fe that precipitates in the apoplast outside the cells (Roschzttardtz et al., 2011), thereby making it available for subsequent membrane transport. In line with this putative role, the foliar application of citric acid to fruit trees has been proven successful in alleviating Fe-induced chlorosis in leaves (Tagliavini et al., 2000; Ălvarez-Fernández et al., 2004), suggesting that apoplastic Fe was made available by this treatment. However, the effect of citrate on Fe accumulation in grains has not yet been tested.

In the present study, N application increased citrate levels in flag leaves of wheat plants growing in the vegetative phase (Figure 17). However, during leaf senescence, citrate levels dropped significantly and the N treatments had no obvious effect at this stage anymore (Figure 17). In the second field trial, the determination of citrate levels in leaf exudates revealed that citrate concentrations increased significantly in exudates collected from senescent leaves (Figure 38), suggesting that citrate was exported out of flag leaves during senescence.

Importantly, the supply of N inhibited this process, since citrate levels were significantly decreased in leaf exudates from plants supplied with N (Figure 38). The additional foliar spray with citric acid was most effective in increasing citrate levels in

leaf exudates from nitrate- or urea-supplied plants (Figure 38). In control plants, the supply of citrate to leaves increased significantly Fe contents in grains, whereas in N-treated plants the additional application of citric acid did not affect Fe accumulation in grains (Figure 27). Also Zn contents in grains tended to be enhanced by citric acid supply in control plants (Figure 29). Since citric acid application to control plants did not increase NA or DMA exudation rates (Figures 32 and 34), it is likely that the positive effect of citrate was not directly related with its influence on the long-distance transport of Fe. Instead, citrate may have facilitated the solubilization of Fe in senescing leaves, making it better mobile and available for retranslocation.

5.4 Conclusion and outlook

In conclusion, the present results in this thesis confirmed that the retranslocation of the micronutrients Fe and Zn from source leaves into grains is obviously stimulated during by senescence. Since the progression of flag leaf senescence turned out to be sensitive to the supplied N form, it is concluded that the choice of a certain N forms represents a tool for fertilizer management when Fe and Zn retranslocation shall be enhanced. While the results presented in this thesis contribute to a deeper understanding between leaf senescence and micronutrient retranslocation, they also require confirmation since in particular the effects of the N forms on retranslocation processes were variable between the two years. This may be due to several reasons, such as fertilization time point, conversion rates of the applied N forms in the soil and soil moisture content influencing additionally the rate of root uptake. Such variables may become less important when N fertilizer application is performed earlier and

mineralization is controlled more tightly. Alternatively, N fertilizers may be applied as leaf sprays. The application of a potent Fe chelator, i.e. citrate, could not be proven to be an effective means to improve Fe or Zn retranslocation from senescent leaves. In fact, the visual analysis and in tendency also the chlorophyll analysis suggested that citrate application induced a re-greening of senescent leaves (Figures 18 and 19). Thus, under the chosen conditions citrate may have stimulated N and C metabolism, thereby preventing citrate from acting as an Fe chelator. To substantiate this possibility, citric acid application trials may be repeated under conditions known to affect Fe availability in leaves such as high apoplastic pH, as found when plants grow on calcareous soils, or elevated P levels in leaves, which promote Fe precipitation in the leaf apoplast.

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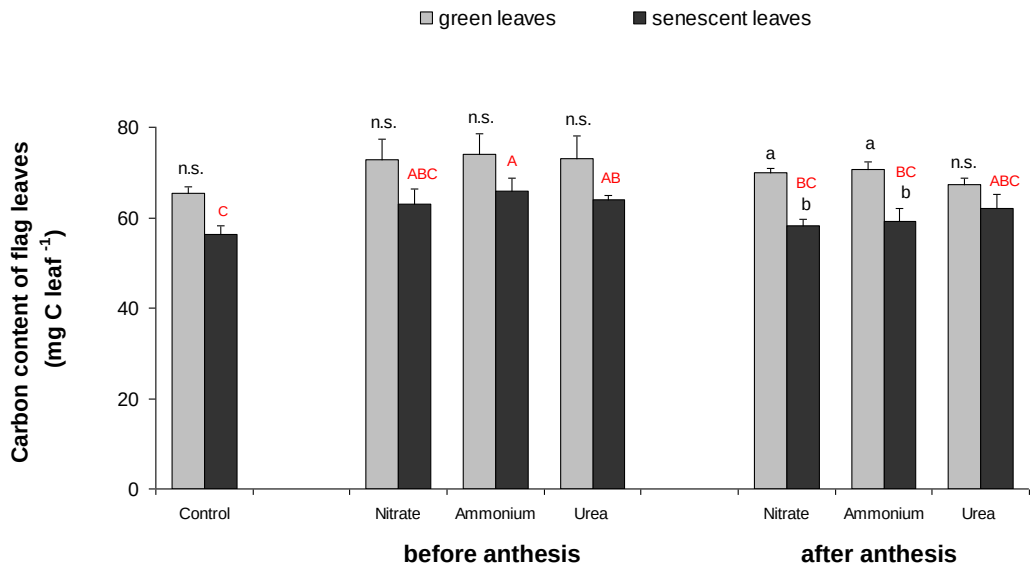
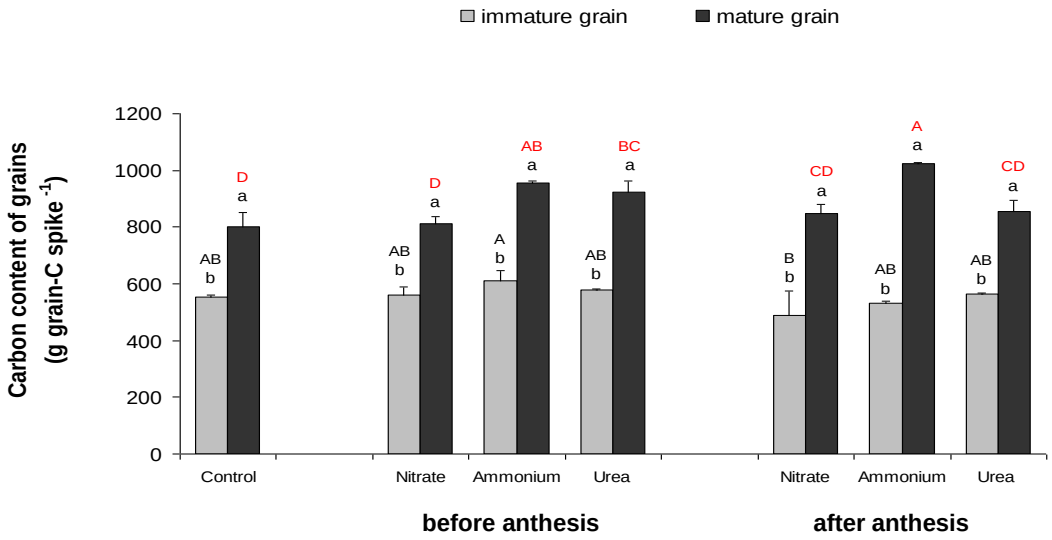
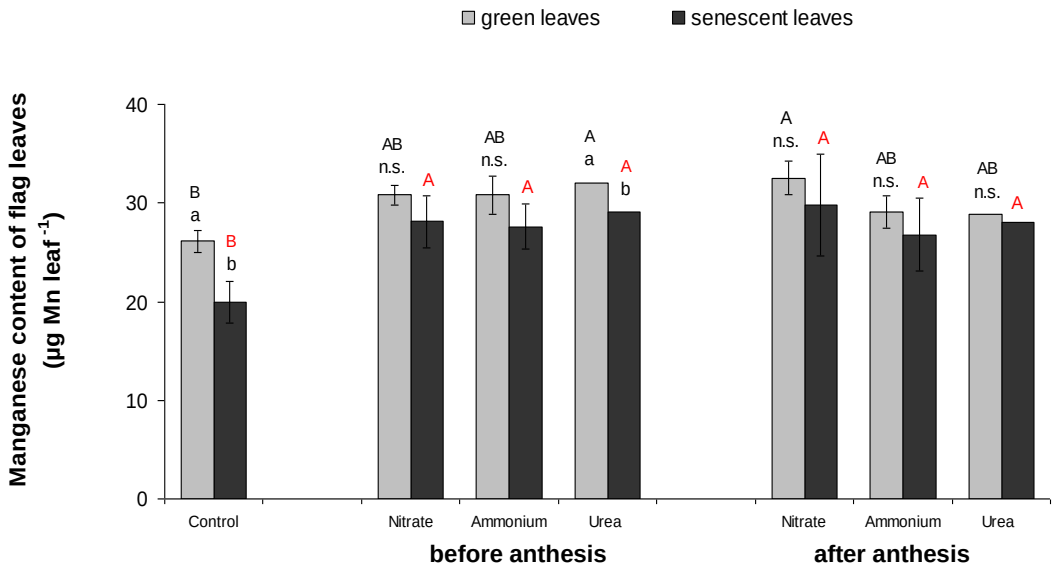
7 APPENDIX



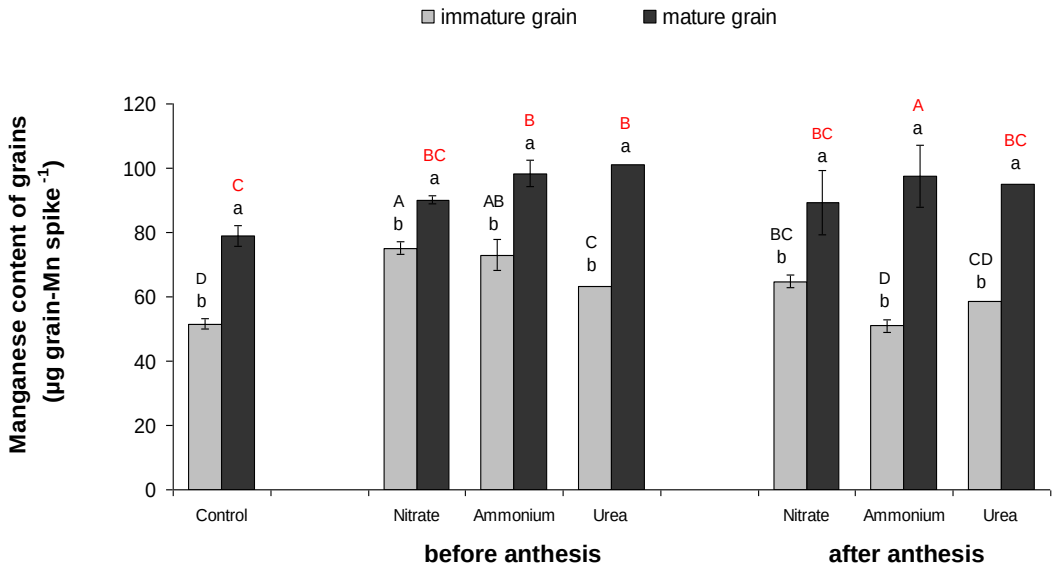
Appendix 1. Effect of N forms on C contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (green leaves) or 85 days (senescent leaves), flag leaves were harvested and the C contents were determined by Elemental Analysis-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference. ns denotes that there is no significant difference.



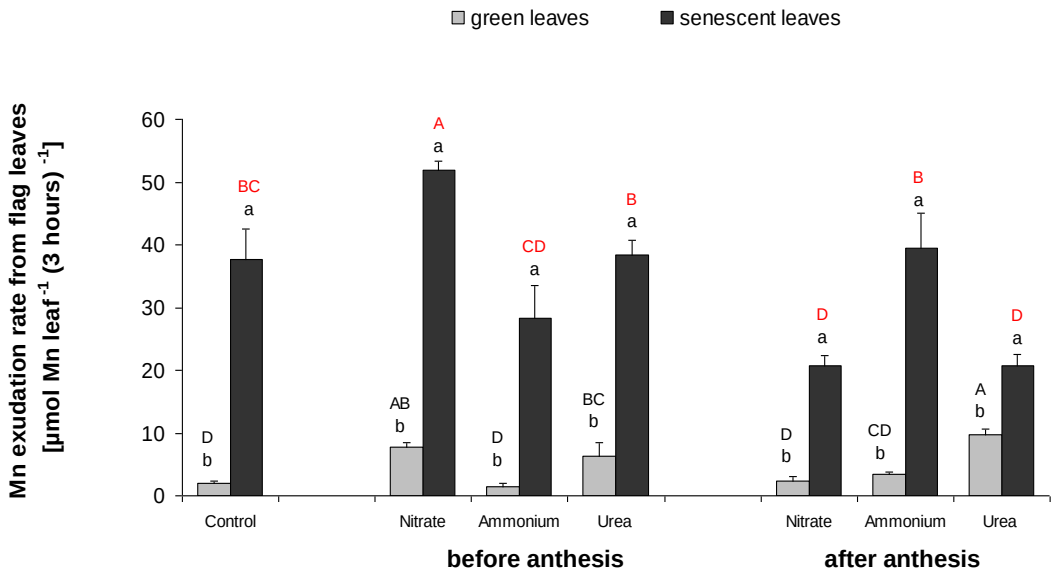
Appendix 2. Effect of N forms on C contents of grains.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (immature grains) or 104 days (mature grains), spikes were harvested and the C contents were determined by Elemental Analysis-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for immature grain and red letters for mature grain), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



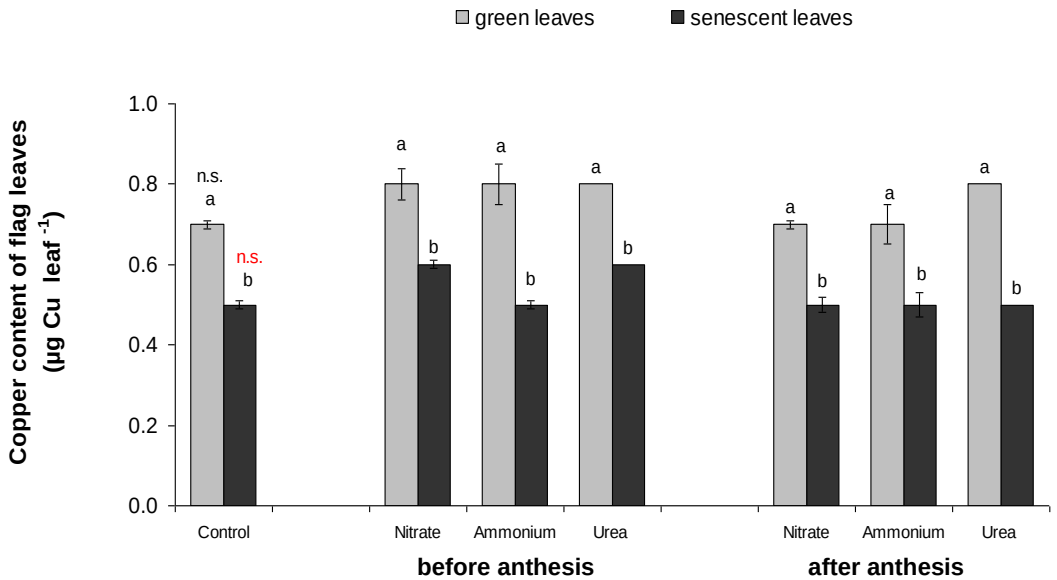
Appendix 3. Effect of N forms on Mn contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (green leaves) or 85 days (senescent leaves), flag leaves were harvested and the Mn contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



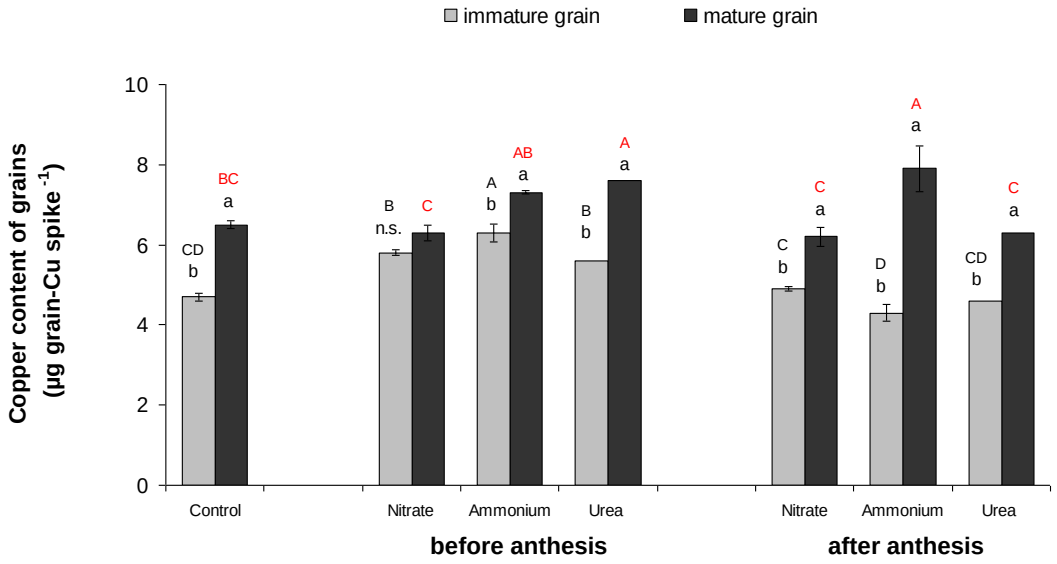
Appendix 4. Effect of N forms on the Mn contents of grains.
Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (immature grains) or 104 days (mature grains), spikes were harvested and grains were collected from single spike, then the Mn contents were determined with ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for immature grain and red letters for mature grain), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



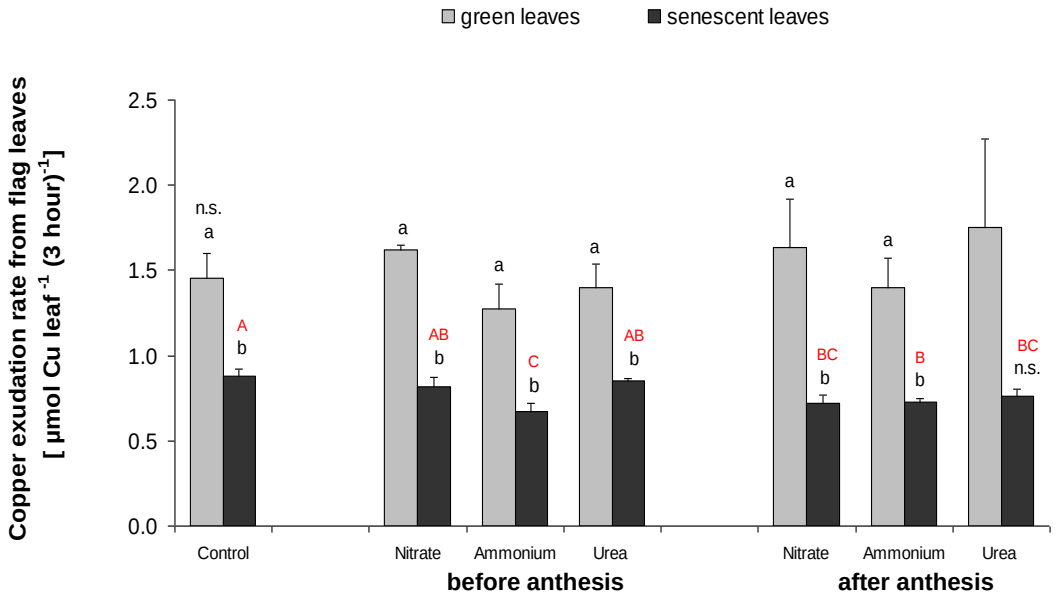
Appendix 5. Effect of N forms on Mn exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). The leaf exudates were collected into 15 mM EDTA solution pH 7.5 from green leaves after 75 days and from senescent leaves after 85 days. The Mn concentration was determined by ICP-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



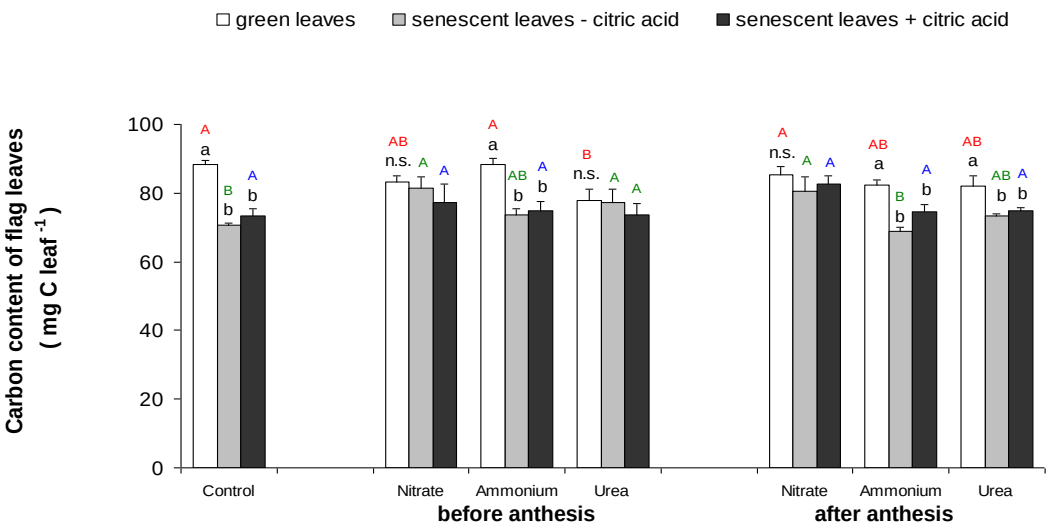
Appendix 6. Effect of N forms on Cu contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). After 75 days (green leaves) or 85 days (senescent leaves), flag leaves were harvested and the Cu contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



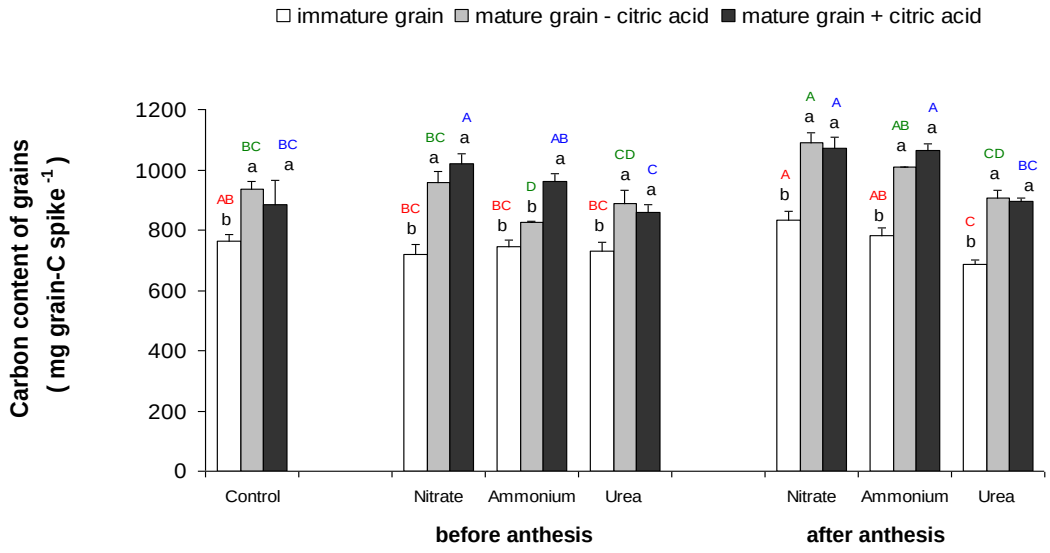
Appendix 7. Effect of N forms on Cu contents of grains.
Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). After 75 days (immature grains) or 104 days (mature grains), spikes were harvested and grains were collected from single spike, then the Cu contents were determined with ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for immature grain and red letters for mature grain), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



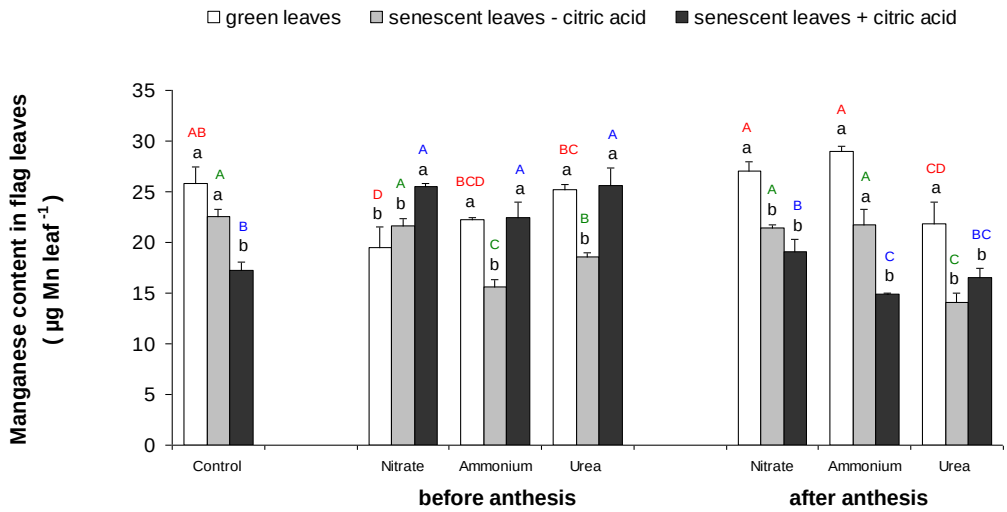
Appendix 8. Effect of N forms on Cu exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). The leaf exudates were collected into 15 mM EDTA solution pH 7.5 from green leaves after 75 days and from senescent leaves after 85 days. The Cu concentrations were determined by ICP-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (black letters for green leaves and red letters for senescent leaves), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



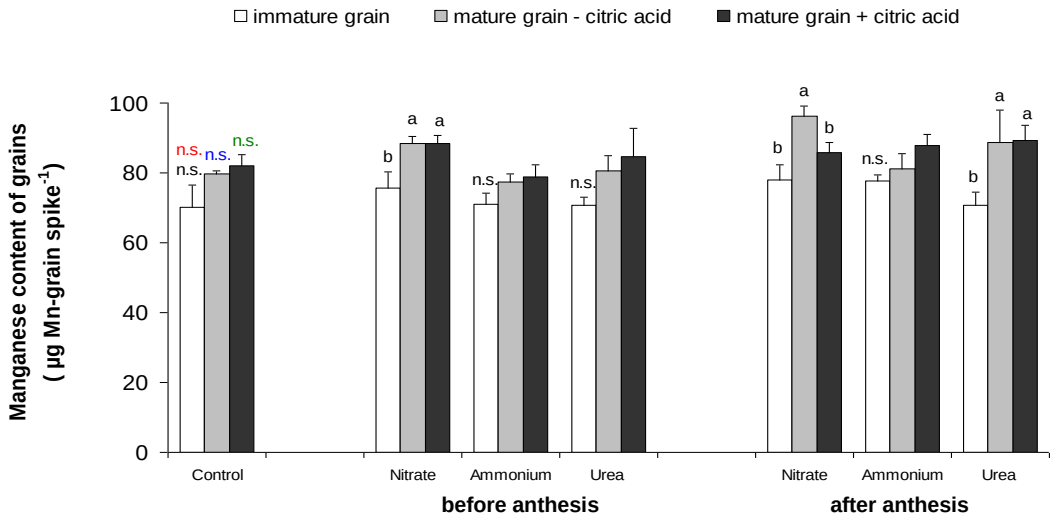
Appendix 9. Effect of N supply and foliar citric acid application on C contents of flag leaves. Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. The flag leaves were harvested and dried, then the C contents were determined by Elemental Analysis-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



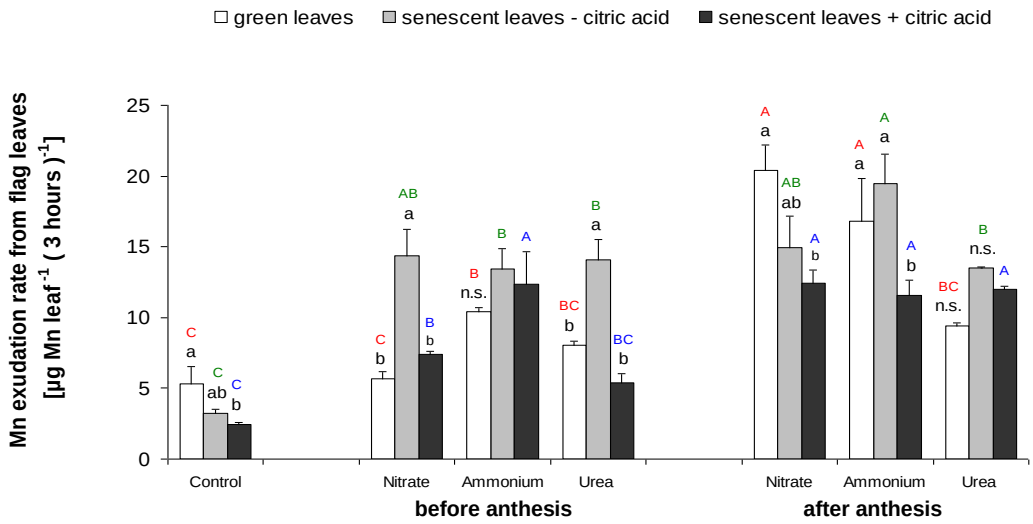
Appendix 10. Effect of N supply and foliar citric acid application on C contents of grains. Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). In addition, foliar application of citric acid was carried out after 85 days and samples were harvested 2 days after spraying. The spikes were harvested after 75 days (immature grains), or 104 days (mature grains). Grains collected from each spike were dried and the C contents were determined by Elemental Analysis-MS. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for immature grain, green letters for mature without citrate and blue letters for mature grain with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



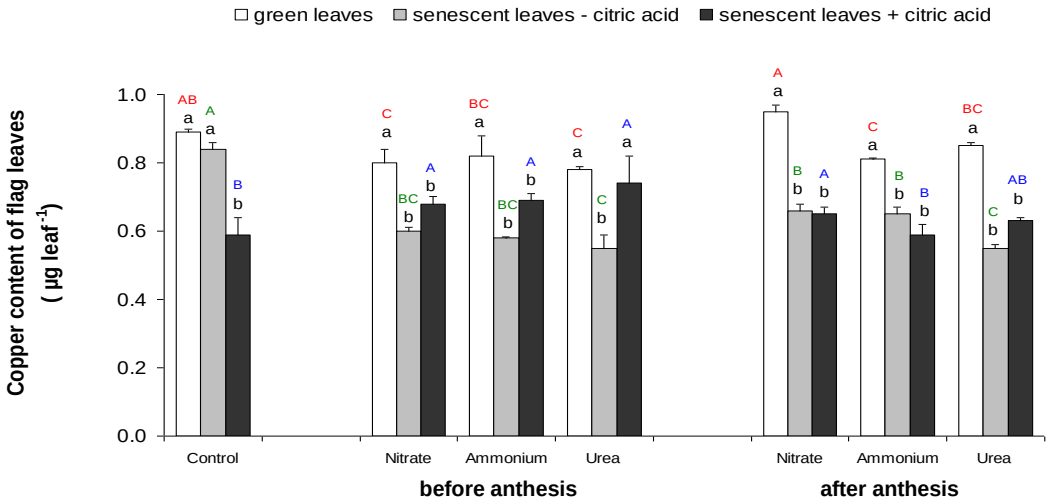
Appendix 11. Effect of N supply and foliar citric acid application on Mn contents of flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying and Mn contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



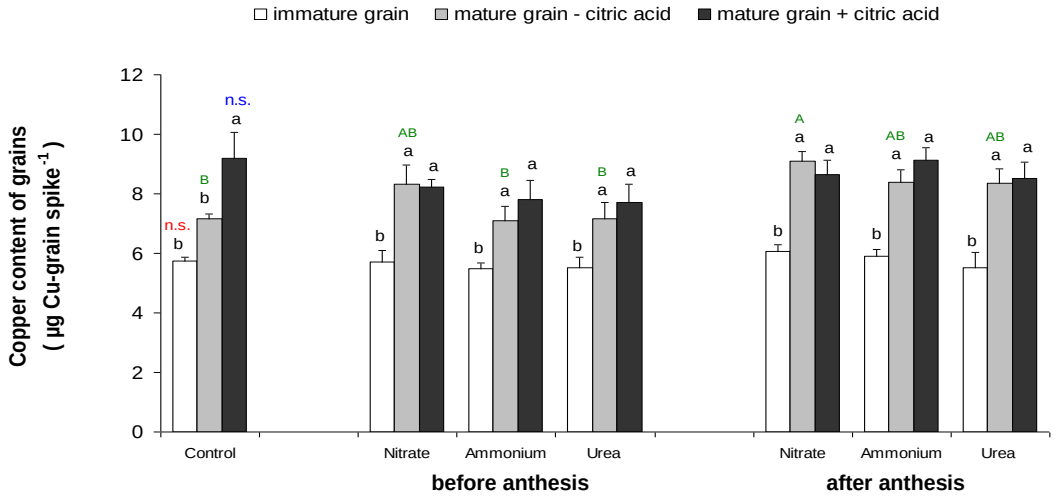
Appendix 12. Effect of N supply and foliar citric acid application on Mn contents of grains.
Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). In addition, foliar application of citric acid was carried out after 85 days and samples were harvested 2 days after spraying. Spikes were harvested after 75 days (immature grains) or 104 days (mature grains) and grains were collected from single spikes and the Mn contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for immature grain, green letters for mature without citrate and blue letters for mature grain with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



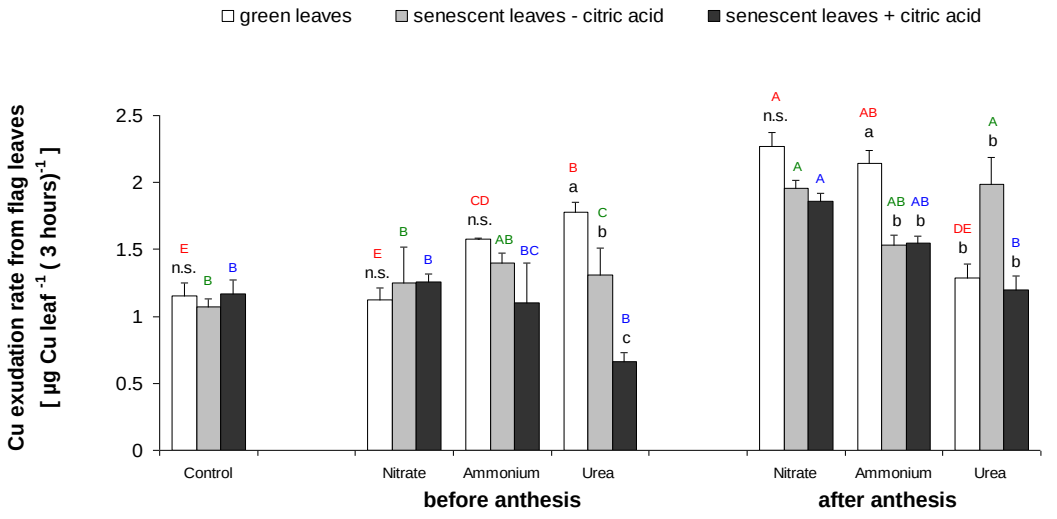
Appendix 13. Effect of N supply and foliar citric acid application on Mn exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. Leaf exudates were collected from single flag leaf into 15 mM EDTA solution pH 7.5 after 75 days (green leaves) or 87 days (senescent leaves) and Mn concentrations were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



Appendix 14. Effect of N supply and foliar citric acid application on Cu contents of flag leaves.
The wheat plants (*Triticum aestivum* L. cv Akteur) were supplied with foliar citric acid (1 g per liter) was at 85 days after planted which have 40% yellowish leaf. Nitrate, ammonium or urea (80kg N ha^{-1}) were supplied either before (EC49) or after the anthesis (EC65). The leaves were harvested after 75 days (green leaves) and 87 days (senescent leaves) 2 days after the citrate sprayed and the Cu contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



Appendix 15. Effect of N supply and foliar citric acid application on Cu contents of grains.
Wheat plants (*Triticum aestivum* cv. Akteur) were supplied with nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after the anthesis (EC65). In addition, foliar application of citric acid was carried out after 85 days and samples were harvested 2 days after spraying. Spikes were harvested after 75 days (immature grains) or 104 days (mature grains) and grains were collected from single spikes and the Cu contents were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for immature grain, green letters for mature grain without citrate and blue letters for mature grain with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.



Appendix 16. Effect of N supply and foliar citric acid application on Cu exudation rates from flag leaves.
Wheat plants (*Triticum aestivum* L. cv Akteur) were fertilized by nitrate, ammonium or urea (80 kg N ha^{-1}) either before (EC49) or after anthesis (EC65). Samples were harvested after 75 days (green leaves) and 87 days (senescent leaves). In addition, foliar application of citric acid (1 g per L) was carried out after 85 days and samples were harvested 2 days after spraying. Leaf exudates were collected from single flag leaf into 15 mM EDTA solution pH 7.5 after 75 days (green leaves) or 87 days (senescent leaves) and Cu concentrations were determined by ICP-OES. Values are means of 4 independent replicates ± SE, and 4 plants per replicate. Capital letters indicate significant differences among treatments in each harvest (red letters for green leaves, green letters for senescent leaves without citrate and blue letters for senescent leaves with citrate), whereas small letters compare the means of the different harvest times in each treatment according to LSD test at P<0.05. ns denotes that there is no significant difference.

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Eidesstattliche Erklärung

Hiermit erkläre ich, dass diese Arbeit von mir bisher weder der Mathematisch-Naturwissenschaftlich-Technischen Fakultät der Martin-Luther-Universität Halle-Wittenberg noch einer anderen wissenschaftlichen Einrichtung zum Zweck der Promotion eingereicht wurde.
Ich erkläre ferner, dass ich diese Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel und Literatur angefertigt habe.

Gatersleben, den Oktober 2012 _____