Quantification of the impact of enhanced urea uptake in cereal grain crops on growth, nitrogen metabolism, yield formation and grain quality

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ZUSAMMENFASSUNG

Harnstoff ist eine wichtige Stickstoffform, die in der landwirtschaftlichen Pflanzenproduktion eingesetzt wird. In Kombination mit Harnstoffdüngemitteln haben Ureaseinhibitoren ein großes Potenzial zur Steigerung des Kornertrags und des Rohprotein-Gehalts gezeigt. Abgesehen von agronomischen Untersuchungen sind physiologische Studien zur Aufnahme von Ureaseinhibitoren durch die Wurzeln und deren Auswirkungen auf den Pflanzenmetabolismus jedoch selten untersucht. Dieses mangelnde Wissen betrifft insbesondere Ureaseinhibitoren, die in die Gruppe der Amide und Ester der Phosphorsäure, wie NBPT und das kürzlich entwickelte 2-NPT, eingeordnet sind. In dieser Studie sollte daher die agronomische Wirksamkeit einer Zugabe von 2-NPT mit Harnstoffdünger parallel zum physiologischen Einfluss von 2-NPT auf den primären Stickstoff- und Kohlenstoffmetabolismus sowie auf den Phytohormon-Haushalt und die Ertragsbildung untersucht werden. Außerdem zeigt diese Studie, ob 2-NPT die Harnstoffaufnahme durch Pflanzenwurzeln hemmt, wenn der unter Harnstoffernährung zugegeben ist. Analyse von möglichen physiologischen Einflüssen nach der Zugabe von 2-NPT zu Getreide werden ebenfalls gezeigt.

Eine erste Reihe von Feldversuchen mit Winterweizen wurde in drei aufeinanderfolgenden Versuchsjahren (2012-2015) an zwei Standorten durchgeführt, die sich in Bodeneigenschaften und Klima unterschieden: Cunnersdorf, ein Standort, der durch seine sandige Bodenstruktur mit geringer Kationenaustauschkapazität bekannt ist, und Gatersleben mit einem vorwiegenden lehmigen Boden und höherem Gehalt an organischen Substanz. Bodenprobenahme und anschließende Analyse der N-Formen zeigten, dass der Ureaseinhibitor 2-NPT die Harnstoffhydrolyse verzögerte, da an beiden Standorten die Zugabe von 2-NPT zu 4-fach höheren Bodenharnstoffkonzentrationen führte als die Anwendung von Harnstoff solo. Die Etablierung einer UPLC-basierten Methode zur Analyse von 2-NPT ermöglichte den Nachweis von 2-NPT im Xylemsaft und seine Anreicherung in Weizenblättern. An beiden Standorten und in allen Versuchsjahren erhöhte die gleichzeitige Anwendung von Harnstoff mit 2-NPT die Translokationsraten von Harnstoff im Xylemsaft. Bei Vorhandensein dieses Ureaseinhibitors in Blättern waren die Harnstoffkonzentrationen im Blatt bis zu 11-fach höher als in Abwesenheit von 2-NPT, während die interne Ureaseaktivität nicht beeinflusst wurde. Bemerkenswert ist, dass die höhere Akkumulation von Harnstoff in Blättern als Folge der Zugabe von 2-NPT nicht zu konsistenten Änderungen in anderen N-Formen einschließlich Ammonium-, Nitrat- oder Aminosäuren und in anderen primären Metaboliten wie Zucker oder organischen Säuren führte. Auch die Phytohormonkonzentrationen blieben von 2-NPT weitgehend unverändert.

Die Kornertraganalyse zeigten, dass 2-NPT den Kornertrag gegenüber dem durch Düngung mit Harnstoff solo erzielten Niveau nur in Cunnersdorf und nur in einem Jahr erhöht wurde. Diese Ertragssteigerung beruhte auf einer größeren Anzahl von ährentragenden Halmen. In allen drei Versuchsjahren in Cunnersdorf erhöhte die Zugabe von 2-NPT mit Harnstoff jedoch den Rohproteingehalt und die gesamt N-Entzüge. Da diese Werte die gleichen waren wie nach der Düngung mit Ammoniumnitrat, wurde als Schlussfolgerung festgelegt, dass 2-NPT die N-Akkumulation und Proteinsynthese während der Kornbefüllungsphase als Folge von verringerten Ammoniakemissionen und einer längeren N-Verfügbarkeit aufgrund einer verzögerten Harnstoffhydrolyse unterstützte. Eine Erklärung für den fehlenden Effekt von 2-NPT in Gatersleben wurde im hohen mineralischen N-Gehalt des Bodens gesehen, der meistens alle N-Düngerbehandlungen unwirksam machte. In diesem ersten Teil der Studie wird die Wirksamkeit des kürzlich entwickelten Ureaseinhibitors 2-NPT in sandigen Böden mit hohem Ammoniakpotenzial und niedrigen bis mäßigen N-Gehalten im Boden hervorgehoben, ohne den Primärstoffwechsel und das Pflanzenwachstum von Weizen zu beeinträchtigen.

Eine weitere Reihe von Experimenten mit einem belüfteten Hydroponiksystem mit Sommergerste wurde nicht nur durchgeführt, um zu bestätigen, ob dieser Ureaseinhibitor von Pflanzen aufgenommen und akkumuliert werden kann, sondern auch um zu untersuchen, ob 2-NPT die Harnstoffaufnahme beeinflussen kann, da der Ureaseinhibitor könnte während der Aufnahme durch Pflanzenwurzeln mit Harnstoff konkurrieren. Der Ureaseinhibitor 2-NPT konnte im Spross und Wurzeln nachgewiesen werden und Analysen in diesen Geweben zeigten eine erhöhte Akkumulation von Harnstoff. Andererseits zeigten ^{15}N-Markiertmessungen, dass die Zugabe von 2-NPT zu Pflanzen, die unter Harnstoffernährung wachsen, zu einer Hemmung der Aufnahmefähigkeit von Wurzeln führen kann, kurz nach ihrer Anwendung in Kombination mit Harnstoff. Dennoch beeinflusste die Zugabe von 2-NPT die interne Ureaseaktivität und die Nährstoffkonzentration der behandelten Pflanzen nicht.

SUMMARY

Urea is a major nitrogen form applied in agricultural plant production. When co-supplied with urea fertilizers, urease inhibitors have shown great potential to increase grain yield and grain protein concentrations. Apart from agronomic investigations, however, physiological studies on the uptake of urease inhibitors by roots and their impact on plant metabolism have remained rare. This lack of knowledge regards in particular urease inhibitors classified in the group of amides and esters of phosphoric acid, such as NBPT and the recently designed 2-NPT. Therefore, this study set out to investigate the agronomic efficacy of 2-NPT application with urea fertilizers in parallel with the physiological impact of 2-NPT on primary nitrogen and carbon metabolism as well as on the phytohormone balance and yield formation in winter wheat. To address the question whether 2-NPT may directly affect urea uptake processes by roots, this study investigated the impact of 2-NPT on uptake and root-to-shoot translocation of urea in hydroponically-grown barley.

A series of field trials with winter wheat was conducted in three consecutive years (2012-2015) at two locations differing in soil properties and climate: Cunnersdorf, a location characterized by its sandy soil structure with low cation exchange capacity, and Gatersleben with a predominantly loamy soil and higher organic matter content. Soil sampling and subsequent analysis of N forms showed that the urease inhibitor 2-NPT was effective in retarding urea hydrolysis, because at both locations 2-NPT application led up to 4-fold higher soil urea levels than application of urea alone. Establishing an UPLC-based method for the analysis of 2-NPT allowed tracing 2-NPT in the xylem sap and its accumulation in wheat leaves. At both sites and in all years, co-application of urea with 2-NPT increased xylem translocation rates of urea. With the presence of this urease inhibitor in leaves, leaf urea concentrations were up to 11-fold higher than in absence of 2-NPT, whereas internal urease activity remained unaffected. Noteworthy, the higher accumulation of urea in leaves as a consequence of 2-NPT application did not result in consistent changes in other N forms including ammonium, nitrate or amino acids and in other primary metabolites such as sugars or organic acids. Also phytohormone concentrations remained largely unaffected by 2-NPT.

Grain yield analysis showed that 2-NPT increased grain yield above the level obtained by fertilization with urea alone only in Cunnersdorf and only in one year. This yield increase was based on a larger number of tillers. However, in all three years at Cunnersdorf, 2-NPT co-application with urea significantly increased grain protein concentrations and the total amount of N accumulated in the above-ground plant biomass. As these levels were the same as achieved by fertilization with ammonium nitrate fertilizer, it was concluded that 2-NPT supported N accumulation and protein synthesis during the grain filling phase as a consequence of decreased ammonia emissions and longer N availability due to retarded urea hydrolysis. An explanation for the lacking effect of 2-NPT in Gatersleben was seen in the high mineral N content of the soil, which mostly rendered all N fertilizer treatments ineffective. This first part of the study emphasizes the efficacy of the recently designed urease inhibitor 2-NPT in sandy soils with high potential for ammonia emissions and low to moderate soil N contents without compromising primary metabolism and plant growth in wheat.

Another set of experiments using an aerated hydroponic system with spring barley was conducted to verify whether 2-NPT can be taken up by roots and translocated to shoots, but also to investigate whether this urease inhibitor affects the capacity of plants to acquire urea, as this urease ihhibitor may compete with urea during the uptake process by plant roots. In both, shoots and roots, 2-NPT was detected and analyses in these tissues showed an increased accumulation of urea. On the other hand, influx measurements of ^{15}N-labeled urea revealed that addition of 2-NPT to plants growing under urea nutrition may decrease the urea uptake capacity of roots. Nonetheless, its addition did not affect the internal urease activity and nutrient concentrations of 2-NPT-treated plants. Thus, this UI when added in conjuction with urea to plants may interfere with the uptake of urea, at least within a short-term after its application to grain cereals.

1 INTRODUCTION

1.1 Nitrogen fertilization in cereal crop production

1.1.1 Importance of cereal grain crops in agricultural plant production

Cereal grain crops have been always considered as one of the most important staple food sources, being cultivated mainly for human nutrition and animal feeding due to their high content of carbohydrates (Köhler & Wieser, 2013) and a considerable amount of proteins, vitamins, minerals and fibre (McKevith, 2004; Sarwar et al., 2013). Moreover, cereal grain crops have been used in the recent years also for the production of renewable energy sources (Kaķītis & Šmits, 2009), in particular via fermentation to biogas or bioethanol (García et al., 2013).

Cereal grain crops belong to the monocot family Poaceae (also known as Gramineae), specifically to the subfamily Pooideae. Among them are some major cereals like the hexaploid bread wheat (*Triticum aestivum* L.), the tetraploid durum wheat (*T. durum* Desf.), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), oat (*Avena sativa* L.) and rice (*Oryza sativa* L.) (Kadereit, 2014). According to FAO (2018), the worldwide surface destined in 2016 to wheat cultivation was approximately 220.1 Mha with a world production of 749.5 Mt, whereas the production of other grain cereals like rice, barley and rye accounted for 740.9, 141.3 and 44.8 Mt, respectively. In Germany, the cultivation of wheat and barley has dominated cereal crop production in the past decades. For instance in 2014, wheat and barley accounted for 53.4% and 22.2%, respectively, of the total cereal grain production in this country, with a harvested area of 32,197 km^2 and 15,737 km^2, respectively, of a total surface of 64,607 km^2 with cultivated cereals (FAO, 2017).

Regarding crop management practices, several attempts have been and are still carried out to increase and stabilize yield formation, which include a plant demand-driven nutrient supply, the fine-tuning of fertilizer supplies to critical plant development stages, the amendment of chemical compounds targeted to manipulate nutrient delivery to plants or plant development, and the genetic improvement of crop cultivars as supported by advanced breeding approaches. Due to the need to stabilize or increase grain yields of crops growing in a changing climate, mineral fertilizer application represents a crucial topic requiring continuous improvement.

1.1.2 Nitrogen and its importance as an essential mineral element required by plants

After oxygen (O), carbon (C) and hydrogen (H), nitrogen (N) is the fourth most abundant element in living organisms (Long et al., 2015). In plants, N is the mineral element taken up from the soil in largest amounts (Taiz & Zeiger, 2010; Hawkesford et al., 2012), thus becoming one of the most limiting elements in plant nutrition (Sirko & Brodzik, 2000). This essential element determines growth and grain production (Hofman & van Cleemput, 2004; Pavlíková et al., 2012), because N is a constituent of many key molecules including amino acids, amides, proteins, nucleic acids, nucleotides, chlorophyll, enzymes, phytohormones and secondary metabolites (Hofman & van Cleemput, 2004; Taiz & Zeiger, 2010; Pavlíková et al., 2012). Approximately 1-5% of the total plant dry matter consists of N (Hawkesford et al., 2012) and in a green plant, about 70% of the leaf N is located in the chloroplasts (Sonnewald, 2014). The greatest requirement for N is the synthesis of amino acids (Long et al., 2015), in which N is first assimilated into glutamate (Glu) and glutamine (Gln) before being incorporated into aspartate (Asp) and asparagine (Asn) or other amino acids (Coruzzi, 2015). Thus, the N supply to cereal crops is usually followed by a significant increase of Gln and Asn concentrations in green leaves (Barunawati et al., 2013).

Considering that N supply strongly affects not only root growth, but also the distribution of the root system in the soil (Lynch et al., 2012; Giehl & von Wirén, 2014), only a balanced N nutrition ensures optimal plant growth (Huber et al., 2012), high grain quality and grain yield (Wiesler, 2012). Additionally, N supply is largely considered as the main factor affecting crude-protein concentration in cereals (Wiesler, 2012). Thus, the manipulation of plant N nutrition represents an important tool to meet plant requirements and to contribute to a sustainable N balance in the plant-soil system.

1.1.3 N availability in soils and main N forms taken up by plants

In most agricultural soils, more than 90% of total soil N is present as organic N, which turns into plant-available N by the action of soil microorganisms through mineralization processes (Hofman & van Cleemput, 2004; George et al., 2012). The latter is part of the N cycle, which also includes nitrogen fixation, nitrification and denitrification, and all these processes contribute to ecosystem balance (Berlicki et al., 2012). Microorganisms, such as bacteria and fungi, play a critical role in mineralizing the soil organic matter, thus making nutrients readily available for uptake by plant roots (Lynch et al., 2012). In the case of N, microorganisms transform N from the organic matter into ammonium, which is further converted to nitrate during nitrification (Hofman & van Cleemput, 2004). According to Hofman & van Cleemput (2004), nitrification consists in a two-step process, in which ammonium is first converted to nitrite, mainly by Nitrosomonas-type bacteria. In a second step, bacteria of the genus Nitrobacter oxidize nitrite to nitrate (Sonnewald, 2014). In contrast, during the anaerobic process of denitrification (Hofman & van Cleemput, 2004), nitrate is reduced to nitrite and then converted via nitrogen oxides to molecular N (Sonnewald, 2014). Interestingly, the availability of N in soils through these processes strongly differs depending on soil properties in particular moisture, temperature and aeration (George et al., 2012), as well as on soil structure (Marschner & Rengel, 2012) texture, pH, and microbial activity (Hawkesford et al., 2012).

N is taken up by plant roots from soils mainly as nitrate (NO_3^-) and/or ammonium (NH_4^+) (Hawkesford et al., 2012). In most agricultural soils, these are the most abundant inorganic N forms and both the cationic and anionic N forms can comprise up to 80% of the total ion uptake by plant roots (Neumann & Römheld, 2012). Typically, both N forms coexist in soils in different amounts, and hence plants are able to take up ammonium as well as nitrate (Britto & Kronzucker, 2005). Because NO_3^- is more mobile in the soil solution than NH_4^+, the anionic N form is usually more available to plants (Hofman & van Cleemput, 2004; Hawkesford et al., 2012) and thus the N form that is predominantly taken up by plant roots (Sonnewald, 2014). However, when N is supplied as ammonium nitrate, NH_4^+ is taken up in preference to NO_3^- (White, 2012), probably due to the lower energy input required for assimilation in plant metabolism (Hofman & van Cleemput, 2004). In fact, uptake studies with Arabidopsis plants showed a preference for ammonium over nitrate, at least as long as external N supplies were in the micromolar range (Gazzarrini et al., 1999). In well-aerated soils, NO_3^- is the major inorganic N form (Britto & Kronzucker, 2005; Neumann & Römheld, 2012) which is generally present in higher concentrations (1-5 mM) than ammonium (20-200 µM) (Hawkesford et al., 2012) due to the rapid oxidation of ammonium (Hofman & van Cleemput, 2004). NH_4^+ in the soil solution can derive from mineralization but also from application of external N sources, which includes the hydrolysis of urea (Hofman & van Cleemput, 2004). The cationic form is usually found at low soil pH, in heathlands and irrigated rice fields (Britto & Kronzucker, 2005). Interestingly, ammonium can even occur in higher concentrations than NO_3^- in unfertilized agricultural soils (Hawkesford et al., 2012). Soil NH_4^+ can go through different processes, i.e. be adsorbed on soil colloids, fixed by clay minerals, fixed by microorganisms, volatilized or oxidized to nitrate (Hofman & van Cleemput, 2004) while an important part is directly used by plants for the biosynthesis of amino acids (Barunawati et al., 2013). The uptake of either ammonium or nitrate has different effects on plant metabolism. Whereas nitrate has been demonstrated to increase cytokinins levels in plants (Rahayu et al., 2005; Garnica et al., 2010), ammonium was found to decrease their main active forms in tobacco plants (Walch-Liu et al., 2000). Finally, when NH_4^+ was supplied instead of NO_3^-, shoot and root biomass were significantly reduced in tobacco plants, which was ascribed to ammonium toxicity triggering i.a. acidification or uncoupling of photophosphorylation, when supplied as the sole N source (Walch-Liu et al., 2000).

1.1.4 N fertilization in agricultural production

In agricultural production, the necessity to reach high grain yields and elevated protein contents requires high fertilizer inputs (Hawkesford, 2014). To ensure consistently high crop yields over time, N but also phosphorus (P) and potassium (K) need to be regularly supplied to agricultural soils (Marschner, 2012). From the supplied N to crops, a considerable amount is withdrawn in harvested products (Hofman & van Cleemput, 2004), which can be then replenished by the application of N fertilizers. Thus, in addition to the inclusion of N_2-fixing crops and the use and amendment of organic N sources (Hofman & van Cleemput, 2004), the main input of N in agriculture relies on the application of mineral fertilizers (Long et al., 2015). Mineral N fertilizers play an important role in ensuring adequate N nutrition of crops, with high relevance for yield and quality (Silva et al., 2017).

The widespread application of N fertilizers to improve crop yields has been strongly implemented during the 19th and 20th centuries (Long et al., 2015). Aside from the small proportion of N available to plants in the soil solution (Marschner & Rengel, 2012), the growing world population and subsequent increasing food demand during the recent decades have generated the need to increase the production and application of N fertilizers worldwide (Ni et al., 2018). Several publications report the extensive use of N fertilizers and their effects on different agricultural crops. For instance, the grain yield as well as the total above-ground plant biomass of wheat plants substantially increased due to N fertilization (Serret et al., 2008). It has been also reported that high N fertilization increased grain protein, due to an elevated synthesis and accumulation of storage proteins (Hawkesford et al., 2012). The effectiveness of N fertilization expresses in higher chlorophyll and N concentrations in leaves (Barunawati et al., 2013). However, problems associated to the excess use of N fertilizers have become also a rising issue. High N fertilizer inputs have negative environmental impacts, which trigger adverse consequences for biodiversity, atmosphere and water quality (Grahmann et al., 2018), resulting from fertilizer leaching (Taiz & Zeiger, 2010) or the volatilization of different N forms (Watson, 2000). Therefore, there is a need to improve N fertilization strategies or even to develop new N fertilizers with a lower adverse environmental impact.

1.2 The utilization of urea in nitrogen fertilization

Urea ($CO(NH_2)_2$; Figure 1-1) was first isolated almost 250 years ago in 1773 after crystallization from urine (Zobel et al., 2015) by Herman Boerhaave. Fifty five years later, Friedrich Wöhler succeeded its synthesis in the laboratory by evaporating an aqueous solution of ammonium cyanate (Wöhler, 1828). Since then this molecule gained deeper interest because it was the first molecule synthesized from inorganic substances (Gerendás et al., 1999; Zobel et al., 2015). Urea is a small-molecular weight (60.06 g mol^{-1}; Sachs et al., 2006; Wang et al., 2008), neutral but polar (Sachs et al., 2006; Kojima et al., 2006; Wang et al., 2008) and hygroscopic substance, which is water soluble and contains 46% N (Watson, 2000) With its central position in N catabolism, urea occurrs ubiquitously in most organisms (Sachs et al., 2006).

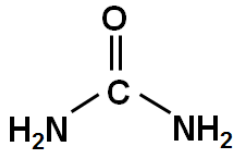

Figure 1-1. Structural formula of urea. The structure of urea ($CO(NH_2)_2$) is represented by a central carbonyl group C=O bound to two amino($–NH_2$) groups.

During the detoxification of nitrogenous compounds by different organisms (Siewe et al., 1998; Liu et al., 2003a), such as urine by animals (containing about 97% urea; Watson, 2000), and through decomposition of dead organisms (Wang et al., 2008), urea is released into the environment (Siewe et al., 1998) and therefore a readily available N source in soils (Siewe et al., 1998; Hawkesford et al., 2012). However, urea concentrations in soils are low (mostly in the µM range) due to the short half-life of this compound (Liu et al., 2003a; Wang et al., 2008) triggered by urea hydrolysis (see section 1.3.1). Therefore, application of urea fertilizers has become another important source of urea in the environment (Liu et al., 2003a).

1.2.1 Urea as a preferred external N source in agricultural plant production

Several advantages over other N fertilizers such as ammonium nitrate or CAN (calcium ammonium nitrate) made urea a desirable fertilizer in agricultural production. Urea fertilizers combine the advantages of being a rapid available N source to plants (Kojima et al., 2006), of being the most concentrated N source available (Watson, 2000; Ni et al., 2018) and of a slow conversion into NO_3^- by soil microorganisms (Kojima et al., 2006). Moreover, apart from having the advantages of safe transportation (Ni et al., 2018), ease handling (Sirko & Brodzik, 2000) and convenient storage (Hofman & van Cleemput, 2004), urea is preferred because of its low cost (Sirko & Brodzik, 2000; Zobel et al., 2015), since it is less expensive to manufacture on a per unit N basis than other N fertilizer forms (Watson, 2000). Urea-N fertilizers can be applied to the soil as granules but also directly to plant leaves. For instance, when urea is foliar applied at anthesis or at later developmental stages, N concentration in grains are promoted (Wang et al., 2008). Despite of some disadvantages of urea like its hygroscopy (Hofman & van Cleemput, 2004), its adverse effects on

seed germination (Watson, 2000) or its volatilization when surface-applied to soils (Bremner, 1995), all these advantages together made urea the most frequently and widely used N fertilizer form in the world (Sirko & Brodzik, 2000; Witte et al., 2002; Hofman & van Cleemput, 2004).

Urea was first used as N fertilizer already more than 80 years ago in 1935, but it has become widely used only about 25 years later (Watson, 2000). Since 1970, the use of urea as a N fertilizer has dramatically increased (Bremner, 1995), and about 10 years ago the use of this N fertilizer increased more than 100-fold compared to the sixties (Glibert et al., 2006). Today, urea is used more than any other N fertilizer in the world (Glibert et al., 2006), which is reflected in the yearly production of approximately 160 Mt just for agricultural use (Zobel et al., 2015). Although urea is the most used N fertilizer worldwide (Figure 1-2A), the most common one in Germany has been CAN (Ni et al., 2014; Figure 1-2B). According to IFA (2017), in 2015 the consumption of urea and CAN in the world was about 51,865 and 3,230 Kt, respectively, whereas in Germany urea and CAN registered a consumption of 437 and 586 Kt, respectively. Interestingly, over a period of 40 years, i.e. from 1975 to 2015 (IFA, 2017), there has been little change in the consumption of NH_4NO_3 and CAN in the world (Watson, 2000; Figure 1-2A). Thus, it is obvious that urea as a N fertilizer meets the economic an managerical requirements in agricultural plant production. Nevertheless, further innovative research is needed to compensate for its disadvantages and to better understand its uptake, transport and metabolism in plants.

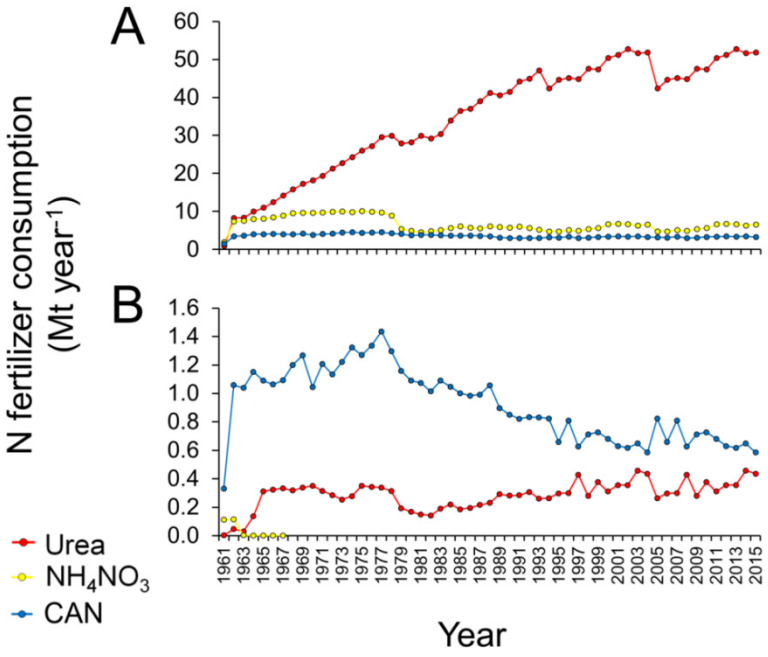

Figure 1-2. Nitrogen fertilizer consumption between 1961 and 2015. Consumption of the most frequently used N fertilizers urea, ammonium nitrate (NH_4NO_3) and calcium ammonium nitrate (CAN) in **(A)** the world and **(B)** Germany over a period of 54 years. Source: IFA (2017).

1.2.2 Urea uptake and translocation in plants

Once urea-N fertilizers are applied to agricultural crops, plant roots could be simultaneously exposed not only to urea, but also to NH_4^+ and NO_3^- ions (Mérigout et al., 2008), because urea is rapidly hydrolyzed to ammonium, which can subsequently be oxidized to nitrate by soil microorganisms (Hofman & van Cleemput, 2004). Therefore, aside from urea itself, it is interesting to know how ammonium or nitrate are taken up by plant roots and how they interact before reaching plant roots. According to Marschner (2012) ammonium can be adsorbed to the negative surface of soil particles, and desorbed by other cations. In contrast, anions like nitrate are usually repelled by the negative charge of soil particles and thus, remain freely mobile in the soil solution. Due to their negative charge, NO_3^- ions require more energy for uptake into root cells, which is mediated by symport with protons (Taiz & Zeiger, 2010). When supplied together at equimolar concentrations, NH_4^+ is taken up in preference over NO_3^- (White, 2012; Hawkesford et al., 2012), although soil concentrations of ammonium are usually lower (<100 µM) than those of nitrate (millimolar range) (White, 2012). Another interaction between these ions can be the inhibition of NO_3^- uptake when the availability of NH_4^+ is increased (Mérigout et al., 2008; White, 2012). Also urea has been shown to interfere with the uptake of NH_4^+ or NO_3^- by plant roots. For instance, in wheat seedlings urea inhibited both NH_4^+ and NO_3^- uptake, whereas NO_3^- promoted the uptake of urea (Mérigout et al., 2008). Finally, whether plant roots prefer the uptake of ammonium (NH_4^+) or ammonia (NH_3) has been also investigated. In solution at 20°C, both forms are in equilibrium at pH close to 9.3 (Gay & Knowlton, 2009) (Hofman & van Cleemput, 2004). Since NH_3 concentrations in most soils (at pH < 9) are very low, NH_4^+ is the prevailing form and preferentially taken up by plant roots (Hawkesford et al., 2012).

Uptake of NH_4^+ from the soil solution by plant roots is mediated by two transport systems, which are known as low- and high-affinity transport systems (LATS and HATS, respectively). At NH_4^+ concentrations typically > 0.5 mM, uptake operates through LATS (Hawkesford et al., 2012). In contrast, HATS usually operate at lower concentrations, typically < 0.5-1.0 mM (Britto & Kronzucker, 2005; Loqué & von Wirén, 2004). On the other hand, uptake of NO_3^- by plant roots is mediated by two nitrate transporter families, i.e. NRT1- and NRT2-type transporters (Hawkesford et al., 2012; Long et al., 2015). In Arabidopsis, major components of the LATS (above 1 mM) are NRT1.1 and NRT1.2 while in the HATS (mostly below 500 µM) NRT2.1 and NRT2.2 are active (Hawkesford et al., 2012). NRT2.1 is the major component of the HATS, induced by NO_3^- and inhibited by high N concentrations in the plant (Mérigout et al., 2008).

For many years, it has been thought that uptake of urea-derived N by plant roots was only in the form of ammonium (Polacco & Holland, 1993; Kojima et al., 2006; Hawkesford et al., 2012). However, in the recent decades it has been investigated whether urea could be taken up by plant roots also as an intact molecule, before being hydrolyzed by urease. Up to date, there is sufficient evidence that proves the direct uptake of this molecule by plant roots (Watson et al., 1994; Gerendás et al., 1998; Watson, 2000; Liu et al., 2003a; Mérigout et al., 2008; Wang et al., 2008; Zanin et al., 2015). For instance, when Mérigout et al. (2008) used mass spectrometry to detect ^{15}N-labeled urea in roots of Arabidopsis plants after 5 min influx of this molecule, they provided evidence for its direct uptake prior to hydrolysis. However, these authors did not prove its uptake by detection of double-labeled urea, i.e. including also ^{14}C-labeled urea. Urea uptake appeared to be independent of the initial plant-N status and was be inhibited by the presence of either NH_4^+ or NO_3^-. Therefore, the fact that urea molecules directly pass through the root cell membrane (Mérigout et al., 2008; Wang et al., 2008) suggested that plants can also use urea as a sole N source (Witte, 2011).

Urea can be taken up by plant roots through dedicated membrane transporters, which can also be distinguished according to their mode of operation and their concentration dependency (Kojima et al., 2006). Passive uptake of urea can be mediated by the major intrinsic proteins (MIPs), also known as water channels or aquaporins (Kojima et al., 2006). MIPs are highly abundant proteins in plant membranes (Taiz & Zeiger, 2010), and some of them can mediate the uptake of urea in a low-affinity manner (Kojima et al., 2006) using the concentration gradient of urea across membranes (Liu et al., 2003a). These MIPs have been classified into four subfamilies, namely i) the plasma membrane intrinsic proteins (PIPs), ii) the tonoplast intrinsic proteins (TIPs), iii) the nodulin 26-like intrinsic membrane proteins (NIPs) and iv) the small basic intrinsic proteins (SIPs) (Kojima et al., 2006; Wang et al., 2008). Among them, TIPs were found to mediate urea transport (Kojima et al., 2006) and some transporter genes have been isolated from the model plant Arabidopsis, corresponding to AtTIP1;1, AtTIP1;2, AtTIP2;1 and AtTIP4;1 (Liu et al., 2003b). Homologous genes were also isolated from other plant species, such as NtAQP1 and NtTIPa in tobacco,

ZmPIP1-5b in maize and CpNIP1 in zucchini (Wang et al., 2008). TIPs are thought to allow plant cells to transport urea from the cytosol into the vacuole either for transient urea storage or for detoxification in case of excess intake or generation of urea (Liu et al., 2003a; Kojima et al., 2006). Finally, it is thought that in plant metabolism (ref to. section 1.2.3) the urea generated in the ornithine cycle might be exported from mitochondria to the cytosol by AQPs that reside in the inner mitochondrial membrane (Kojima et al., 2006).

Active transport of urea into roots of higher plants is mediated by the high-affinity transporter gene DUR3, which is induced under N deficiency but also by its substrate (Bohner et al., 2015), as long as other N sources are absent (Mérigout et al., 2008). DUR3 is a secondary active urea transporter belonging to the SSS (sodium solute symporters) family (Liu et al., 2003a). To date, this high-affinity urea transporter located in the plasma membrane has been identified in several plants, such as AtDUR3 in Arabidopsis, ZmDUR3 in maize and OsDUR3 in rice (Liu et al., 2003a; Wang et al., 2012; Liu et al., 2015). DUR3 proteins permeate besides urea also its structural homolog thiourea which carries a sulfur (S) atom in place of oxygen (Liu et al., 2003a). AtDUR3 mediates the transport of urea in a H^+-dependent manner (proton/urea symporter), and its gene expression has been observed to be upregulated under N deficiency. With a fairly low K_m value of approx. 3 µM, it transports urea even at very low external urea concentrations (Liu et al., 2003a). In addition, its gene expression was not induced when NH_4NO_3 was supplied together with urea in the nutrient solution, suggesting that NH_4NO_3 inhibited its transcription (Mérigout et al., 2008). Taking together, whenever urea concentrations in the soil solution are high, uptake through low-affinity transport systems as represented by MIP-type transporters will prevail, whereas the high-affinity transporter DUR3 will take advantage at low urea concentrations, allowing the direct uptake of this valuable N source (Kojima et al., 2006).

1.2.3 Urea metabolism in plants

Considering that N is taken up from soils mainly as NO_3^- or NH_4^+ (Hawkesford et al., 2012; ref. to section 1.1.3) and that urea can be also directly taken up by plant roots as an intact molecule (Mérigout et al., 2008; ref. to section 1.2.2), it remains important to know how all these three molecules are assimilated after their absorption by plant roots. While NO_3^- is a readily mobile ion within the whole plant, it can be stored in vacuoles of roots and shoots (Hawkesford et al., 2012). Assimilation of NO_3^- (Figure 1-3) involves its reduction to NH_4^+ by the sequential action of two enzymes. NO_3^- is first reduced to nitrite (NO_2^-), which is a highly reactive and potentially toxic ion, by the action of the cytoplasmatic enzyme nitrate reductase (NR) (Taiz & Zeiger, 2010; Hawkesford et al., 2012; Long et al., 2015). NR is the main protein containing molybdenum (Mo) in plant tissues (Taiz & Zeiger, 2010). Subsequently, the generated NO_2^- is immediately transported from the cytoplasm to the chloroplasts in leaves or plastids in roots, where NO_2^- is converted into NH_4^+ by the action of nitrite reductase (NiR) (Taiz & Zeiger, 2010; Hawkesford et al., 2012; Long et al., 2015). Ammonium is then incorporated into organic compounds into amino acids. In order to avoid the accumulation of NH_4^+ and thus NH_4^+ toxicity, it is rapidly converted into glutamine (Gln) and glutamate (Glu) by the sequential action of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Taiz & Zeiger, 2010). Glu is exported to the cytoplasm and transaminated to aspartate (Asp) by aspartate aminotransferase (Asp-AT). Asp is subsequently converted into asparagine (Asn) by the action of asparagine synthetase (AS) (Taiz & Zeiger, 2010) for the synthesis of other amino acids, ureides, amines, peptides, nucleic acids, proteins, chlorophylls or phytohormones as well as other N-containing compounds such as membrane constituents (Pavlíková et al., 2012; Hawkesford et al., 2012).

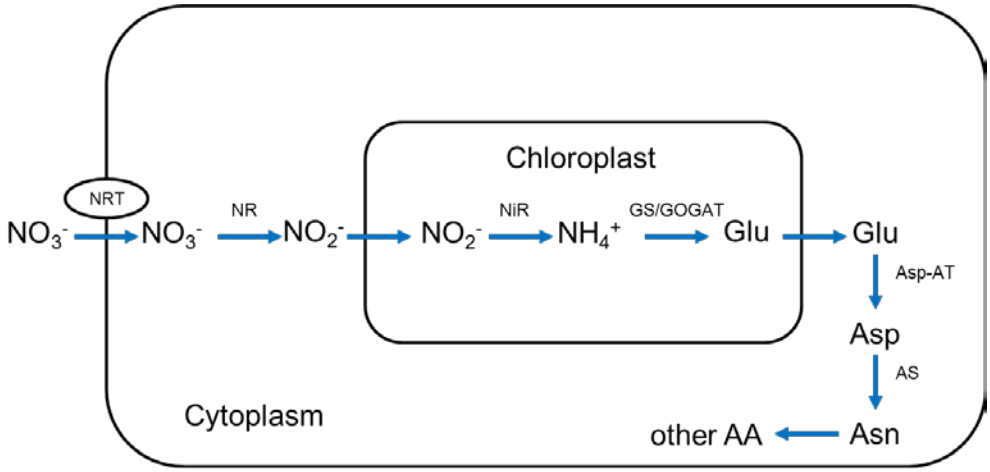

Figure 1-3. Nitrate assimilation in cells by the action of different enzymes. Nitrate (NO_3^-) enters mesophyll cells through nitrate transporters (NRT). Once in the cytoplasm, nitrate is reduced to nitrite (NO_2^-) by the action of nitrate reductase (NR). After transport into the chloroplast, NO_2^- is reduced to ammonium (NH_4^+) by nitrite reductase (NiR). The resulting NH_4^+ is converted to glutamine (Gln) and glutamate (Glu) by glutamine synthetase (GS) followed by the action of glutamate synthase (GOGAT). Cytoplasmatic Glu is transaminated to aspartate (Asp) by aspartate aminotransferase (Asp-AT). Finally, aspartate is converted into asparagine (Asn) by the action of asparagine synthetase (AS), which is then used for the synthesis of other amino acids (AA). Adapted from Taiz & Zeiger (2010).

Ammonium derived from uptake by plant roots is usually assimilated near the site of absorption, where it can be rapidly stored in the vacuoles in case of excessive amounts were taken up (Taiz & Zeiger, 2010). Several routes have been described for the assimilation of this cation (Taiz & Zeiger, 2010). Among these , the GS/GOGAT pathway is considered the main route for NH_4^+ assimilation in plants (Coruzzi, 2015). Figure 1-4A shows the assimilation of NH_4^+ through GS and GOGAT, where ammonium is converted to Gln and Glu (Hofman & van Cleemput, 2004). According to Taiz & Zeiger (2010), Glu is first combined with NH_4^+ by the action of GS to form Gln. Increased levels of Gln stimulate the activity of GOGAT, which transfers the amide group ($-NH_2$) of Gln to 2-OG (2-oxoglutarate) resulting in two molecules of Glu. In the other pathway, GDH catalyzes the conversion of Glu to NH_4^+ and 2-OG, which is a reversible process (Figure 1-4B; Taiz & Zeiger, 2010). Once ammonium is assimilated either into Gln or Glu through one of these pathways, N is incorporated into other organic compounds via transamination reactions (Sirko & Brodzik, 2000; Hofman & van Cleemput, 2004; Taiz & Zeiger, 2010).

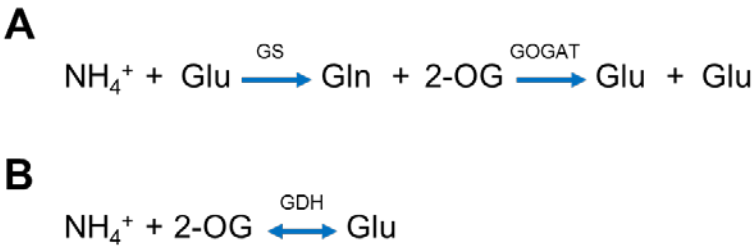

Figure 1-4. Ammonium assimilation in plant cells is mediated by the action of different enzymes. Two different pathways catalyze the assimilation of ammonium (NH_4^+). **(A)** In the GS/GOGAT pathway, NH_4^+ is combined with glutamate (Glu) to form glutamine (Gln) by the action of glutamine synthetase (GS). Afterwards, glutamate synthase (GOGAT) transfers the amide group of Gln to 2-oxoglutarate (2-OG) yielding two molecules of Glu. **(B)** NH_4^+ is converted with 2-OG to one molecule of Glu by the action of glutamate dehydrogenase (GDH). Depending on the glutamate and ammonium availability, GDH can also catalyze the deamination of glutamate. Adapted from Taiz & Zeiger (2010).

In the case of urea, it is important to note that this compound is present in plants not only as a consequence of its uptake by roots from the soil solution (Klein & Tauböck, 1927; Witte, 2011) but also as a result of N metabolism (Witte, 2011). Urea is present in plants in the micro- to millimolar range (Bohner et al., 2015), deriving from two major biochemical pathways, namely from arginine (Arg) degradation and from ureide catabolism (Sirko & Brodzik, 2000; Follmer, 2008; Wang et al., 2008). With regard to the major biochemical pathways that generate urea in plants, Arg catabolism including the arginase reaction is reported as the major urea-synthesis route in plants (Gerendás et al., 1999), taking place in mitochondria (Hawkesford et al., 2012). The corresponding pathway (Figure 1-5) has been also described as ornithine (Orn) or urea cycle (Gerendás et al., 1999; Kojima et al., 2006) and is of general importance for urea synthesis (Broadley et al., 2012). After protein degradation, arginine (Arg) which is an important metabolite involved in the transport and storage of nitrogenous compounds in plants, is catabolized in mitochondria into Orn and urea by the action of arginase (Polacco & Holland, 1993; Hildebrandt et al., 2015). Arginase (EC 3.5.3.1) is the only enzyme in higher plants able to generate urea *in vivo* (Witte, 2011). Arginase-derived urea is then exported to the cytosol (Mérigout et al., 2008; Hawkesford et al., 2012), probably through an AQP (Witte, 2011; ref. to section 1.2.2). Once in the cytosol, urea needs to be maintained at low concentrations to avoid toxicity (Broadley et al., 2012) and therefore is rapidly hydrolyzed to NH_4^+ by endogenous urease (Mérigout et al., 2008; Kojima et al., 2006). Finally, the resulting NH_4^+ is re-assimilated by cytosolic GS using Glu from Orn catabolism as substrate, and Arg-N is incorporated into Gln (Witte, 2011).

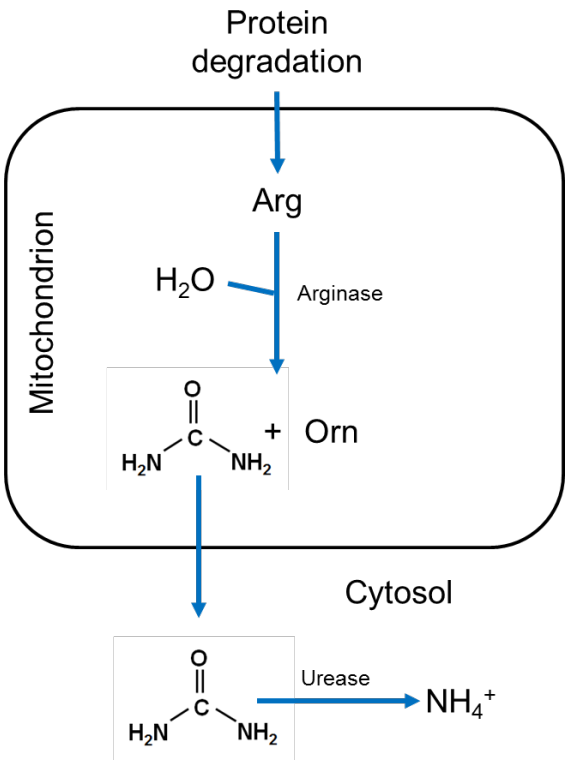

Figure 1-5. The arginase reaction generates urea in higher plants. After protein degradation, arginine (Arg) in mitochondria is converted into urea ($CO(NH_2)_2$) and ornithine (Orn) by the action of the enzyme arginase. Arginase-derived urea is then exported to the cytosol and rapidly hydrolyzed by endogenous urease to ammonium (NH_4^+), which is subsequently re-assimilated. Adapted from Witte (2011).

The other major biochemical pathway for urea biosynthesis in higher plants results from the catabolism of purines or ureides, finding among them the intermediates allantoin and allantoate (Todd & Polacco, 2006; Witte, 2011). These ureides represent the major N transport form employed by some leguminous plant species and are used mainly for long-distance translocation of N (Stebbins & Polacco, 1995; Mérigout et al., 2008). For instance in some tropical N_2-fixing legumes like soybean and cowpea, ureides play a crucial role in transporting organic nitrogenous compounds to the shoots (Todd et al., 2006). Nonetheless, it has been also reported that the transport of hghly aminated metabolites can be carried out in the form of Asn and Gln (Todd et al., 2006; Todd & Polacco, 2006). Regarding the intermediates involved in the catabolism of ureides (Figure 1-6), degradation of nucleic acids generates purine in plant cells, which is then converted into xanthine by the action of nucleotidases or nucleosidases. Xanthine dehydrogenase catalyzes the conversion of xanthine into uric acid in the cytosol (Long et al., 2015). Later, uric acid is catabolized to allantoin, which is subsequently degraded to allantoate by allantoinase in the endoplasmic reticulum. Allantoin and allantoate are released into the xylem and transported to shoots, where urea is formed but rapidly catabolized to ammonium (Taiz & Zeiger, 2010; Long et al., 2015). Interestingly, urease may not be required for allantoin breakdown (Gerendás et al.,1999). Finally, urea-derived NH_4^+ can be assimilated by the GS/GOGAT cycle as described before (Mérigout et al., 2008; Figures 1-3 and 1-4).

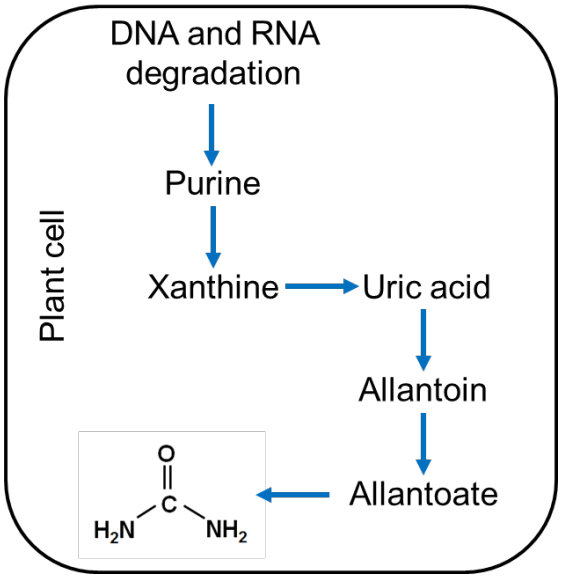

Figure 1-6. Ureide catabolism as another major biochemical pathway that generates urea in higher plants. In root and shoot cells, DNA and RNA degradation generates purine. Xanthine is formed from purine by the action of nucleotidases or nucleosidases. Uric acid is synthesized from xanthine and catabolized to allantoin and allantoate. Allantoate generates urea, which is finally degraded to ammonium and further assimilated by the GS/GOGAT cycle. Adapted from Wang et al. (2008).

1.3 Importance of urease for plants and implications associated to its enzymatic activity

1.3.1 The enzyme urease and its activity in soil and role in plant metabolism

Agricultural soils are biologically active and therefore urea-N from fertilization can be rapidly degraded (Zobel et al., 2015). Because urea is the most frequently used N fertilizer worldwide (ref. to section 1.2.1), its enzymatic hydrolysis is a process of great agricultural importance (Follmer, 2008). Typically, urea is degraded within a few days after its application to soils (Watson, 2000), which will depend mainly on the climatic conditions as well as on certain soil properties. Urea hydrolysis is carried out by urease (EC 3.5.1.5; urea amidohydrolase), which is a metalloenzyme that catalyzes its conversion to carbon dioxide (CO_2) and ammonia (NH_3) (Gerendás et al., 1999; Sirko & Brodzik, 2000; Follmer, 2008). However, this process can be also catalyzed by urea amidolase (Wang et al., 2008; Krajewska, 2009; Kumar, 2015).

Plant urease was first discovered in soybean (*Glycine max* L. Merr.) more than 100 years ago by Takeuchi in 1909 (Krajewska, 2009), and it was the first enzyme to be purified from the same plant species by Sumner in 1926 (Sumner, 1926). Urease is biosynthesized in many different organisms (Wang et al., 2008), occurring not only in plants, bacteria and fungi (Sachs et al., 2006; Shah & Soomro, 2012) but also in algae and invertebrates (Sirko & Brodzik, 2000; Krajewska, 2009), being therefore described as an ubiquitous enzyme (Sirko & Brodzik, 2000; Zobel et al., 2015). Since urease activity occurs widely in soil and aquatic environments (Mobley & Hausinger, 1989), it is not rare that urea concentrations in natural soils or in lakes are usually low (Kojima et al., 2006). Aside from its crucial role in nitrogen catabolism after protein degradation (Sirko & Brodzik, 2000; Wang et al., 2008), urease has been shown to play an important role in N metabolism during germination, allowing the utilization of seed-protein reserves during this early developmental stage (Polacco & Holland, 1993; Sirko & Brodzik, 2000). There, urea may even have a role as defense compound against phytopathogens, due to its insecticidal activity (Kumar, 2015; Follmer, 2008).

Urease is assumed to be located only in the cytosol (Sirko & Brodzik, 2000), since some proteins required for its activation are found to be cytosolic (Witte, 2011). Although urea is a readily-available substrate for its activation (Krajewska, 2009), urease is not necessarily induced by this compound as shown in potato leaves (Witte et al., 2002). Its activation is a complex process, which requires several accessory proteins (Follmer, 2008) and depends essentially on nickel (Ni) (Polacco & Holland, 1993; Follmer, 2008). In fact, urease is the only known Ni-containing enzyme in plants (Taiz & Zeiger, 2010) and up to date this is the only essential function of Ni proven in higher plants (Brown et al., 1987). Regarding the structure of urease (Figure 1-7A), this enzyme contains two Ni atoms in its active center (Zobel et al., 2015). According to Krajewska (2009), both Ni atoms are bridged by a carbamylated lysine (Lys). Furthermore, one atom of Ni is pentacoordinated and the other one is hexacoordinated, both bound to two molecules of histidine (His), respectively. Additionally, the hexacoordinated Ni is bound to one molecule of aspartate (Asp). Both Ni ions are bridged by an hydroxyl group and each Ni atom binds one molecule of water (H_2O). Finally, another molecule of H_2O is located towards the opening of the active site (Figure 1-7B), where substrates like urea or other analogue molecules are typically bound.

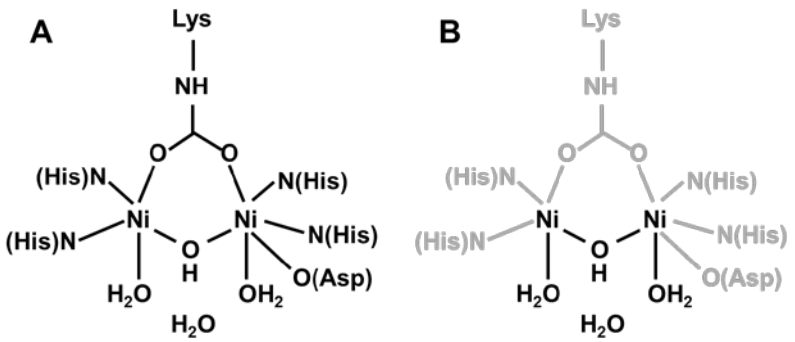

Figure 1-7. Chemical structure of the urea binding site in the metalloenzyme urease. (A) In its centre, urease contains two nickel (Ni) atoms, which are bridged by a carbamylated lysine (Lys). Furthermore, the pentacoordinated Ni ion (on the left) is bound to two molecules of histidine (His), whereas the hexacoordinated ion (on the right) is additionally bound to one molecule of aspartate (Asp). Both Ni ions are bridged by an hydroxyl group and each one of them binds one molecule of water (H_2O). Another molecule of H_2O is located towards the opening of the active site, as indicated in bold in **(B)**. Adapted from Krajewska (2009).

Urease activity in soils correlates positively with microbial biomass and therefore depends mainly on organic matter and water content (Zanin et al., 2015). In plants, enzymatic hydrolysis of urea occurs in the same way as in soils (Figure 1-8). First, urea is hydrolyzed to one molecule of NH_3 and carbamate (Sachs et al., 2006). Subsequently, due to the unstability of carbamate (Witte et al., 2002), a second non-enzymatic reaction occurs (Witte, 2011) in which carbamate is spontaneously decomposed yielding CO_2 and a second molecule of NH_3 (Mobley & Hausinger, 1989; Berlicki et al., 2012). As a consequence of this hydrolysis in soils, NH_4^+ can be adsorbed by the soil matrix, be maintained as exchangeable cation, be converted into NO_3^- during the nitrification process or be volatized as NH_3 (Zanin et al., 2015). Thereby, the release of NH_3 as a volatile product of the urease reaction (Witte et al., 2002) may result in adverse effects for agriculture and for the environment (ref. to following section 1.3.2; Upadhyay, 2012; Kumar, 2015).

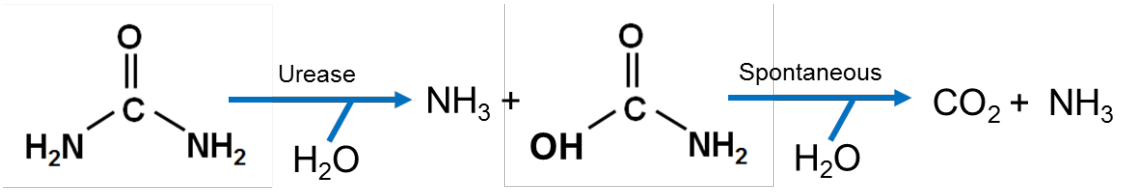

Figure 1-8. Enzymatic hydrolysis of urea by action of the metalloenzyme urease. Urease catalyzes the hydrolysis of urea, yielding ammonia (NH_3) and carbamate. This process is followed by the spontaneous hydrolysis of carbamate to carbon dioxide (CO_2) and a second molecule of ammonia. Adapted from Hausinger (2004) and Witte (2011).

1.3.2 Agricultural implications of urease activity

After soil application of urea-N fertilizers and upon enzymatic urea hydrolysis by urease, protons from the soil solution are consumed (Watson, 2000) triggering a pH increase. The extent of this increase depends on the soil buffer capacity (Hofman & van Cleemput, 2004) but also on soil temperature and its water content (Koelliker & Kissel, 1988). This transient rise in pH may lead to NH_3 volatilization (Bremner, 1995), which can be potentially high if the soil does not have enough adsorption capacity to bind the released NH_3 in the form of NH_4^+ (Hucke et al., 2009) and if urea fertilizers are surface applied (Hofman & van Cleemput, 2004). Since urea-N is widely used in agriculture, ammonia emissions can result in a relevant source of pollution (Buerkert et al., 2012) not only as a consequence of N fertilization but also when generated from livestock waste (Krajewska, 2009). Then, ammonia emissions cause damages to human and animal health as well as to the vegetation and eutrophies water bodies (Ni et al., 2018). High NH_3 concentrations may negatively affect seed germination and the development of plant seedlings under unfavorable climatic conditions (Krogmeier et al., 1989a; Bremner, 1995; Hucke et al., 2009).

Different approaches have been proposed to reduce the negative implications of urea hydrolysis in agriculture. For instance, the first proposed idea was to minimize or prevent the adverse effects of NH_3 volatilization by its immediate incorporation into the soil irrigation or tillage (Sommer et al., 2004) (Hofman & van Cleemput, 2004; Jones et al., 2007). In fact, after application and incorporation of urea at 10 cm depth in a silt loam soil, ammonia losses were reduced to 0% of applied N (Rochette et al., 2013). Nonetheless, a mechanical incorporation may disturb the crop and in some cases, it is not economically reasonable (Schraml et al., 2016). Other approches to reduce these problems refer to the coating of urea fertilizers with sulfur to reduce the dissolution rate of fertilized granules or they refer to the acidulation of urea fertilizers by using phosphoric or nitric acid (Bremner, 1995). Jones et al. (2007) referred also to the possibility of avoiding urea fertilizer application, preferring instead other N fertilizers like CAN, UAN (urea ammonium nitrate) or ammonium sulfate ($(NH_4)_2SO_4$), while Bremner (1995) insisted on the use of urea fertilizers by proposing the treatment of urea granules in combination with inorganic salts. However, all these methods are not widely used. Because urea fertilizers are generally surface applied, an approach which is receiving considerable attention in the past 30-45 years (Bremner, 1995; Watson, 2000) is the addition of urease inhibitors (ref. to following section 1.4) for a controlled release of N from urea-containing fertilizer granules (Jones et al., 2007). When combined with urea fertilizers, urease inhibitors can decrease soil urease activities and thus retard urea hydrolysis and subsequent losses by N volatilization (Bremner, 1995; Hofman & van Cleemput, 2004).

1.4 Urease inhibitors designed for agriculture

In well-drained soils, urea is usually hydrolyzed within 10-14 d at most (Hofman & van Cleemput, 2004). Since urea is the most used N fertilizer worldwide (ref. to section 1.2.1), it is crucial to develop solutions that reduce its hydrolysis and subsequent N losses by ammonia (Hucke et al., 2009). One approach has been the search for compounds that inhibit the urease activity (Freney et al., 1993). By retarding the activity of soil urease at the soil surface, urea could reach deeper soil layers before its hydrolysis (Phongpan et al.,1995; Hucke et al., 2009), and thus been taken up by plant roots as an intact molecule (Zanin et al., 2015, ref. to section 1.2.2). Hence, ammonia emissions may decrease (Freney et al., 1993) as ammonia can be easier buffered and adsorbed to the soil matrix in form of ammonium (Hucke et al., 2009). Such compounds are known as urease inhibitors (UIs), and when applied together or combined with urea fertilizers (UIs can efficiently inhibit the urease activity for 1-8 weeks depending on the environmental conditions (Zobel et al., 2015; Abalos et al., 2014). The application of UIs has shown to be a successful strategy to reduce ammonia losses (Wang et al., 2008; Zanin et al., 2015; Schraml et al., 2016). and to improve crop productivity and nitrogen use efficiency (NUE). For instance, a meta-analysis reported a grand mean increase of approx. 8 and 13% for both crop productivity and NUE, respectively (Abalos et al., 2014).

In recent years, the use of UIs has become of great importance for agriculture. With introduction of the new fertilizer ordinance in Germany starting from 1st February 2020, the use of urea fertilizers will require co-application of UIs or immediate incorporation (within < 4 h) (BGBl, 2017). Therefore, the search and development of new UIs are of particular interest and must meet several requirements, like a cost-effective production and an adequate storage stability. In addition, UIs must not interact with urea (Zobel et al., 2015), they must be safe for the environment, not be phytotoxic, be highly active and specific for urease, be stable and thus non-volatile and their solubility and diffussivity must be similar to those of urea (Watson, 2000). Depending on the type of UI, UIs usually retard urea hydrolysis by blocking the active center of the urease (ref. to section 1.3.1; Schraml et al., 2016). The latter can be carried out by interaction with a key functional group of urease, changing the conformation of its active site (Watson, 2000) or by forming a chelated complex with one of the Ni ions located in the active site of the urease (Upadhyay, 2012).

Up to date, more than 14.000 different compounds have been tested and some of them patented or proposed as UIs, mainly in USA and Germany (Bremner, 1995; Watson, 2000; Kiss & Simihăian, 2002; Hucke et al., 2009). According to Krajewska (2009), the best investigated among them are i) urea analogues like hydroxyurea, formamide, thiourea, ethylurea or methylurea, ii) thiols like β-mercaptoethanol, iii) acylhydroxamic acids like the acetohydroxamic acid, iv) amides and esters of phosphoric acid (ref. to following section 1.4.1), v) boron compounds like boric acid, butylboronic acid, phenylboronic acid or 4-bromophenylboronic acid, vi) heavy metal ions like Hg^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Pb^{2+} or Co^{2+}, vii) bismuth compounds like Bi(EDTA) or $Bi(Cys)_3$ and viii) quinones like 1,4-benzoquinone, 2,5-dimethyl-1,4-benzoquinone or tetrachloro-1,4-benzoquinone. However, different authors classify these different UIs into four major categories according to their chemical structures (Mobley & Hausinger, 1989; Watson, 2000; Upadhyay, 2012), namely i) sulphydryl reagents like sodium chloride or fluoride ions, which react with mercapto groups (Watson, 2000), ii) hydroxamates like caprylohydroxamic acid, which forms a complex with one Ni ion of the active site (Mobley & Hausinger, 1989; Watson, 2000), iii) thiolic compounds, which react directly with the active center of the enzyme (Upadhyay, 2012) and iv) structural analogues of urea, which have similar structures to urea, compete for the same active site (Watson, 2000) and have been considered as the most effective UIs (Upadhyay, 2012).

1.4.1 Experiences with most effective urease inhibitors

On the search for potential UIs to reduce the rate of urea hydrolysis, investigations classified the group of amides and esters of phosphoric acid as the strongest urease inhibitors (Krajewska, 2009). Among them, the most potent soil UIs are the phosphorodiamidates, phosphorotriamides and thiophosphorotriamides (Watson, 2000; Berlicki et al., 2012), which all are structural urea analogues (Watson, 2000; ref. to section 1.2), bearing a phosphorus (P) atom instead of the carbon (C) in the urea molecule. To this group belong the UIs N-(n-butyl)thiophosphoric triamide, commonly known as NBPT or NBTPT and phenylphosphorodiamidate, commonly known as PPD or PPDA (Figure 1-9; Watson, 2000).

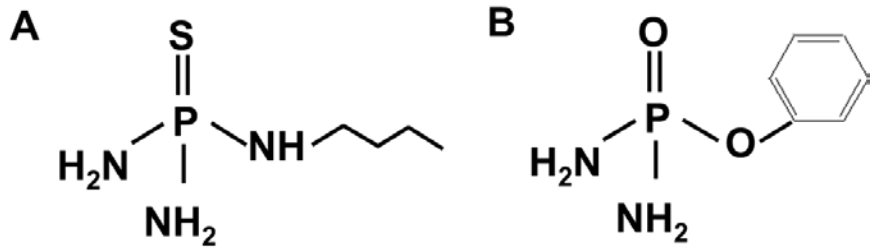

Figure 1-9. Chemical structures of most common urease inhibitors belonging to amides and esters of phosphoric acids family. Structures of two common urease inhibitors from the thiophosphorotriamide and phosphorodiamidate family. **(A)** N-(n-butyl) thiophosphoric triamide, commonly known as NBPT or NBTPT and **(B)** phenylphosphorodiamidate, commonly known as PPD or PPDA. Adapted from Krajewska (2009) and Zobel et al. (2015).

The urease inhibitor NBPT was developed in USA in 1984 (Zobel et al., 2015) and was first introduced to the market more than 20 years ago by the International Minerals and Chemical Corporation IMC Global Inc. (Watson, 2000), being the only UI commercially available and sold under the trade name of Agrotain® (Koch Agronomic Services LLC, Wichita, USA) (Watson, 2000; Hucke et al., 2009; Silva et al., 2017). Due to its instability (Hucke et al., 2009), NBPT is formulated as a green liquid, which must be combined with the urea fertilizer prior to its application (Watson, 2000). To block the active center of the urease, this thiophosphoric triamide needs to be converted to its oxygen analogue N-(n-butyl) phosphoric triamide (NBPTO), which actually carries out the urease inhibition (Hendrickson & Douglass, 1993; Watson, 2000). It is assumed that the latter also causes difficulties for the effectiveness of this UI. In fact, NBPT has shown to be less effective in retarding urea hydrolysis in rice grown in flooded soils because there was not sufficient oxygen for its conversion; thus urea was hydrolyzed before NBPT conversion to NBPTO (Freney et al., 1993; Phongpan et al., 1995). Nonetheless, numerous reports have shown its benefits. For instance, a meta-analysis that included 47 reviewed studies confirmed its effectiveness in different crops by slowing down urea hydrolysis, reducing ammonia volatilization and thus enhancing N availability (Silva et al., 2017). Furthermore, after testing this UI in 16 different soils with a wide range of different chemical and physical properties, NBPT showed also to reduce NH_3 losses, with an observed inhibitory effect even 9 d after application (Watson et al., 1994). Its inhibitory effect has been found to act for up to 14 d (Shah & Soomro, 2012). Nevertheless, its effectiveness appeared to be conditioned by some soil properties like organic matter content and pH as well as the soil potential for NH_3 losses (Watson et al., 1994; Silva et al., 2017), probably due to the need of conversion to its oxygen analogue (Watson et al., 1994). Among different inhibitors reviewed in 12 different studies, which included also some nitrification inhibitors, a meta-analysis showed that NBPT had the strongest effect on yield, promoting it by approx. 10% (Abalos et al., 2014). However, this positive effect obtained with NBPT may not be consistent in all soils, especially when N is not a limiting factor (Silva et al., 2017). Aside from the inconvenience of its required conversion to NBPTO, other drawbacks have been reported for this UI. For example, Zanin et al. (2015) observed in hydroponically-grown maize that, when NBPT was present in the nutrient solution, urea uptake capacity was inhibited, probably as a consequence of the competition between NBPT and urea.

On the other hand, PPD was the first UI developed by SKW Stickstoffwerke Piesteritz GmbH in Germany in 1979, which was never commercially available in the market (Zobel et al., 2015), probably because it was not agronomically economical (Byrnes et al., 1983). Different studies reported beneficial effects also after application of this UI. For instance, by testing the retardation of urea hydrolysis by application of PPD to 15 soils, it was observed that urea hydrolysis was substantially retarded in all soils 2 d after its application and in seven of these soils even up to 10 d after addition of this UI (Martens & Bremner, 1984). Moreover, the addition of PPD to urea fertilizer applied to flooded soils has been shown to delay urea hydrolysis and to reduce NH_3 volatilization losses considerably (Byrnes et al., 1983). However, the addition of this UI to urea fertilizers has been shown also to have some drawbacks for certain crops, such as an increase of necrotic lesions in soybean leaves (Krogmeier et al., 1989a, b). Therefore, there is still a need to develop other UIs with better performances than these previously synthesized amides or esters of phosphoric acid (NBPT or PPD). Notably, UIs should not only be effective in different soils and show better stability but also not damage crops. Finally, the use of these new UIs should be economically reasonable. Currently, costs for NBPT treatment per ton of urea mount up to 66-68 US $ t^{-1} urea (Watson, 2000).

1.4.2 The urease inhibitor 2-NPT

While the development of new UIs with better effectiveness, lower production costs and less harmful effects on crops and the environment require several years of data collection for their registration and authorization (Watson, 2000), different UIs have been developed and tested in Germany between 2001 and 2006. Among others, these efforts resulted in the introduction of N-(2-nitrophenyl) phosphoric acid triamide (2-NPT, $C_6H_9N_4O_3P$) as one of the most promising UIs (Schraml et al., 2016) due to its significant effect on the reduction of NH_3 emissions from urea-fertilized soils (Ni et al., 2014; Schraml et al., 2016). This novel UI, which has been developed by SKW Stickstoffwerke Piesteritz GmbH in 2004 (Zobel et al., 2015; Ni et al., 2018), belongs to the group of amides and esters of phosphoric acid (ref. to section 1.4.1) and thus is also a structural analogue of urea (Figure 1-10).

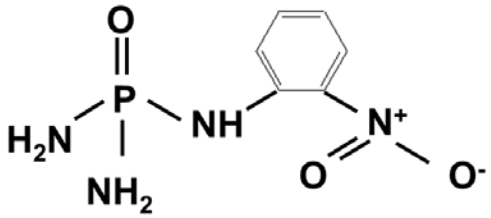

Figure 1-10. Chemical structure of the urease inhibitor 2-NPT. Chemical structure of the urease inhibitor N-(2-nitrophenyl) phosphoric acid triamide (2-NPT, $C_6H_9N_4O_3P$). A central phosphorus atom binds to three amides, and one of them is coordinated by a nitrophenyl group. Adapted from Zobel et al. (2015).

As a new UI, 2-NPT has been approved in Germany as well as in the European Union and introduced to the market under the trade name of PIAZUR® (Zobel et al., 2015), and since autumn 2016 2-NPT is commercialized as PIAGRAN® pro. This fertilizer combines urea (46% as ureic N) with the urease inhibitor 2-NPT, which has been formulated as a yellow powder (SKW Stickstoffwerke Piesteritz GmbH, 2018) coating the urea granules at low density (Zobel et al., 2015), i.e. to 0.075% based on the total N amount. The addition of 2-NPT to urea fertilizers is easily carried out by conventional methods (Hucke et al., 2009), and the resulting granules have an average diameter of 3.5 mm (SKW Stickstoffwerke Piesteritz GmbH, 2018). Treating urea granules with 2-NPT implies a final cost of approx. 40-50 € t^{-1} above the commercial price of urea alone (Hahn, 2018, personal communication[1]). 2-NPT is characterized by a high storage stability (Zobel et al., 2015; Schraml et al., 2016; SKW Stickstoffwerke Piesteritz GmbH, 2018), even higher than that of NBPT (Ni et al., 2018). Up to date, there are no available reports about the specific mode of action of 2-NPT and its action as a structural analogue of urea within the active site of urease. However, it is known that its mode of action is carried out by blocking the active center of urease (SKW Stickstoffwerke Piesteritz GmbH, 2018; ref. to section 1.3.1), likely in a similar way as described recently for NBPT (Mazzei et al., 2017). It is assumed that 2-NPT is degraded later without triggering adverse effects on urease (Ni et al., 2018).

Despite of the fact that 2-NPT was designed as an UI not only to reduce N losses after co-application with urea fertilizers, but also to reduce the NH_3 emissions from livestock wastes (Hucke et al., 2009), many studies conducted in the recent years focused on plant production. For instance, Ni et al. (2014) observed a significant reduction (up to 89%) in NH_3 emissions after application of urea + 2-NPT to winter wheat, which were similar to the emissions reached after application of CAN. Although 2-NPT has been reported to retard urea hydrolysis up to 14 d (SKW Stickstoffwerke Piesteritz GmbH, 2018), an incubation study has shown that its inhibitory effect, measured by reducing NH_3 emissions (approx. 90% compared to urea alone), lasted even up to 19 d after its application (Ni et al., 2018). Moreover, these authors observed that urea-N was still present even after 20 d in the soil treated with 2-NPT. In a different study, Schraml et al. (2016) observed promoted grass yield after fertilization with 2-NPT as a consequence of retarded urea hydrolysis and thus mitigated ammonia losses by 70-100% right after application for up to 7 d.

Taken together, co-application of 2-NPT to urea fertilizers appears to be a highly promising alternative to avoid the rapid hydrolysis of urea and to enhance N availability to crops. Nevertheless, several open questions need to be addressed, when using this novel UI. Among these questions, it is important to know how an increased urea uptake

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by crops will affect their growth and yield formation, whether plant-N metabolism will be affected or how overall N uptake and translocation are altered by the enhaced uptake of urea. Interesting to know is also whether the UI can be taken up by plant roots, and if this is the case, to address whether 2-NPT will accumulate in plant tissues and affect N metabolism. Finally, there is the possibility that grain yield or protein concentrations in grains of cereals will be promoted by the addition of this UI.

1.5 Aim of this study

To date, studies on 2-NPT were almost exclusively conducted by the German fertilizer company SKW Stickstoffwerke Piesteritz GmbH in Lutherstadt Wittenberg that focused mainly on the agronomic performance of this new UI (Hucke et al., 2009). In field experiments with winter wheat or ryegrass, 2-NPT has been described to effectively reduce urea hydrolysis and subsequent NH_3 emissions (Ni et al., 2014; Schraml et al., 2016). However, little is known about the physiological effects of 2-NPT on N uptake and N metabolism in plants, considering that 2-NPT is a urea analogue and that urea is continuously synthesized in plants via the arginase reaction (Witte, 2011; ref. to section 1.2.3). Since Zanin et al. (2015) reported an inhibition of the urea uptake capacity of maize roots after application of NBPT, another structural analogue of urea, the question arose whether 2-NPT is also affecting N uptake and N translocation in cereals and whether it can be taken up by roots.

The first chapter of the present thesis presents field experiments conducted with winter wheat at two different locations in Germany during three subsequent cropping years to clarify i) to what extent the fertilization of urea with the UI alters the total N uptake, ii) how the UI influences N metabolism and plant growth, and iii) which are the effects of an enhanced urea-based N nutrition on yield formation and grain quality of field-grown winter wheat. Thus, the overall aim of this first set of investigations was to study the effects of a urea fertilizer in combination with 2-NPT on growth, development, metabolism, yield and grain quality of wheat. In the second chapter, investigations were carried out with hydroponically-grown spring barley to address the questions how 2-NPT interferes with N uptake and N translocation under urea nutrition and whether there are short-term effects of 2-NPT on the uptake capacity of different N forms. For this purpose, ^{15}N-labeled N forms were employed.

CHAPTER 1

Impact of urea fertilization in combination with urease inhibitor 2-NPT on physiological parameters, yield and N uptake of winter wheat

CHAPTER 1: IMPACT OF UREA FERTILIZATION IN COMBINATION WITH UREASE INHIBITOR 2-NPT ON PHYSIOLOGICAL PARAMETERS, YIELD AND N UPTAKE OF WINTER WHEAT

2 CHAPTER 1: INTRODUCTION

In recent years urease inhibitors (UI) have become very important for nitrogen (N) fertilization, since they can delay urea hydrolysis ($CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2NH_3$), a process which takes place in the presence of water, where urea is rapidly hydrolyzed to ammonium (NH_4^+) by the activity of ubiquitous soil urease (urea amidohydrolase, EC 3.5.1.5) (Martens & Bremner, 1984; Sirko & Brodzik, 2000). Urease is a nickel-dependent enzyme (Watt & Ludden, 1999; Boer et al., 2014), which is known to be very stable in soils even after the decay of soil microorganisms (Liu et al., 2003a). Urea is the most dominant form of mineral N fertilizer used worldwide (Follmer, 2008; IFA, 2017). However, surface-applied urea is vulnerable to losses by ammonia (NH_3) volatilization (Watson et al., 1994). Depending on the pH surrounding the urea granules and a range of other factors, NH_4^+ may be transformed and emitted in the volatile form NH_3 (Hofman & van Cleemput, 2004). Urease inhibitors can reduce the concentration of NH_4^+ in the soil solution resulting in a lower potential for NH_3 volatilization and a higher N availability for the plants (Trenkel, 2010; Cruchaga et al., 2011).

Owing to the need to reduce NH_3 emissions after application of urea fertilizers (Phongpan et al., 1995; Zanin et al., 2015), different UI have been patented and tested so far in several crop species (Gill et al., 1999). It has been reported, that most effective UIs belong to the group of amides and esters of phosphoric acid (Krajewska, 2009), finding among them NBPT (N-(n-butyl)thiophosphoric triamide), which has been deeply studied in view of the fact that it was the first commercial UI sold worldwide to reduce urea hydrolysis (Gill et al., 1999; Shah & Soomro, 2012; Zanin et al., 2015; Silva et al., 2017). The efficacy of NBPT was tested recently by Zanin et al. (2015), who conducted hydroponic experiments with maize observing that urea concentrations in roots and shoots were significantly increased when this UI was supplied to the nutrient solution in the presence of urea. Other UIs, like PPD (phenylphosphorodiamidate), have been also intensively studied, resulting in the same way in increased urea concentrations in soybean leaf tissues as reported by Krogmeier et al. (1989a, 1989b) and in retarded urea hydrolysis followed by a reduction of NH_3 emissions in rice (Byrnes et al., 1983). Nevertheless, these two UIs, considered as the most successful UIs developed so far (Phongpan et al., 1995), showed also negative responses in some plants. Cruchaga et al. (2011) reported the appearance of leaf-tip scorch and necrosis in pea plants, associated with an increased urea concentration in leaves after addition of NBPT. Moreover, the addition of NBPT to the nutrient solution repressed the uptake rate of urea and N accumulation in maize plants as observed by Zanin et al. (2015). Thus, new UIs are required to cover an increasing demand for stabilized urea-based fertilizers, which should be also effective by inhibiting the activity of the urease, allowing plants to take advantage of a longer presence of urea in the soil.

Recently, a new UI named N-(2-nitrophenyl)-phosphoric acid triamide (2-NPT) has been developed by the fertilizer company SKW Stickstoffwerke Piesteritz GmbH in Lutherstadt Wittenberg (Hucke et al., 2009). Recent studies with 2-NPT reported mitigation of NH_3 losses up to 89% after application of this UI, when compared to application of urea alone (Ni et al., 2018). Similarly, a previous investigation carried out in field conditions with perennial ryegrass reported that addition of 2-NPT combined with a urea fertilizer successfully inhibited soil urea hydrolysis, thus reducing ammonia volatilization losses by 70-100% after using UI concentrations between 0.75 and 1.5 g 2-NPT kg^{-1} soil (Schraml et al., 2016). Also Ni et al. (2014) conducted field experiments but with winter wheat over three years to evaluate the reduction of NH_3 emissions by 2-NPT after application of urea and reported 48-89% lower NH_3 volatilization when compared to unamended urea treatment. Nonetheless, little is known about the physiological effects of the UI 2-NPT in plants. Hence, in this first chapter results will be presented of field experiments conducted over three cropping seasons at two different locations in Germany. Experiments were designed to investigate the impact of urea-based N fertilization in combination with the new UI 2-NPT on plant-N metabolism, phytohormone regulation, grain yield formation and grain quality of field-grown winter wheat.

3 MATERIALS AND METHODS

3.1 Plant material and growth conditions

3.1.1 Plant material

Winter wheat (*Triticum aestivum* L.) cv. Akteur, which has been produced by the Deutsche Saatveredelung AG (Wusterhausen/Dosse, Germany) and introduced in the market in 2003 was chosen due to its high grain yield and protein concentration as well as to its proven winter hardness and pathogen tolerance, allowing cv. Akteur to be sown late (Gienapp et al., 2012; Sacher et al., 2014; Deutsche Saatveredelung AG, 2016).

3.1.2 Experimental site description

Field experiments were carried out over 3 subsequent winter wheat seasons (2012/13, 2013/14 and 2014/15) at two German locations: i) Cunnersdorf in Saxony (51°21'56" N, 12°33'41" E) by SKW Stickstoffwerke Piesteritz GmbH and ii) Gatersleben in Saxony-Anhalt (51°49'23" N, 11°17'13" E) at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK).

Weather conditions

Climatic data have been chronologically recorded for Cunnersdorf (1987-2016) and Gatersleben (1953-2016) with annual average temperatures of 9.4 and 9.0°C and annual precipitations of 612.0 and 490.3 mm, respectively. Weather information was recorded at both experimental sites over the whole cropping season. Data corresponding to soil and air temperature were measured at 2 m above the soil surface. Monthly precipitations are shown in Figure 3-1A for Cunnersdorf and Figure 3-1B for Gatersleben.

At both experimental sites, the first crop year 2012/13 was characterized by a long snowy and cold winter period with average air temperatures measured between January and March of -0.5°C and -0.4°C at Cunnersdorf and Gatersleben, respectively. Consequently, this triggered a later start in the vegetation period, wherein precipitations as snow or as rainwater during this first season were 64% and 19% higher in Cunnersdorf and 75% and 33% higher in Gatersleben, when compared with total precipitations during the following seasons 2013/14 and 2014/15, respectively. Moreover, different precipitations during spring were observed in all years, when 2012/13 was a very wet year, 2014/15 was an average year and 2014/15 had a dry spring period at both locations. Similar average air temperatures were recorded at both experimental sites in each cropping season, averaging in the first year 9.0°C and 8.8°C in Cunnersdorf and Gatersleben, respectively, 10.5°C at both locations during the second year, while in 2014/15 10.7°C and 10.0°C were recorded at Cunnersdorf and Gatersleben, respectively. Elevated precipitations were recorded for Cunnersdorf in 2012/13 being 170 mm higher than in Gatersleben. However, similar amounts of precipitations at both experimental sites were registered during the two following years.

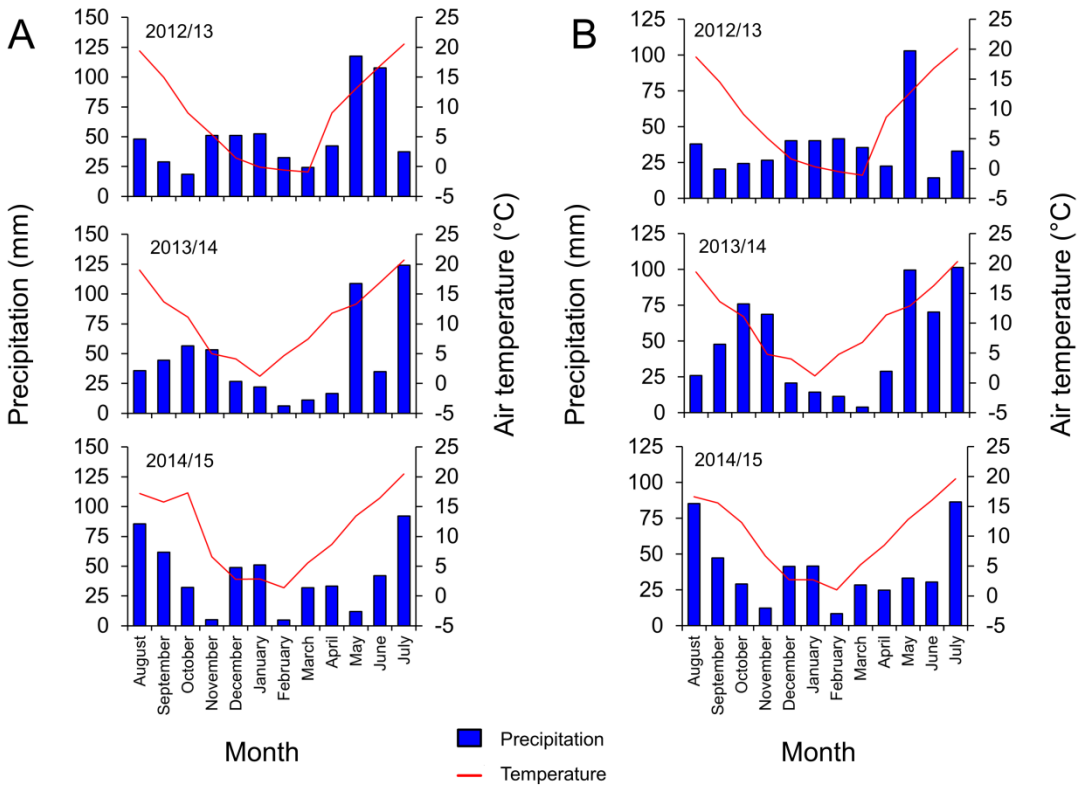


Figure 3-1. Weather data at Cunnersdorf and Gatersleben in 2012/13, 2013/14 and 2014/15. Monthly precipitations and air temperatures in each cropping year are shown in **(A)** for Cunnersdorf and in **(B)** for Gatersleben. Bars and lines indicate precipitations and temperatures, respectively.

Soil properties

Soils in Cunnersdorf consist of 30-60 cm sandy loess, which evolved during the Weichsel Ice Age, covering the stray sand-containing boulder clay, originated from the Saale Ice Age and thus, these soils are classified as sandy clayey to loamy soil type with a soil value of 49-53 (Bräutigam & Kleinstäuber, 1996). In contrast, soils in Gatersleben are mainly black and classified as silty to clayey loam soil type, with a soil value of 70-97. Other differences between both soils are presented in Table 3-1.

Table 3-1. Soil characteristics at Cunnersdorf and Gatersleben sites. Soil urease activity determined according to Tabatabai & Bremner (1972) and Kandeler & Gerber (1988). CEC, cation exchange capacity.

Soil parameter	Location	
	Cunnersdorf	Gatersleben
pH value	6.4	7.0
CEC (cmol kg^{-1})	6.6	21.0
Humus content (%)	1.5	3.0
Organic carbon (%)	1.1	1.8
Urease activity (mg NH_4-N kg $soil^{-1}$ h^{-1})	17.4	16.7

Representative soil samples from 0-30 cm and 30-60 cm depth were taken at both experimental sites before the experiments and analyzed for minerals by certified laboratories (Agrolab Boden- und Pflanzenberatungsdienst GmbH, Oberdorla, Germany; Bio-Chem agrar Labor für biologische und chemische Analytik GmbH, Machern, Germany), as listed in Table 3-2.

Table 3-2. Total mineral contents or concentrations in soils at Cunnersdorf and Gatersleben before and during each winter wheat cropping. Soil samples were collected in Cunnersdorf on 08/Nov./2012, 23/Apr./2014 and 06/Mar./2015. Nevertheless, soil samples for determination of N_{min} at this location were collected on **(A)** 10/Mar./2013, **(B)** on 13/Feb./2014 and **(C)** on 10/Mar./2015. In Gatersleben samples were collected on 27/Feb./2012, 08/Jun./2013, 14/Jan./2014 and 27/Feb./2015. n.d., not determined.

Location / Measured parameters	Cropping year			
	2012	2013	2014	2015
Cunnersdorf				
Total N_{min} (kg N ha^{-1})	n.d.	13.4 **(A)**	19.8 **(B)**	12.6 **I**
Phosphorus (mg 100 g^{-1})	5.3	n.d.	6.5	10.9
Potassium (mg 100 g^{-1})	12.1	n.d.	13.4	14.5
Magnesium (mg 100 g^{-1})	12.4	n.d.	13.5	17.0
Boron (mg kg^{-1})	0.52	n.d.	n.d.	0.45
Copper (mg kg^{-1})	1.7	n.d.	n.d.	1.3
Iron (mg 100 g^{-1})	n.d.	n.d.	n.d.	9.3
Manganese (mg kg^{-1})	77	n.d.	n.d.	30
Sodium (mg kg^{-1})	n.d.	n.d.	n.d.	n.d.
Zinc (mg kg^{-1})	2.9	n.d.	n.d.	1.8
Sulfur (mg kg^{-1})	n.d.	n.d.	n.d.	n.d.
Gatersleben				
Total N_{min} (kg N ha^{-1})	18.0	45.0	55.0	38.0
Phosphorus (mg 100 g^{-1})	14.4	5.9	14.1	11.2
Potassium (mg 100 g^{-1})	6.8	8.6	9.0	15.0
Magnesium (mg 100 g^{-1})	8.0	11.6	7.5	9.4
Boron (mg kg^{-1})	1.27	0.98	n.d.	n.d.
Copper (mg kg^{-1})	4.2	1.6	n.d.	n.d.
Iron (mg 100 g^{-1})	n.d.	2.4	n.d.	n.d.
Manganese (mg kg^{-1})	140	92	n.d.	n.d.
Sodium (mg kg^{-1})	n.d.	10	n.d.	n.d.
Zinc (mg kg^{-1})	n.d.	2.2	n.d.	n.d.
Sulfur (mg kg^{-1})	n.d.	28.3	n.d.	n.d.

3.1.3 Sowing of plant material

Winter wheat seeds were sown using a seed drill machine with 2.5 m working width at Cunnersdorf (Hege PN, Lichtenstein, Germany) and at Gatersleben (Amazone D8 25 spezial, Hasbergen-Gaste by Osnabrück, Germany) in September/October according to common agricultural practice for the chosen cultivar with a sowing density of 280-420 seeds m^{-2} and a depth of approximately 2-4 cm (Landesforschungsanstalt für Landwirtschaft und Fischerei, 2006), as shown in Table 3-3.

Table 3-3. Sowing dates for winter wheat at Cunnersdorf and Gatersleben including sowing density, day of emergence and preceding crops over the 3 cropping seasons.

Cultivation data	Cropping year		
	2012/13	2013/14	2014/15
Cunnersdorf			
Sowing date	02/Oct./2012	01/Oct./2013	30/Sep./2014
Sowing density (seeds m^{-2})	300	300	280
Emergence	15/Oct./2012	11/Oct./2013	09/Oct./2014
Preceding crop	oat	oat	oat
Gatersleben			
Sowing date	25/Oct./2012	28/Oct./2013	15/Oct./2014
Sowing density (seeds m^{-2})	420	420	420
Emergence	01/Nov./2012	04/Nov./2013	20/Oct./2014
Preceding crop	oilseed rape	oilseed rape	oilseed rape

3.1.4 Treatments and fertilization

The field experiments were set up with 4 N treatments corresponding to: i) Control, ii) Ammonium nitrate (NH_4NO_3), iii) Urea or iv) Urea + UI, as shown in Table 3-4. According to the BBCH scale describing phenological stages (Meier et al., 2009), which follows the principle of the code developed for cereals by Zadoks et al. (1974), fertilizers were applied on the soil surface of experimental plots when plants were in the middle of the tillering phase (BBCH 25) and a second fertilization was conducted when plants were starting the stem elongation phase (BBCH 32). Every time 90 kg N ha^{-1} were applied to meet the high N demands of this crop.

Control plants were not fertilized with N, while ammonium nitrate (Grupa Azoty Zakłady Azotowe "Puławy" S.A., Puławy, Poland) that contained 34% N was used as positive control. Urea fertilizer as well as urea containing the UI 2-NPT were supplied by SKW Stickstoffwerke Piesteritz GmbH (Lutherstadt Wittenberg, Germany), and both contained 46% N. The addition of the UI 2-NPT to urea granules was carried out by SKW Stickstoffwerke Piesteritz GmbH at a concentration of 0.075% related to the N concentration of the urea fertilizer. This UI was added by coating 2-NPT formulated as powder onto the surface of urea granules, not affecting the size of the granules, according to Schraml et al. (2016).

Table 3-4. Nitrogen fertilizer application to winter wheat at Cunnersdorf and Gatersleben over the 3 cropping seasons.

Treatments	N fertilization (kg N ha^{-1})	
	BBCH 25	BBCH 32
Control	0	0
NH_4NO_3	90	90
Urea	90	90
Urea + UI	90	90

3.1.5 Crop protection

Prior to sowing, seeds in Cunnersdorf and in Gatersleben were coated with Arena C (Syngenta Agro GmbH, Maintal, Germany) at a dose of 2 mL kg^{-1}, containing 25 g L^{-1} fludioxonil and 5 g L^{-1} tebuconazole to reduce the incidence of pathogens at the early developmental stages.

In the three experimental years, different pesticides were used during crop development against weeds, insects or fungi as well as some growth regulators. Table 3-5 indicates the date of application, the corresponding developmental stage at the day of application, the chemical composition of each product and the dose of the applied chemical.

Table 3-5. Plant protection at Cunnersdorf and Gatersleben over the 3 years of field experiments with winter wheat. Applied pesticides were produced by **(A)** DuPont, Wilmington, USA; **(B)** Bayer AG, Leverkusen, Germany; **(C)** BASF SE, Ludwigshafen am Rhein, Germany; **(D)** Syngenta Agro GmbH, Maintal, Germany; **I** Lotus Agrar GmbH, Mannheim, Germany; **(F)** Adama Agricultural Solutions Ltd., Tel Aviv, Israel; **(G)** KeMiChem-Swiss GmbH, St. Margrethen, Switzerland; **(H)** Cheminova Deutschland GmbH & Co. KG, Stade, Germany; **(I)** UPL limited, Mumbai, India; **(J)** Agro Trade Handelsgesellschaft mbH, Lauschied, Germany; **(K)** Kwizda Agro GmbH, Vienna, Austria.

Date of application	BBCH	Action	Pesticide	Chemical composition	Amount
Cunnersdorf					
24/Oct./2012	12	herbicide	Absolute® M **(A)**	flupyrsulfuron methyl 5.56% + diflufenican 44.4%	180 g ha^{-1}
02/May/2013	31	growth regulator	CCC 720® **(B)**	chlormequat chloride 720 g L^{-1}	1.0 L ha^{-1}
08/May/2013	32	fungicide	Capalo® **I**	metrafenone 75 g L^{-1} + epoxiconazole 62.5 g L^{-1} + fenpropimorph 200 g L^{-1}	1.0 L ha^{-1}
08/May/2013	32	fungicide	Cirkon® **(F)**	prochloraz 400 g L^{-1} + propiconazole 90 g L^{-1}	0.8 L ha^{-1}
08/May/2013	32	fungicide	Zenit® M **(D)**	fenpropidin 750 g L^{-1}	0.4 L ha^{-1}
17/May/2013	37	herbicide	MCPA-I 500® SL **(G)**	MCPA 500 g L^{-1}	1.25 L ha^{-1}
17/May/2013	37	insecticide	Fastac® SC **I**	alpha-cypermethrin 100 g L^{-1}	0.1 L ha^{-1}
06/Jun./2013	51	fungicide	Aviator® Xpro **(B)**	bixafen 75 g L^{-1} + prothioconazole 150 g L^{-1}	1.0 L ha^{-1}
06/Jun./2013	51	fungicide	Fandango® **(B)**	prothioconazole 100 g L^{-1} + fluoxastrobin 100 g L^{-1}	1.0 L ha^{-1}
24/Jun./2013	69	fungicide	Prosaro® **(B)**	tebuconazole 125 g L^{-1} + prothioconazole 125 g L^{-1}	1.0 L ha^{-1}
24/Jun./2013	69	insecticide	Karate Zeon® **(D)**	lambda-cyhalothrin 100 g L^{-1}	0.08 L ha^{-1}
25/Oct./2013	12	herbicide	Absolute® M **(A)**	flupyrsulfuron methyl 5.56% + diflufenican 44.4%	180 g ha^{-1}
11/Apr./2014	31	fungicide	Capalo® **I**	metrafenone 75 g L^{-1} + epoxiconazole 62.5 g L^{-1} + fenpropimorph 200 g L^{-1}	1.0 L ha^{-1}
11/Apr./2014	31	growth regulator	Moddus® **(B)**	trinexapac-ethyl 250 g L^{-1}	0.3 L ha^{-1}
11/Apr./2014	31	growth regulator	CCC 720® **(B)**	chlormequat chloride 720 g L^{-1}	0.5 L ha^{-1}
29/Apr./2014	35	fungicide	Zenit® M **(D)**	fenpropidin 750 g L^{-1}	0.75 L ha^{-1}
05/May/2014	37	fungicide	Input Xpro® **(B)**	spiroxamine 250 g L^{-1} + bixafen 50 g L^{-1} + prothioconazole 100 g L^{-1}	1.25 L ha^{-1}
02/Jun./2014	65	fungicide	Matador® **(H)**	tebuconazole 225 g L^{-1} + triadimenol 75 g L^{-1}	1.0 L ha^{-1}

02/Jun./2014	65	insecticide	Karate Zeon® **(D)**	lambda-cyhalothrin 100 g L^{-1}	0.08 L ha^{-1}
20/Oct./2014	13	herbicide	Absolute® M **(A)**	flupyrsulfuron methyl 5.56% + diflufenican 44.4%	180 g ha^{-1}
05/Nov./2014	21	insecticide	Perfekthion® **I**	dimethoat 400 g L^{-1}	0.7 L ha^{-1}
22/Apr./2015	31	fungicide	Capalo® **I**	metrafenone 75 g L^{-1} + epoxiconazole 62.5 g L^{-1} + fenpropimorph 200 g L^{-1}	1.1 L ha^{-1}
22/Apr./2015	31	fungicide	Cirkon® **(F)**	prochloraz 400 g L^{-1} + propiconazole 90 g L^{-1}	0.3 L ha^{-1}
22/Apr./2015	31	growth regulator	Moddus® **(B)**	trinexapac-ethyl 250 g L^{-1}	0.3 L ha^{-1}
22/Apr./2015	31	growth regulator	CCC 720® **(B)**	chlormequat chloride 720 g L^{-1}	0.4 L ha^{-1}
08/May/2015	37	herbicide	U46® M fluid **I**	MCPA 500 g L^{-1}	1.0 L ha^{-1}
08/May/2015	37	herbicide	Lodin® EC **(I)**	fluroxypyr 180 g L^{-1}	0.5 L ha^{-1}
21/May/2015	55	fungicide	Aviator® Xpro **(B)**	bixafen 75 g L^{-1} + prothioconazole 150 g L^{-1}	0.75 L ha^{-1}
21/May/2015	55	fungicide	Fandango® **(B)**	prothioconazole 100 g L^{-1} + fluoxastrobin 100 g L^{-1}	0.75 L ha^{-1}
Gatersleben					
24/Apr./2013	30	herbicide	Taipan® **(J)**	fluroxypyr 200 g L^{-1}	0.9 L ha^{-1}
24/Apr./2013	30	herbicide	Ralon® Super **I**	fenoxaprop-P-ethyl 69 g L^{-1} + mefenpyr-diethyl 75 g L^{-1}	1.0 L ha^{-1}
05/Jun./2013	37	fungicide	Talius® **(A)**	proquinazide 200 g L^{-1}	0.25 L ha^{-1}
31/Mar./2014	31	herbicide	Axial® 50 **(D)**	pinoxaden 50 g L^{-1} + cloquintocet-mexyl 12.5 g L^{-1}	1.2 L ha^{-1}
31/Mar./2014	31	herbicide	Biathlon® **I**	tritosulfuron 714 g kg^{-1}	70 g ha^{-1}
31/Mar./2014	31	herbicide	Starane® XL **(J)**	fluroxypyr 100 g L^{-1} + florasulam 2.5 g L^{-1}	0.75 L ha^{-1}
02/Jun./2014	39	fungicide	Fandango® **(B)**	prothioconazole 100 g L^{-1} + fluoxastrobin 100 g L^{-1}	0.75 L ha^{-1}
02/Jun./2014	39	fungicide	Aviator® Xpro **(B)**	bixafen 75 g L^{-1} + prothioconazol 150 g L^{-1}	0.75 L ha^{-1}
02/Jun./2014	39	fungicide	Tebusha® **(B)**	tebuconazole 250 g L^{-1}	1.0 L ha^{-1}
02/Jun./2014	39	insecticide	Decis® **(B)**	deltamethrin 25 g L^{-1}	0.3 L ha^{-1}
21/Apr./2015	37	herbicide	Dirigent® SX® **(A)**	metsulfuron methyl 14.3% + tribenuron methyl 14.3%	35 g ha^{-1}
12/May/2015	39	herbicide	Biathlon® 4D **I**	tritosulfuron 714 g kg^{-1} + florasulam 54 g kg^{-1}	70 g ha^{-1}
09/Jun./2015	55	fungicide	Fandango® **(B)**	prothioconazole 100 g L^{-1} + fluoxastrobin 100 g L^{-1}	0.65 L ha^{-1}
09/Jun./2015	55	fungicide	Aviator® Xpro **(B)**	bixafen 75 g L^{-1} + prothioconazole 150 g L^{-1}	0.65 L ha^{-1}

3.2 Experimental setup

3.2.1 Experimental design of the field trials

As previously shown in Table 3-4 (see section 3.1.4), field experiments were carried out at both locations with 4 N treatments, each one with 4 replicates in a Latin square and set up in 2 identical duplicates, allowing in one of them to conduct distructive measures including leaf and xylem sap harvests as well as other measurements for physiological parameters, whereas the other duplicate was designated for capturing yield parameters, since these plots were not affected by sampling and measurements (Figure 3-3). Field plots in Cunnersdorf were set up each with a size of 25.0 m^2 (10.0 x 2.5 m^2) while in Gatersleben plots were slightly bigger with a size of 30.0 m^2 (10.0 x 3.0 m^2). To avoid border effects on plant traits, experimental plots were surrounded by strips of winter wheat.

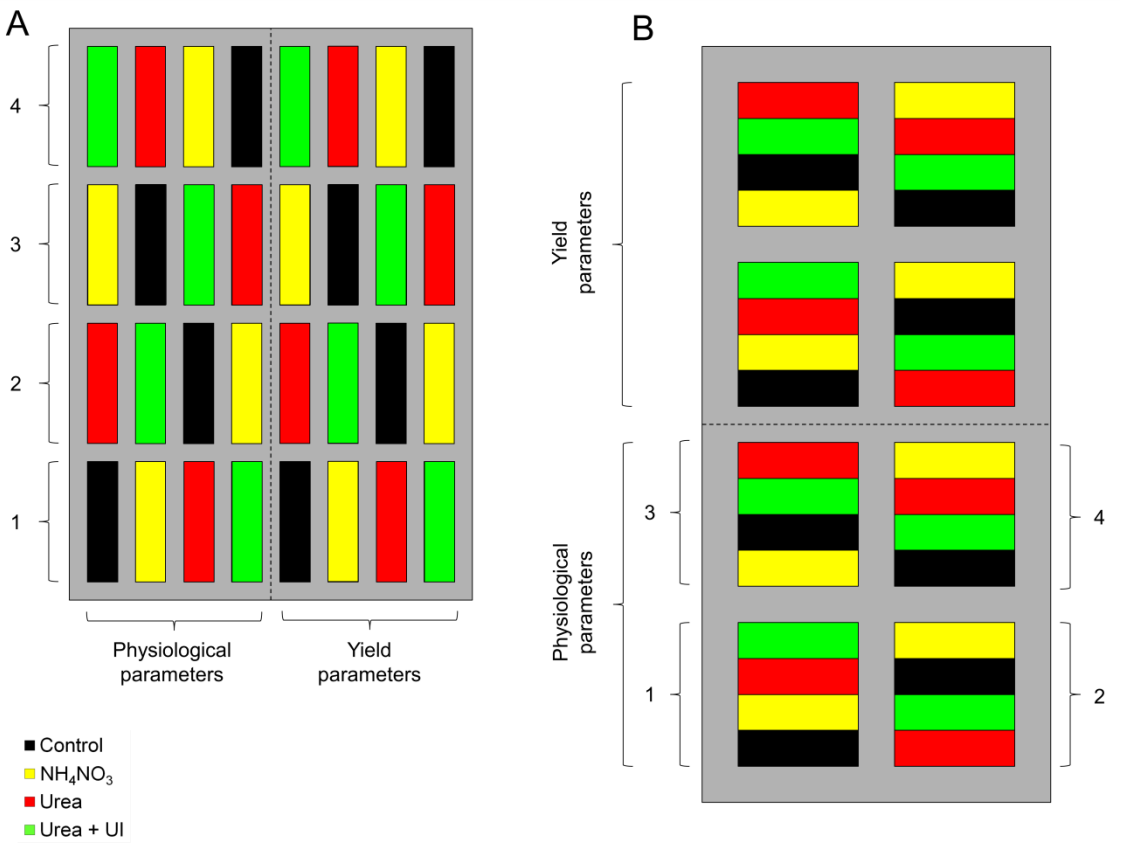


Figure 3-3. Field plot design at Cunnersdorf and Gatersleben. Set-up of latin square plots in Cunnersdorf **(A)** and Gatersleben **(B)**. In grey color are border strips, consisting of non-harvested wheat. Numbers indicate replicates (n=4).

3.2.2 Harvest plan of xylem exudates and leaves after fertilization

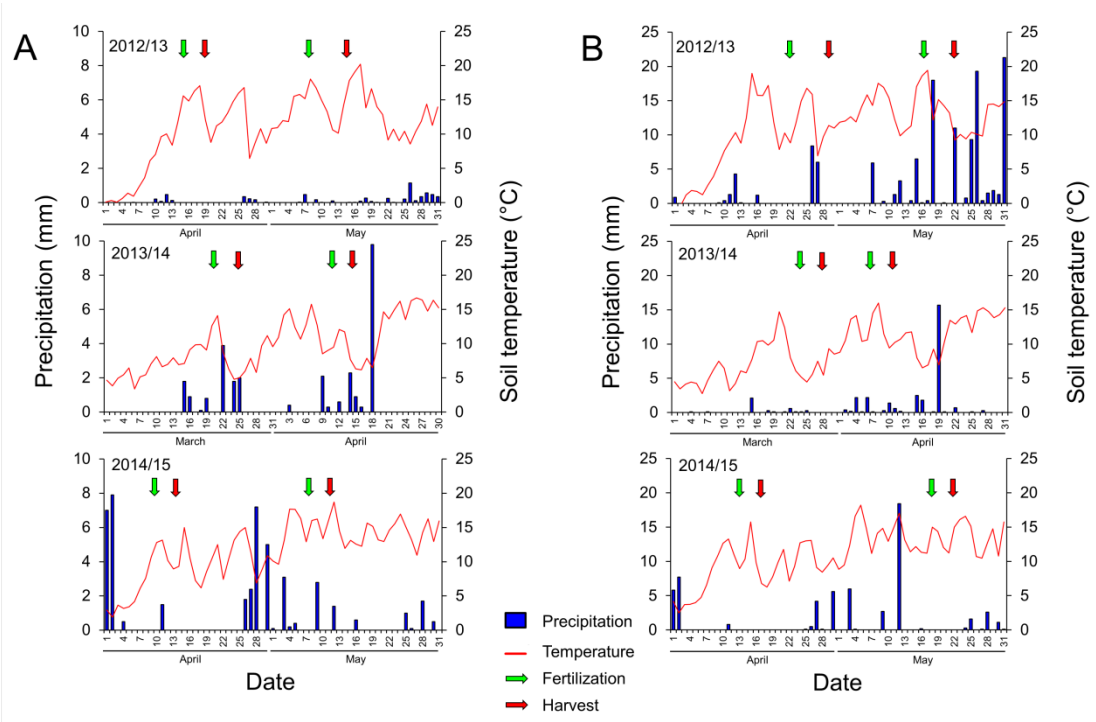
As already mentioned before in section 3.1.4, plants were fertilized at two fertilization time points, i.e. BBCH 25 and BBCH 32 (T1 and T2, respectively). Few days after each fertilization time point, xylem exudates and leaf samples were collected for further analysis (Table 3-6). Xylem exudates were collected to determine translocation rates of different N forms (urea, ammonium, nitrate as well as amino acids) and of phytohormones (auxins, cytokinins, abscisic acid, phaseic acid and salicylic acid). On the other hand, analysis in leaves included determination of different N forms, macro- and micronutrients, chlorophyll concentrations as well as concentrations of phytohormones, sugars (fructose, glucose and sucrose) and organic acids (malate, fumarate, citrate and isocitrate). Furthermore, leaf samples were harvested 1 d before xylem sap collection at each location to determine the activity of the enzyme urease. The xylem translocation rate of the UI 2-NPT and its concentration was also determined in xylem exudates and leaf samples, respectively.

Table 3-6. Fertilization time points and corresponding xylem exudate and leaf sampling dates at Cunnersdorf and Gatersleben over the 3 years of field experiments. Fertilization or harvest time points are indicated with T1 for the first one and with T2 for the second one. Total days indicate the period between fertilization time point and the corresponding date of xylem sap collection or leaf harvest.

Event	Cropping year					
	2012/13		2013/14		2014/15	
	T1	T2	T1	T2	T1	T2
Cunnersdorf						
Fertilization	15/Apr./2013	08/May/2013	21/Mar./2014	11/Apr./2014	10/Apr./2015	08/May/2015
Xylem sap and leaf sampling	19/Apr./2013	15/May/2013	25/Mar./2014	15/Apr./2014	14/Apr./2015	12/May/2015
Total days	4	7	4	4	4	4
Gatersleben						
Fertilization	22/Apr./2013	17/May/2013	24/Mar./2014	07/Apr./2014	13/Apr./2015	18/May/2015
Xylem sap and leaf sampling	29/Apr./2013	22/May/2013	28/Mar./2014	11/Apr./2014	17/Apr./2015	22/May/2015
Total days	7	5	4	4	4	4

3.2.3 Daily climatic data during fertilization and harvest time points

In general, comparable middle air temperatures were registered between fertilization and harvest time points at both experimental sites over the 3 cropping seasons (Figure 3-4). Nevertheless, stronger differences in precipitations between locations were found mainly during 2012/13, where almost no precipitation was recorded in Cunnersdorf (Figure 3-4A) at either time point (0.0 and 0.3 mm at T1 and T2, respectively) but strong rainfalls were registered in Gatersleben (Figure 3-4B) at both time points (14.4 and 29.5 mm at T1 and T2, respectively). Furthermore, higher precipitations were recorded in Cunnersdorf during the second trial year with 7.7 and 3.8 mm whereas in Gatersleben only 0.4 and 2.5 mm fell at T1 and T2, respectively. During the third season 2014/15, there were precipitations in Cunnersdorf but not in Gatersleben.



► **Figure 3-4.** (For description of this Figure refer to next page).

◄ **Figure 3-4. Climate data at Cunnersdorf and Gatersleben between fertilization and harvest time points over the three cropping years 2012/13, 2013/14 and 2014/15.** Precipitation and soil surface temperature as well as fertilization and harvest time points are shown in **(A)** for Cunnersdorf and in **(B)** for Gatersleben. Bars and lines indicate precipitations and temperatures, respectively. Green and red arrows indicate fertilization and harvest time points, respectively.

3.3 Determination of major plant-available N forms in soil

A few days after each fertilization time point (Table 3-7) and also at the end of each cropping season, a total of 10 representative soil samples per plot were taken from 0-30 cm depth at both locations to determine the content of the major plant-available N forms, namely urea, ammonium and nitrate. Samples were homogenized and kept on ice until preparation for analysis.

Table 3-7. Fertilization time points and corresponding soil sampling dates at Cunnersdorf and Gatersleben over the 3 seasons of field experiments. Fertilization time points are indicated by T1 and T2. Total days indicate the period between fertilization time point and the corresponding soil sampling date.

Event	Cropping year					
	2012/13		2013/14		2014/15	
	T1	T2	T1	T2	T1	T2
Cunnersdorf						
Fertilization	15/Apr./2013	08/May/2013	21/Mar./2014	11/Apr./2014	10/Apr./2015	08/May/2015
Soil sampling	18/Apr./2013	14/May/2013	24/Mar./2014	14/Apr./2014	13/Apr./2015	11/May/2015
Total days	3	6	3	3	3	3
Gatersleben						
Fertilization	22/Apr./2013	17/May/2013	24/Mar./2014	07/Apr./2014	13/Apr./2015	18/May/2015
Soil sampling	28/Apr./2013	21/May/2013	27/Mar./2014	10/Apr./2014	16/Apr./2015	21/May/2015
Total days	6	4	3	3	3	3

Measurements were carried out at SKW Stickstoffwerke Piesteritz GmbH (Cunnersdorf, Germany) by using a Continuous Flow Analyzer San++ (Skalar Analytical B.V., Breda, Holland), which operates according to the principle of continuous flow analysis for fully automatic sample determination.

3.3.1 Preparation and extraction of soil samples

Major plant-available N forms present in soil were extracted by following the methods described in VDLUFA (1991a, b), which were slightly modified by changing the extraction agent from $CaCl_2$ to a stronger one, namely KCl as reported by Kuderna et al. (1993). The extraction consisted of shaking soil samples for 1 h with an overhead shaker in 1 N KCl (VWR International, LLC., Radnor, USA) after addition of urease inhibitor NBPT (N-(n-butyl) thiophosphoric triamide, as Agrotain®, Koch Agronomic Services, Wichita, USA). To stabilize urea during the extraction, this urease inhibitor was added in a proportion of 40 mg soil : 1000 mL 1 N KCl and diluted for the extraction by 1:4 (soil mass : volume of the extraction solution). Prior to the determination of major N forms, samples were microfiltered using 0.45 µm syringe filters (Minisart® RC25, Sartorius AG, Göttingen, Germany). Filtered samples were then processed according to manufacturer's extraction procedures as indicated for urea determination in "chemical method no. 612" (Skalar Analytical B.V., 2005), for ammonium determination in "chemical method no. 155" (Skalar Analytical B.V., 2013b) and for nitrate determination in "chemical method no. 461" (Skalar Analytical B.V., 2013a).

3.3.2 Determination of urea in soil samples

Filtrates to be analyzed for urea were first acidified with sulfuric acid and mixed with diacetyl monoxime and thiosemicarbazide (both from VWR International, LLC., Radnor, USA), the latter to intensify the reaction color. Subsequently, samples were heated at 90°C, cooled down at room temperature and measured spectrophotometrically at 520 nm immediately after the reaction.

3.3.3 Determination of ammonium in soil samples

Determination of ammonium was based on the Berthelot reaction, in which ammonia generated after hydrolysis of urea reacted with sodium nitroprusside reagent (VWR International, LLC., Radnor, USA), hypochlorite and salicylate forming an indophenol-color complex which was then measured spectrophotometrically at 660 nm.

3.3.4 Determination of nitrate in soil samples

To determine nitrate, soil extracts were placed into a reduction column, where nitrate was reduced into nitrite. Under acid conditions Griess-Ilosvay's nitrite reagent (Merck KGaA, Darmstadt, Germany) containing sulfanilic acid, 1-naphthylamine and acetic acid was added to form a red diazo dye, which was then measured spectrophotometrically at 540 nm.

3.4 Xylem exudate collection and measurements

Xylem exudate collection followed the principle described by Taiz & Zeiger (2010), which consists of cutting plant shoots from rooted plants close to the soil surface allowing a continuous xylem sap flow from the cut stem for some time. For this purpose, a few days after each fertilization time point 25 plants plot^{-1} were cut 2-3 cm above the soil at early morning hours by using a disinfected stainless steel scalpel (Figure 3-5A) and by removing remaining leaves (Figure 3-5B). The cut section of the stem was cleaned with distilled water and dried with paper tissue (Takei et al., 2001). Silicon tubes of 4-5 cm length (Rotilabo®-Silikonschlauch, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were placed fitting with the corresponding cut section diameter of the hypocotyl, allowing the accumulation of xylem exudates (Figures 3-5C, D). Then, silicon tubes were covered with aluminum foil (Rotilabo®-Typ R 100, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) to protect xylem exudates from contamination and from UV light.

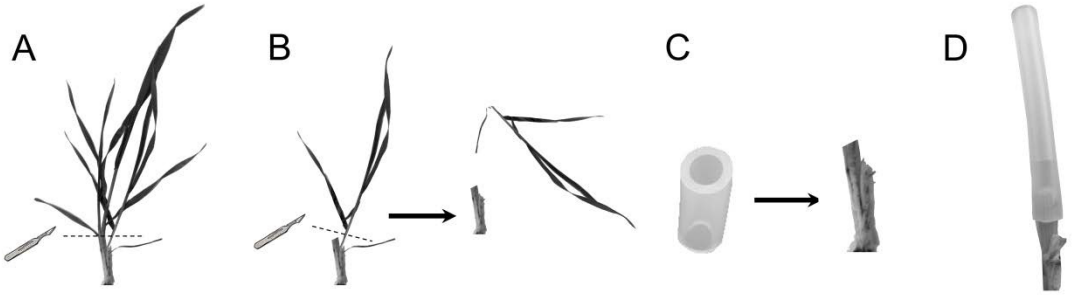


Figure 3-5. Scheme of xylem exudate extraction and collection. Procedure to obtain xylem exudates, wherein **(A)** shows shoot separation from the stem, **(B)** removal of remaining leaves from the main stem, **(C)** placement of silicon tubes which fit to the diameter of hypocotyls and **(D)** resulting stem with placed silicon tube which is covered with aluminum foil to protect xylem sap from UV light and contamination. Dotted lines represent cut sections.

Additionally, examined plants were watered the day before the xylem sap collection as shown in Table 3-8. Number of sampled plants per plot as well as start-end time points at the beginning and at the end of xylem exudate collection were documented to calculate xylem exudation rates. Xylem sap was kept on ice during collection and transport. Afterwards samples were maintained at -20°C until analysis.

Table 3-8. Amount of water given to plants during the collection of xylem exudates at Cunnersdorf and Gatersleben over the three years of field experiments. Harvest time points are indicated by T1 and T2. Plants were irrigated the day before with indicated volumes of tap water (in L m^{-2}).

Location	Cropping year					
	2012/13		2013/14		2014/15	
	T1	T2	T1	T2	T1	T2
Cunnersdorf	0	10	5-8	5-8	5-8	5-8
Gatersleben	0	10	10	10	10	10

3.4.1 Determination of urea in xylem exudate

Urea in xylem exudates was determined colorimetrically based on the method proposed by Kyllingsbæk (1975) as described by Kojima et al. (2007), in which a red-colored product results after heating urea in combination with two reagents. For this analysis, 1 mL of a color development reagent containing 1.28 mM thiosemicarbazide (Merck KgaA, Darmstadt, Germany), 4.60 mM diacetyl monoxime (Merck KgaA, Darmstadt, Germany), 6.6% H_2SO_4 (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 14.6 µM iron(III) chloride hexahydrate (Merck KgaA, Darmstadt, Germany) and 0.006% H_3PO_4 (Laborchemie Apolda GmbH, Apolda, Germany) was added to 120 µL xylem sap and incubated at 99°C and 750 rpm for 15 min. Thereafter, samples were cooled down on ice for 5 min and kept under dark conditions until absorbance measurement at 540 nm with a spectrophotometer (Genesys™ 10S UV-VIS, Thermo Fisher Scientific Inc., Waltham, USA).

3.4.2 Determination of ammonium and amino acids in xylem exudates

To determine ammonium and amino acids in xylem exudates, samples were first derivatized by using 3 ppm ACQ (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, IPK-Gatersleben, Germany), which is a fluorescent reagent proposed by Cohen et al. (1993) that allows detection of amino acids in an accurate manner, by forming highly stable urea compounds with fluorescence emission that can be measured at 400 nm.

ACQ was dissolved in acetonitrile (Th. Geyer GmbH & Co. KG, Renningen, Germany) and incubated at 55°C and 300 rpm for 10 min. For the derivatization step, 20 µL of each sample were pipeted into microcentrifuge tubes containing 160 µL of 0.2 M boric acid buffer (AppliChem GmbH, Darmstadt, Germany), pH 8.8 adjusted with KOH (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Then, 20 µL of ACQ were added to each tube, shortly vortexed and incubated at 55°C for 10 min. Subsequently, derivatized samples were centrifuged at 8,000 rpm and 4°C for 1 min. 180 µL of the supernatant were transferred into 96-well polypropylene sample collection plates (Waters Corporation, Milford, USA) and kept at 4°C before analytical separation, which was carried out using UPLC (ACQUITY UPLC® H-Class System, Waters Corporation, Milford, USA) composed by a quaternary solvent manager (QSM), a sample manager – flow through needle (SM-FTN), a column heater (CH) and a fluorescent detector photodiode array detector (PDA).

For the separation step, xylem sap samples were placed into the SM-FTN at 8°C and the column (Acquity UPLC® BEH C18, 2.1 x 100 mm, 1.7 µm, Waters Corporation, Milford, USA) was heated to 55°C during a flow rate of 0.7 mL min^{-1}. The QSM was composed of 4 solutions for the mobile phase gradient: i) 100% eluent A for amino acids analysis (AccQ-Tag Ultra, Waters Corporation, Milford, USA) ii) 1:25 acetonitrile:LC-MS water (Th. Geyer GmbH & Co. KG, Renningen, Germany), pH 2.6 adjusted with formic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), iii) 100% LC-MS water and iv) 100% acetonitrile. PDA detection was at 266 nm for excitation and 473 nm for emission. Before amino acid and ammonium separation (total run per sample of 10.2 min), Eluent A and 100% LC-MS water (1:9) were used to equilibrate the column for 30 min. Obtained chromatograms were quantified using the Empower Software (Waters Corporation, Milford, USA).

3.4.3 Determination of nitrate in xylem exudates

Determination of nitrate in xylem exudates was based on the methodology described by Cataldo et al. (1975) with slight modifications, where a yellow-colored product results after increasing the pH of a complex formed under low pH conditions by nitration of salicylic acid to nitrosalicylic acid. For this analysis, 5 µL aliquots of each xylem sap sample were transferred into 2 mL microcentrifuge tubes and mixed with 160 µL salicylic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) dissolved in concentrated H_2SO_4 (AppliChem GmbH, Darmstadt, Germany) and incubated on ice for 20 min. Subsequently, 1.8 mL of 4 M NaOH were added very carefully to each sample, thus increasing the pH above 12. Samples were cooled down to room temperature for 15 min until absorbance measurements at 410 nm with a spectrophotometer (Genesys™ 10S UV-VIS, Thermo Fisher Scientific Inc., Waltham, USA).

3.4.4 Determination of phytohormones in xylem exudates

Determination of phytohormones in xylem exudates followed the principle described by Kojima et al. (2009) with some modifications to improve their detection.

Extraction of phytohormones from xylem exudates

For this analysis, 200 µL aliquots from each sample were mixed with 200 µL of a methanol solution containing labeled phytohormones used as internal standards (OlChemim s.r.o., Olomouc, Czech Republic). To the resulting mixed solution 800 µL deionized water containing 1% formic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added and vortexed for 30 seconds.

Samples were evaporated at 50°C for 30 min using a vacuum centrifuge composed by a rotational vacuum concentrator with infrared heating system (RVC 2-33 IR, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and an ice condenser (Alpha 2-4 LD plus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) to remove the methanol from internal standards. Samples were centrifuged at 14,000 rpm and 4°C for 10 min, before using supernatants for a solid-phase extraction (SPE) cleanup procedure.

Cleanup

For the cleanup of samples, Oasis® HLB (hydrophilic-lipophilic-balanced) 30 µm reversed-phase sorbent columns (Waters Corporation, Milford, USA) were first conditioned with 1 mL methanol (Th. Geyer GmbH & Co. KG, Renningen, Germany) containing 0.1% formic acid and then equilibrated with 2 mL deionized water containing 0.1% formic acid by using a vacuum manifold (CHROMABOND®, Macherey-Nagel GmbH & Co. KG, Düren, Germany). Extracted sample solutions were added onto the HLB columns, then microcentrifuge tubes were rinsed with 200 µL deionized water containing 0.5% formic acid and columns were washed 3 times with 2 mL of the latter solution. Samples in columns were eluted with 600 µL methanol containing 0.1% formic acid and 600 µL acetonitrile (Th. Geyer GmbH & Co. KG, Renningen, Germany) containing 5% ammonia (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Samples were evaporated under vacuum centrifugation at 50°C until dryness.

Measurement of phytohormones

Pellets obtained after evaporation were dissolved in 10 µL methanol containing 1% formic acid, by using a vortex and ultrasonic bath for 3 min (Transsonic 820/H, Elma Schmidbauer GmbH, Singen, Germany). Afterwards, samples were centrifuged at 14,000 rpm and 4°C for 10 min and transferred to a 384-well polypropylene plate with square shape (Acquity UPLC®, Waters Corporation, Milford, USA) and covered with an adhesive seal, consisting in a polyolefin film with a synthetic rubber adhesive.

Phytohormones in samples were separated using a UPLC-MS (ACQUITY UPLC® System, Waters Corporation, Milford, USA), by heating a column (Acquity UPLC® BEH C18, 2.1 x 100 mm, 1.7 µm, Waters Corporation, Milford, USA) to 40°C during a total run of 7 min with a flow rate of 400 µL min^{-1}. Obtained chromatograms were quantified and processed using MassLynx™ Mass Spectrometry 4.1 Software (Waters Corporation, Milford, USA).

3.4.5 Determination of 2-NPT in xylem exudates

Determination of the urease inhibitor N-(2-nitrophenyl)-phosphoric acid triamide (2-NPT) in xylem exudates was established at the IPK Gatersleben, according to methodological experiences described in the section 3.5.9 for the detection of this urease inhibitor in leaves. For this analysis, xylem exudate samples were first centrifuged at 4°C and 14,000 rpm for 15 min and 40 µL supernatants were transferred directly into 1.5 mL vials and kept at 4°C until analysis.

Separation and detection were carried out by ultra-performance liquid cromatography coupled to mass spectrometry (UPLC-MS/MS), by using an Agilent 1290 Infinity LC System (Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany), which was connected to a high sensitive Agilent 6400 Series Triple Quadrupole LC/MS System (QQQ 6490, Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany). A high capacity ion exchange analytical column (Dionex IonPac™ AS11-HC, 2 x 250 mm, Thermo Fisher Scientific Inc., Waltham, USA) coupled to a guard column (Dionex IonPac™ AG11-HC, 2 x 50 mm, Thermo Fisher Scientific Inc., Waltham, USA) was heated at 45.0°C during a total run of 10 min per sample with a flow rate of 0.4 mL min^{-1}. The gradient was established by using 2 eluents, namely 100% LC-MS water containing 0.1% formic acid and 100% acetonitrile containing 0.5% formic acid. Tryptophan (AppliChem GmbH, Darmstadt, Germany) was added to each sample as internal standard. Obtained data were extracted by using the Agilent MassHunter Workstation Software revision B.03.01 (Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany).

3.5 Leaf sample collection and measurements

Highly transpiring leaf samples were collected by filling 10 fully expanded leaves (per plot) into 15 mL scintillation vials (Zinsser Analytic GmbH, Frankfurt am Main, Germany). Immediately after, leaf samples were kept under liquid N and later during transport on dry ice and finally at -80°C for further analysis.

Prior to analysis, leaf samples were homogenized by grinding them under liquid N using a milling machine (Schwingmühle MM 400, Retsch Technology GmbH, Haan, Germany). For some analyses, part of the resultant material was freeze-dried (P25K2S50, Dieter Piatkowski Forschungsgeräte, Munich, Germany), another part was dried at 65°C (Heraeus®, Kendro Laboratory Products GmbH, Hanau, Germany) and the rest kept frozen at -80°C for further measurements.

3.5.1 Determination of total nitrogen concentration in leaves

To determine the total N concentration in leaves, 1.5-2.0 mg dried ground leaf samples were weighed in tin capsules, which were then sealed and analyzed using an elemental analyzer (Euro EA 3000, HEKAtech GmbH, Wegberg, Germany). Under concentrated oxygen conditions, samples were oxidized at 990°C resulting in CO_2, H_2O and NO_x as reaction products. Afterwards NO_x was reduced to N_2 and residual oxygen was bound by Cu granules in a column producing gas containing N_2, CO_2, H_2O, and SO_2, followed by separation and quantification using gas chromatography. Detection of N was carried out by a thermal conductivity detector. As calibration standard for C, H, O, N and S, BBOT (HEKAtech GmbH, Wegberg, Germany) was used. Obtained values were processed using the Callidus Software 5.1 (EuroVector S.p.A., Milan, Italy).

3.5.2 Determination of urea concentrations in leaves

Urea concentrations in leaves were determined following the same principle as described above for measurements in xylem exudates (see section 3.4.1). For this analysis, to each 50 mg freeze-dried ground leaf sample 1 mL of 10 mM formic acid (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was added and mixed using a vortexer (Vortex-Genie 1, Scientific Industries Inc., New York, USA). Afterwards, leaf samples were centrifuged for 15 min at 13,200 rpm and 4°C. Supernatants of formic acid extracts were maintained on ice during the analysis. A total of 30 µL from each sample extract were mixed by using a ThermoMixer® Comfort (Eppendorf AG, Hamburg, Germany) with 1 mL of a color development reagent (see section 3.4.1) and incubated at 99°C and 750 rpm for 15 min. Immediately after, samples were cooled down on ice for 5 min and kept under dark conditions until absorbance measurement at 540 nm with a spectrophotometer (Genesys™ 10S UV-VIS, Thermo Fisher Scientific Inc., Waltham, USA). Urease enzyme present in leaf samples was deactivated by freezing and boiling plant extracts as reported by Witte et al. (2002).

3.5.3 Determination of amino acid concentrations in leaves

Amino acids in leaves were extracted by adding 1 mL of 80% methanol HPLC (Th. Geyer GmbH & Co. KG, Renningen, Germany) to approximately 50 mg fresh weight ground leaf material. Samples were incubated at 80°C for 60 min, cooled down at room temperature for 15 min and centrifuged at 14,000 rpm and 4°C for 5 min. Supernatants were transferred to new microcentrifuge tubes and evaporated at 50°C to dryness using a vacuum centrifuge (see section 3.4.4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Pellets were resuspended in 250 µL LC-MS water (ChemSolute®, Th. Geyer GmbH & Co. KG, Renningen, Germany), derivatized and analyzed as described before for xylem exudates in section 3.4.2.

3.5.4 Determination of ammonium and nitrate concentrations in leaves

Extraction of ammonium and nitrate from leaves

Ammonium and nitrate concentrations in leaves were determined by adding 1 mL of an extraction buffer containing chloroform (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and methanol HPLC-grade (Th. Geyer GmbH & Co. KG, Renningen, Germany) at a proportion of 1:1 (v/v) to approximately 50 mg (dry weight) ground leaf material. Leaf samples were mixed at 400 rpm and 4°C for 30 min by using a ThermoMixer® Comfort (Eppendorf AG, Hamburg, Germany). Afterwards, to each sample 300 µL LC-MS water (ChemSolute®, Th. Geyer GmbH & Co. KG, Renningen, Germany) were added before mixing them carefully after with a vortex. Once mixed, samples were centrifuged at 14,000 rpm and 4°C for 10 min. Supernatants were transferred to new microcentrifuge tubes and then evaporated by using a vacuum centrifuge (see details in section 3.4.4) at 35°C for 120 min. Pellets were dissolved in 200 µL LC-MS water by using a vortex and an ultrasonic bath (Transsonic 820/H, Elma Schmidbauer GmbH, Singen, Germany). Before separation, samples were derivatized as described before in section 3.4.2, but for this analysis 80 µL 0.2 M boric acid buffer and 10 µL ACQ were added to each 10 µL sample.

Separation and detection of ammonium and nitrate

Separation and detection of ammonium and nitrate in leaf samples were carried out by using an ion chromatography system (Dionex™ ICS-5000+, Thermo Fisher Scientific Inc., Waltham, USA). This system was connected to a conductivity detector (Dionex™ ICS-5000+ CD Conductivity Detector, Thermo Fisher Scientific Inc., Waltham, USA), at a triple quadrupole LC/MS system (QQQ 6490, Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany) and to a flow pump (Pharmacia LKB Pump P-1, GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

A high capacity ion exchange analytical column (Dionex IonPac™ AS11-HC, 2 x 250 mm, Thermo Fisher Scientific Inc., Waltham, USA) coupled to a guard column (Dionex IonPac™ AG11-HC, 2 x 50 mm, Thermo Fisher Scientific Inc., Waltham, USA) was heated at 37.0°C for a total run time of 40 min per sample with a flow rate of 400 µL min^{-1}. The gradient was established by using 100% LC-MS water and produced using 4 mM potassium hydroxide as eluent (Dionex™ EGC KOH, Thermo Fisher Scientific Inc., Waltham, USA). Obtained chromatograms were analyzed by using the Dionex™ Chromeleon™ 7.1 SR1 Chromatography Data System (CDS) software (Thermo Fisher Scientific Inc., Waltham, USA).

3.5.5 Determination of urease activity in leaves

Urease activity in leaves was determined according to Witte & Medina-Escobar (2001), who highlighted to analyze leaf samples as fresh as possible to detect enzyme activity. A total of 5 most recent fully expanded leaves were harvested per plot at each experimental location, as shown in Table 3-9. Fresh samples were carefully rolled and introduced in 2.0 mL Eppendorf® Safe-Lock microcentrifuge tubes (Eppendorf AG, Hamburg, Germany) and kept on ice until analysis.

Table 3-9. Fertilization time points and corresponding leaf sampling dates for determination of urease activity in leaves at Cunnersdorf and Gatersleben over 3 years of field experiments. Fertilization time and leaf sampling time points are indicated by T1 and T2. Total days indicate the period between fertilization time point and corresponding leaf sampling date.

Event	Cropping year					
	2012/13		2013/14		2014/15	
	T1	T2	T1	T2	T1	T2
Cunnersdorf						
Fertilization	15/Apr./2013	08/May/2013	21/Mar./2014	11/Apr./2014	10/Apr./2015	08/May/2015
Leaf sampling	18/Apr./2013	14/May/2013	24/Mar./2014	14/Apr./2014	13/Apr./2015	11/May/2015
Total days	3	6	3	3	3	3
Gatersleben						
Fertilization	22/Apr./2013	17/May/2013	24/Mar./2014	07/Apr./2014	13/Apr./2015	18/May/2015
Leaf sampling	28/Apr./2013	21/May/2013	27/Mar./2014	10/Apr./2014	16/Apr./2015	21/May/2015
Total days	6	4	3	3	3	3

Protein extraction procedure

Leaf samples were shortly rinsed with distilled water and cut into 1.0-1.5 cm pieces. To approximately 100 mg leaf material weighed in 2.0 mL microcentrifuge tubes, 500 µL of an extraction buffer containing 50 mM pH 7.5 sodium phosphate buffer (Ausubel et al., 1994), 50 mM sodium chloride (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 1 mM EDTA iron(III) sodium salt (Sigma-Aldrich Co., St. Louis, USA) and 1.5% (w/v) PVP-25 (polyvinylpyrrolidone 25, Serva Electrophoresis GmbH, Heidelberg, Germany) were added. Furthermore, to each sample 5 µL of 10 mM DTT (dithiothreithol, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 5 µL of 0.1 mM PMSF (phenylmethylesulfonyl fluoride, AppliChem GmbH, Darmstadt, Germany) were added. The protein extraction was carried out by using an overhead stirrer machine (RZR 2040, Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) with a pestle fitting to the bottom of microcentrifuge tubes for 3 min avoiding sample heating. Extracts were first centrifuged at 4°C and 13,200 rpm for 10 min and subsequently, approximately 400 µL of supernatants were centrifuged in new microcentrifuge tubes at 4°C and 13,200 rpm for 20 min. Resulting purified protein extracts were kept under liquid N during analysis and later at -20°C for further protein determination.

Analysis procedure and measurement

Interfering agents like DTT were removed by passing 100 µL purified protein extracts through Micro Bio-Spin™ columns (Bio-Rad Laboratories Inc., Hercules, USA), which were first centrifuged at 2,700 rpm for 1 min after adding 500 µL gel filtration buffer containing Sephadex™ G-25 Medium (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) dissolved in the extraction buffer without PVP-25, DTT and PMSF. Micro Bio-Spin™ columns containing the extracts were centrifuged at 4°C and 2,700 rpm for 2 min.

To 90 µL of each resulting clarified extract 1 µL 5 M urea (Sigma-Aldrich Co., St. Louis, USA) was added, mixed by using a vortexer and incubated in a ThermoMixer® Comfort (Eppendorf AG, Hamburg, Germany) at 50°C for 2 min. The analysis involved 4 time points every 20 min, which started from time point 1 (0 min) with an aliquot of 20 µL from these incubated extracts, which was transferred to new microcentrifuge tubes containing 980 µL distilled water. Immediately after, to each sample extract 100 µL phenol nitroprusside reagent containing phenol (Roti®-Phenol, Carl

Roth GmbH + Co. KG, Karlsruhe, Germany) and sodium nitroprusside dihydrate (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were added as well as 200 µL hypochloride reagent pH 12.0 containing sodium hydroxide (Carl Roth GmbH + Co. KG, Karlsruhe, Germany), sodium monohydrogen phosphate (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and 12% sodium hypochlorite solution (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). Sample extracts from each time point were incubated in a shaking water bath (Schüttelwasserbad THERMOLAB® 1086, GFL Gesellschaft für Labortechnik mbH, Burgwedel, Germany) at 50°C for 15 min. Absorbance was spectrophotometrically measured at 636 nm (Genesys™ 10S UV-VIS, Thermo Fisher Scientific Inc., Waltham, USA). As calibration standard 400 µM ammonium chloride (Merck KgaA, Darmstadt, Germany) was used.

3.5.6 Determination of macro- and micronutrient concentrations in leaves

Macro- and micronutrient concentrations in leaves were determined by adding 2 mL nitric acid (Bernd Kraft GmbH, Duisburg, Germany) to approximately 70 mg dried leaf sample weighed into 15 mL PTFE digestion tubes (polytetrafluoroethylene, MLS GmbH, Leutkirch im Allgäu, Germany). Leaf samples were digested under pressure by using a high performance microwave reactor (Ultraclave 4, MLS GmbH, Leutkirch, Germany) and then diluted with de-ionized water into 15 mL Greiner centrifuge tubes (Sigma-Aldrich Co., St. Louis, USA). Nutrient concentrations in leaves were analyzed by inductively-coupled plasma optical emission spectrometry technique (iCAP 6500 dual OES spectrometer, Thermo Fisher Scientific Inc., Waltham, USA). An ICP multi-element standard solution (Certipur®, Merck KgaA, Darmstadt, Germany) was used as standard.

3.5.7 Determination of chlorophyll concentrations in leaves

Determination of chlorophyll concentrations in leaves was carried out according to Moran & Porath (1980) with slight modifications. Approximately 30 mg fresh leaf biomass were incubated with 1.8 mL DMF (N,N-dimethylformamide, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) at 4°C for 24 h under dark conditions. Once leaf samples were bleached, supernatants were transferred to glass cuvettes and absorbance was spectrophotometrically measured (Genesys™ 10S UV-VIS, Thermo Fisher Scientific Inc., Waltham, USA) at 647 and 664 nm using DMF as a blank. Chlorophyll concentrations were calculated following the formulae proposed by Moran (1982).

3.5.8 Determination of phytohormones in leaves

Determination of phytohormones in leaf samples was carried out with the same chemicals and instruments as those used for xylem exudates (section 3.4.4).

Extraction of phytohormones from leaf samples

To determine phytohormones in leaf samples, 1 mL water and methanol (in a proportion of 30:70) containing 0.5% formic acid were added to approximately 50 mg fresh weight of ground leaf material. After adding 2 steel balls with a diameter of 3 mm (Grade 40, KGM Kugelfabrik Gebauer GmbH, Fulda, Germany), samples were vortexed for 30 seconds and sonicated by using an ultrasonic bath (Transsonic 820/H, Elma Schmidbauer GmbH, Singen, Germany) at 4°C for 15 min.

Extraction of phytohormones from leaf tissues was carried out by using an overhead shaker (Reax 2, Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) at 4°C for 60 min. Samples were centrifuged at 14,000 rpm and 4°C for 10 min, and supernatants were transferred into new 2 mL microcentrifuge tubes, which were kept at 4°C during the extraction. This extraction step was repeated a second time using the same leaf samples, not discarded in the previous step, and both supernatants were combined. Methanol was evaporated from the samples using a vacuum centrifuge (see details in section 3.4.4) at 50°C for approximately 30 min, until approx. 600 µL remained in each microcentrifuge tube.

After evaporation, 1 mL deionized water containing 0.5% formic acid was added to each sample extract. Samples were vortexed for 30 seconds and sonicated in an ultrasonic bath at 4°C for 15 min. Samples were placed for a second time in the overhead shaker at 4°C for 60 min, being afterwards centrifuged at 14,000 rpm and 4°C for 10 min. Supernatants were combined with those from the previous step. Labeled phytohormones dissolved in methanol were used as internal standards (OlChemim s.r.o., Olomouc, Czech Republic), adding 100 µL to each leaf sample extract. To evaporate methanol, samples were evaporated by using a vacuum centrifuge at 50°C for 5 min, until 1.6 mL remained, which were used afterwards for a solid-phase extraction (SPE) cleanup procedure.

Cleanup

For the cleanup step of samples, Oasis® HLB (hydrophilic-lipophilic-balanced) and MCX (mixed-mode, reversed-phase/strong cation-exchange) 30 µm sorbent columns (Waters Corporation, Milford, USA) were used in 2 cleanup steps. For the first one, HLB reversed-phase cartridges were conditioned with 1 mL methanol containing 0.1% formic acid and equilibrated with 2 mL deionized water containing 0.1% formic acid. Sample extracts were added into the column, rinsing microcentrifuge tubes afterwards with 200 µL of the latter solution. Columns were washed with 2 mL deionized water containing 0.1% formic acid and then eluted with 1.2 mL 90% methanol containing 0.1% formic acid. Methanol present in samples was evaporated using a vacuum centrifuge until 120 µL were left over. To each sample extract, 880 µL deionized water containing 0.1% formic acid was added, vortexed for 30 seconds and sonicated for 2 min.

The second cleanup step was carried out using MCX columns. For this, cartridges were also conditioned and equilibrated with the same chemicals and amounts as those used for HLB columns. Sample extracts were added into the column, rinsing microcentrifuge tubes afterwards with 200 µL deionized water containing 0.1% formic acid. Then, columns were washed with 1 mL using the latter solution and then eluted with 1.2 mL methanol containing 0.1% formic acid. Obtained extracts were kept at 4°C and used for determination of acidic and neutral phytohormones (auxins, abscisic acid and salicylic acid). For determination of basic phytohormones (cytokinins), columns after last elution were washed with 1 mL deionized water containing 1% formic acid and, immediately after, eluted first with 600 µL 60% methanol containing 5% ammonia and then eluted with 600 µL 60% acetonitrile containing 5% ammonia.

Measurement of phytohormones

After cleanup steps, sample extracts were evaporated with a vacuum centrifuge at 50°C until dryness. Residues obtained after evaporation were dissolved in 10 µL 50% methanol containing 0.5% formic acid and vortexed. Afterwards, to each sample extract 40 µL deionized water were added, vortexed and incubated in an ultrasonic bath for 2 min. Subsequently, samples were centrifuged at 14,000 rpm and 4°C for 10 min and transferred to a 384-well polypropylene plate with square shape, covered with an adhesive seal and separated by using UPLC-MS, as described before in section 3.4.4 for measurements in xylem exudates.

3.5.9 Determination of 2-NPT in leaves

Determination of the urease inhibitor N-(2-nitrophenyl)-phosphoric acid triamide (2-NPT) in leaves was newly established at IPK Gatersleben. For this analysis, to approximately 30 mg freeze-dried ground leaf sample 1 mL ethanol 80% (v/v) was added. Samples were incubated for 30 min at room temperature and centrifuged at 4°C and 14,000 rpm for 15 min. Supernatants were transferred into new microcentrifuge tubes and concentrated at 40°C by using a vacuum centrifuge (see section 3.4.4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Pellets were dissolved in 50 µL acetonitrile containing 0.1% formic acid by using an ultrasonic bath (Transsonic 820/H, Elma Schmidbauer GmbH, Singen, Germany) for 3 min at room temperature. Separation and detection of 2-NPT in leaf samples were carried out as described previously in section 3.4.5.

3.5.10 Determination of sugar concentrations in leaves

Concentrations of soluble sugars in leaves were determined by adding 1 mL 80% (v/v) ethanol to approximately 50 mg fresh ground leaf sample. Samples were vortexed and incubated at 80°C for 30 min by using a ThermoMixer® Comfort (Eppendorf AG, Hamburg, Germany). Afterwards, samples were centrifuged at 4°C and 14,000 rpm for 10 min before transferring supernatants into new 2.0 mL microcentrifuge tubes. Prior to the analysis, samples were

concentrated at 50°C by using a vacuum centrifuge (see section 3.4.4, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and dissolved in 300 µL deionized water. Sugars were measured at 340 nm by using an PowerWave™ HT microplate spectrophotometer (BioTek® Instruments, Inc., Winooski, USA), after adding to each sample a buffer containing 100 mM imidazole hydrochloride (pH 6.9), 5 mM magnesium chloride, 2.25 mM NAD, 1 mM ATP and 4 auxiliary enzymes, which were sequentially added (glucose-6-phosphate dehydrogenase, hexokinase, phosphoglucoisomerase and β-fructosidase). Obtained data for sucrose, glucose and fructose were processed using the KC4™ Data Analysis Software 3.4 Rev 21 (BioTek® Instruments, Inc., Winooski, USA).

3.5.11 Determination of organic acid concentrations in leaves

Extraction of organic acids from leaf samples, namely malate, citrate, fumarate and isocitrate from the tricarboxylic acid cycle (TCA), was carried out following the methods described briefly by Tognetti et al. (2007) with some modifications. For this analysis, approximately 50 mg of ground dry leaf material were added to1 mL of an extraction buffer containing chloroform (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and methanol HPLC-grade (Th. Geyer GmbH & Co. KG, Renningen, Germany) in a proportion of 1:1 (v/v), allowing the extraction of both lipophilic and hydrophilic substances. Leaf samples were mixed at 300 rpm and 4°C for 20 min by using a ThermoMixer® Comfort (Eppendorf AG, Hamburg, Germany). Similar to the methods described before for the determination of ammonium and nitrate in leaf samples (see section 3.5.4), 300 µL LC-MS water were added to each sample before gently mixing for 30 seconds by using a vortex. Subsequently, samples were centrifuged at 14,000 rpm and 4°C for 10 min, transferring afterwards the supernatants to new microcentrifuge tubes. Samples were concentrated by using a vacuum centrifuge (see details in section 3.4.4) at 40°C to dryness. Pellets were dissolved in 200 µL LC-MS water by using a vortex and an ultrasonic bath. Separation and detection of malate, citrate, fumarate and isocitrate were carried out as described before in section 3.5.4.

3.6 Determination of yield and quality parameters

At the end of the ripening stage (BBCH 99), winter wheat plots were harvested at both experimental sites (Table 3-10) by using a plot harvester with a working width of 1.6 or 1.5 m for Cunnersdorf or Gatersleben, respectively (Cunnersdorf: C-85 Parzellenmähdrescher, Haldrup GmbH, Ilshofen, Germany; Gatersleben: Wintersteiger Classic, Wintersteiger AG, Ried, Austria), which considered only the core of each plot (Cunnersdorf: 1.6 x 8.0 m²; Gatersleben: 1.5 x 7.0 m²). Grain and straw samples were dried on a drying system using a heated air flow (Hoopman Equipment & Engineering B.V., Aalten, The Netherlands) for 72 h.

Table 3-10. Grain and straw harvest dates at Cunnersdorf and Gatersleben over the 3 years of field experiments.

Harvest date at experimental site	Cropping year		
	2012/13	2013/14	2014/15
Cunnersdorf	15/Aug./2013	23/Jul./2014	03/Aug./2015
Gatersleben	16/Aug./2013	08/Aug./2014	30/Jul./2015

3.6.1 Grain yield

Grain yield was determined by weighing dried grains collected on each plot, considering its dry matter content. All grain yields were based on 14% standard moisture content for better comparability and expressed as dt ha^{-1}.

3.6.2 Spiked tillers per square meter

At each experimental site, spiked tillers from each plot were quantified at the middle of the fruit development stage (BBCH 75). For this yield parameter, spiked tillers were counted from 3 randomly chosen 1 m-long rows per plot considering the space between rows, and the number was related to m².

3.6.3 Grains per spike

The amount of grains per spike was calculated from following yield parameters: i) grain yield (kg m^{-2}), ii) thousand kernel weight (kg) and iii) spiked tillers (number m^{-2}).

3.6.4 Thousand kernel weight

After cleaning grains from rests of straw and other elements, thousand kernel weight (TKW) was determined by using a high speed seed counter (CONTADOR, Pfeuffer GmbH, Kitzingen, Germany).

3.6.5 Straw yield

Straw yield was determined by weighing dried straw collected per plot, considering its dry matter content, similarly as for grain yield determination. All straw yields were based on 100% standard dry mass for better comparability and expressed as dt ha^{-1}.

3.6.6 Determination of crude-protein concentration and sedimentation

Once dried, grain samples were milled by using a vibratory disc mill (RS200, Retsch Technology GmbH, Haan, Germany). Crude-protein concentration and sedimentation in grains were determined by using a whole grain analyzer, which works with a near-infrared transmittance technology (Infratec™ 1241, FOSS, Hilleroed, Denmark).

3.6.7 Determination of total N accumulated in above-ground organs

Total N accumulated in above-ground organs was calculated based on N concentrations in dried grains and straw. For this, N concentration in these organs was determined by weighing 200 mg material for analysis by a CN-Analyzer (vario Max, Elementar Analysensysteme GmbH, Langenselbold by Hanau, Germany).

3.7 Statistical analyses

Data were analyzed by analysis of variance (ANOVA), and treatment means were compared by using Tukey test at $P<0.05$ ($n=4$). Statistical analyses were performed using Statgraphics Centurion XV software version 15.2.05 (Statgraphics Technologies, Inc., The Plains, USA).

4 RESULTS

4.1 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on the content of major plant-available N forms in the soil

At both experimental sites and over the three cropping years, soil samples were taken a few days after each fertilization time point (see Methods 3.3; Table 3-7) to prove the effectiveness of the urease inhibitor 2-NPT in the soil. Contents of major plant-available N forms present in the soil, namely urea, ammonium and nitrate were determined for each treatment. The addition of the urease inhibitor 2-NPT in combination with urea fertilizer was expected to prolong the availability of urea in the soil. Furthermore, by supplying these combined fertilizers, contents of ammonium in the soil were expected to be reduced as a consequence of decreased urea hydrolysis.

Amounts of N present in the soil as urea, ammonium or nitrate are shown for Cunnersdorf and Gatersleben in Figure 4-1A and 4-1B, respectively. At both experimental sites, very low amounts of urea-N, which did not exceed 1.5 kg N ha^{-1}, were found under control conditions without N fertilization. After fertilization with ammonium nitrate, urea-N remained low, reaching exceptionally 63.2 kg N ha^{-1} in Gatersleben in 2014/15 at T1. As expected, after urea supply contents of soil urea were strongly increased at each time point and at both sites in all three years. Urea contents were even up to almost 4-fold higher after addition of the UI 2-NPT. Nevertheless, urea contents in the soil were not always significantly increased by the addition of 2-NPT when compared to urea alone, as observed in Cunnersdorf during the first year at T1 as well as during 2014/15 at T2.

In Gatersleben in 2012/13, soil samples were collected 6 or 4 d after the first or second fertilization time point, respectively. Late sampling dates and higher rainfalls between fertilization and soil sampling dates (14.4 and 29.5 mm at T1 and T2, respectively) were probably responsible for urea being more diluted and close to the detection limit and for lacking differences between urea alone and urea with 2-NPT. It is assumed that under high precipitations, urea and the UI are rapidly dissolved in the soil, which would decrease UI concentrations relative to those of urea. Then, moist conditions may accelerate urea hydrolysis and may result in similar urea-N contents as observed during the first trial year. Although lower precipitations were registered in the following years for Gatersleben as compared to Cunnersdorf and despite the fact that shorter periods between fertilization and sampling dates were realized, UI increased urea contents in the soil only at T2 in the last two years (Figure 4-1).

At both experimental sites, contents of NH_4-N in the soil were very low without N fertilization. However, under fertilization with NH_4NO_3, amounts of soil NH_4-N strongly increased, mounting up to > 93 kg NH_4-N ha^{-1} at T2 in Cunnersdorf in the third year and at Gatersleben up to > 230 kg NH_4-N ha^{-1} at T2 during 2013/14. It should be kept in mind that this elevated value resulted from extraction with KCl of the Gatersleben soil that has a high CEC. Therefore it is not surprising that this value exceeds by far the amount of fertilized ammonium. Interestingly, under fertilization with urea alone, NH_4-N contents in the soil were much higher than when 2-NPT was added, especially in Cunnersdorf. When plots were fertilized with urea and 2-NPT, NH_4-N contents in the soil were 36.7 and 32.6% less at T1 and T2 during 2012/13 in Cunnersdorf, respectively, when compared to urea alone. The same was also observed in the two following years with 21.8 and 24.0% and 3.4 and 60.4% less NH_4-N (at T1 and T2) during 2013/14 and 2014/15, respectively.

In Gatersleben, an effect of 2-NPT on reduced ammonium release in the soil was observed just in tendency, when plots with UI had 11.2 and 14.0% less soil NH_4^+ at T1 and T2, respectively, during 2012/13, or 35.9% (T1) and 33.0% (T2) less during 2013/14 or 5.4% less at T1 during the last year. However, at T2 during 2014/15 in Gatersleben, no reduction in soil ammonium contents was observed. Taken together, urea and ammonium analysis successfully proved the effectiveness of the UI 2-NPT at the Cunnersdorf site, whereas in Gatersleben the UI was effective only in maintaining higher urea levels at T2 in the last two years.

NO_3-N contents in the soil were highest after fertilization with NH_4NO_3 at either location, where they mostly varied between 30 and 120 kg NO_3-N ha^{-1}, apart from exceptionally high levels in Gatersleben at T2. In the other treatments, soil nitrate mostly remained < 25 kg NO_3-N ha^{-1}. In general, the UI 2-NPT had no significant effect on the nitrate-N content in the soil.

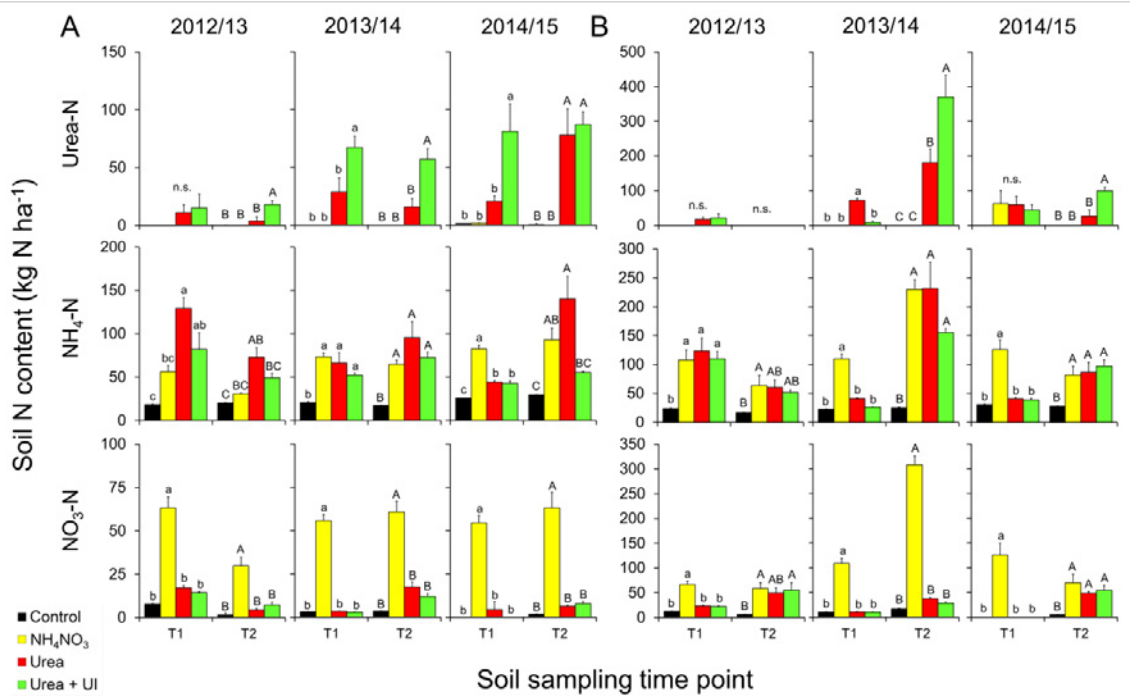


Figure 4-1. Influence of fertilization with different N forms (±UI) on the content of major plant-available N forms in the soil. Contents of N forms are shown for **(A)** Cunnersdorf and **(B)** in Gatersleben at the first (T1) and second (T2) soil sampling time point over the three cropping years 2012/13, 2013/14 and 2014/15. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

4.2 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on the plant nutritional status of winter wheat

While several studies have shown that supplementation of 2-NPT to urea fertilizers improves grain yield and grain N accumulation, almost nothing is known on the uptake of 2-NPT by plants and its putative effect on plant metabolism and the plant nutritional status. Therefore, xylem sap and leaves of field-grown wheat were analyzed for the occurrence of 2-NPT and for possible effects of 2-NPT on leaf N metabolism.

4.2.1 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on xylem exudation rates and translocation rates of 2-NPT

Xylem sap samples were collected between 4 and 7 d after each fertilization time point at both experimental locations, as indicated previously in Table 3-6, and xylem exudation rates were calculated for each location (Figure 4-2). It is important to mention that no xylem exudate could be collected at the first harvest time point during the first trial year at Cunnersdorf (Figure 4-2A), because the soil was too dry and soil temperature was too low. In addition, there was not much growth of the plants due to the long-lasting frost and snowy winter during the year 2012/13 at this location. At Cunnersdorf, xylem exudation rates in 2013/14 and 2014/15 were between 6 and 16 µL h^{-1} $plant^{-1}$ without significant differences between fertilization treatments. These exudation rates were somewhat less than those reported by Simpson et al. (1983), who measured exudation rates of about 30 µL h^{-1} $plant^{-1}$ in wheat plants, probably under warmer or more humid conditions. However, there was an important difference between years, since in 2012/13 xylem exudation rates at T2 were between 38 and 77 µL h^{-1} $plant^{-1}$ and thus, more than 3-fold higher than in the following years. The reason for these high rates was probably due to the way of collecting xylem exudates, since at Cunnersdorf during 2012/13, plants were irrigated with 10 L m^{-2} before sampling while in later years only 5-8 L m^{-2} were given (Table 3-8).

At Gatersleben, xylem exudates collected at T2 during 2012/13 (Figure 4-2B) were also up to 8-fold higher than those collected at any other harvest time point. Except for T1 in 2012/13, irrigation at this location was always at 10 L m^{-2}. Notwithstanding a lacking correlation between irrigation and xylem sap volumes, the soils in Gatersleben

remained usually wet due to higher clay contents, relative to the sandy soil in Cunnersdorf. These differences restrict the direct comparison of translocation rates of any substance between 2012/13 (T1) and the two subsequent years.

Xylem exudate samples were analyzed by UPLC-MS/MS to investigate whether the UI 2-NPT was taken up by roots and translocated to shoots. Measurements indicated that the UI was taken up and translocated in the xylem sap at both experimental sites in all three crop years (Figure 4-2). As expected, the UI could be detected only in those plants, which were fertilized with urea in combination with 2-NPT. Higher translocation rates at T2 in 2012/2013 were due to the higher xylem transport rates. In general, translocation rates of the UI were higher at T2 than at T1, which might be related to a higher uptake capacity and plant demand for N, rather than an accumulation of the UI in the xylem sap. Interestingly, translocation rates of 2-NPT were much higher in Cunnersdorf than in Gatersleben, which may be related to a lower concentration of the free i.e. plant-available fraction of 2-NPT in the Gatersleben soil.

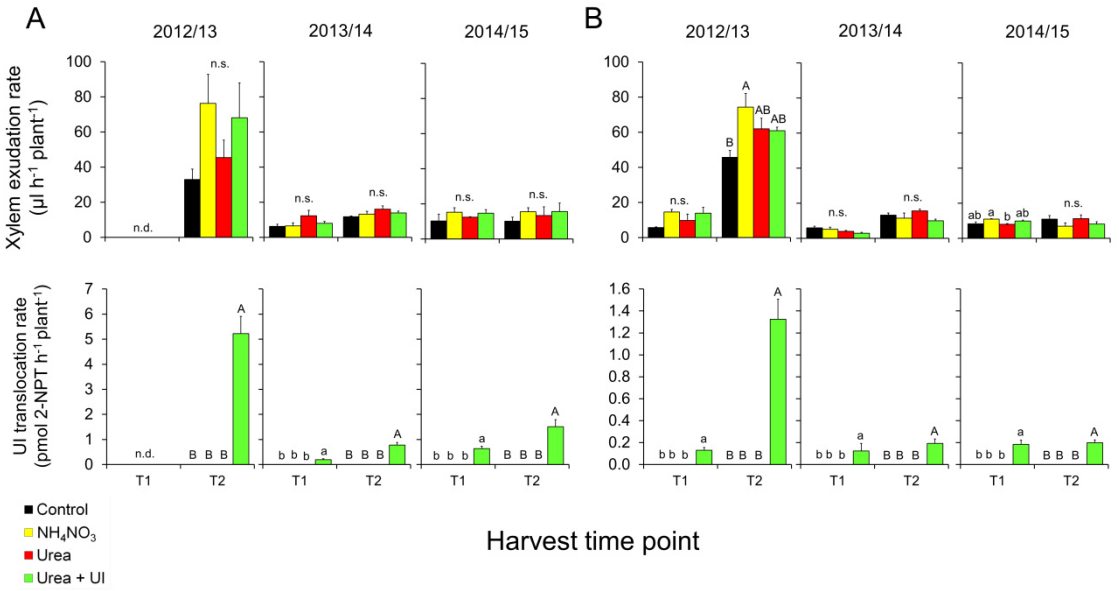


Figure 4-2. Influence of fertilization with different N forms (±UI) on xylem exudation rates and on translocation rates of the urease inhibitor 2-NPT in the xylem sap. Results are shown for the first (T1) and second (T2) harvest time point in **(A)** Cunnersdorf and in **(B)** Gatersleben over the three cropping years 2012/13, 2013/14 and 2014/15. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant n.d.; not determined).

4.2.2 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on concentrations of 2-NPT, chlorophyll and nitrogen in leaves

To address the question whether the translocated UI 2-NPT also accumulated in leaves, leaf samples were harvested and analyzed by UPLC-MS/MS (Figure 4-3). In agreement with higher translocation rates of the UI in xylem exudates, UI concentrations in leaves were higher in Cunnersdorf than in Gatersleben and also higher at T2, when compared to concentrations at T1, the latter indicating that the UI accumulated over time in leaves. Furthermore, the UI 2-NPT could not be detected when 2-NPT was not supplied. Additionally, 2-NPT concentrations in Cunnersdorf were higher in 2013/14 than in 2012/13 and in 2014/15 higher than in 2013/14, which was not the case in Gatersleben. As indicated by low standard deviations, the reason for this was not a problem of variability in sampling or analytical detection but may be related to seasonal effects and the soil type, as the higher CEC in Gatersleben may bind more 2-NPT and thus decrease its availability to plants.

Chlorophyll concentrations in leaves were determined (Figure 4-3) to verify whether urea (±UI) was metabolically converted as well as ammonium or nitrate in leaves. At T1 in both experimental locations, chlorophyll concentrations in leaves were similar and not significantly different between non-fertilized and N-fertilized plants in all three years. Chlorophyll concentrations in leaves in Cunnersdorf at T2 were slightly higher than at T1 (around 0.5 mg g^{-1} FW higher), when highest concentrations were observed after fertilization with ammonium nitrate, suggesting a more rapid metabolic conversion by the plant. On the other hand, chlorophyll concentrations in Gatersleben at T2 were very similar between non-fertilized and N-fertilized variants (Figure 4-3B), probably because at this relatively early developmental stage control plants could take up enough nitrogen from the soil even without N fertilization. Only

during the third cropping year an influence of N fertilization on chlorophyll concentrations in leaves was observed at this location, where concentrations were twofold higher when N fertilization took place. During the three experimental years, no correlation was observed between the concentration of 2-NPT and leaf chlorophyll, neither in Cunnersdorf nor in Gatersleben. Thus, 2-NPT accumulation in leaves had no impact on chlorophyll formation.

In general, chlorophyll and nitrogen concentrations in leaves mostly follow a similar trend (Torres et al., 2002; Mattiello et al., 2015), thus a comparable pattern was expected here after N fertilization. This expectation held true. N concentrations in leaves (Figure 4-3A) were not different between control and N-fertilized plants at both locations after the first fertilization time point. In agreement with chlorophyll concentrations, significant differences were observed at T2 in Cunnersdorf, where non-fertilized plants reached N concentrations of around 30 mg g^{-1} DW and N-fertilized plants of around 50 mg g^{-1} DW.

When comparing chlorophyll and N concentrations in leaves at Gatersleben, both followed a similar trend. Only at T2 in 2012/13, leaf N concentrations were higher when plants were fertilized with N, which did not hold true for chlorophyll concentrations at this time point. Interestingly, in Cunnersdorf concentrations of N in leaves were approx. 7 and 20% higher under fertilization with ammonium nitrate than with urea, as observed at T2 during the first and third trial years, respectively. However, in presence of the UI 2-NPT leaf concentrations of this nutrient were also significantly higher that those observed for plants under urea fertilization, and reached similar concentrations as under ammonium nitrate. This might be related to a slower release of urea-N and higher absorption of N when urea hydrolysis was retarded by 2-NPT.

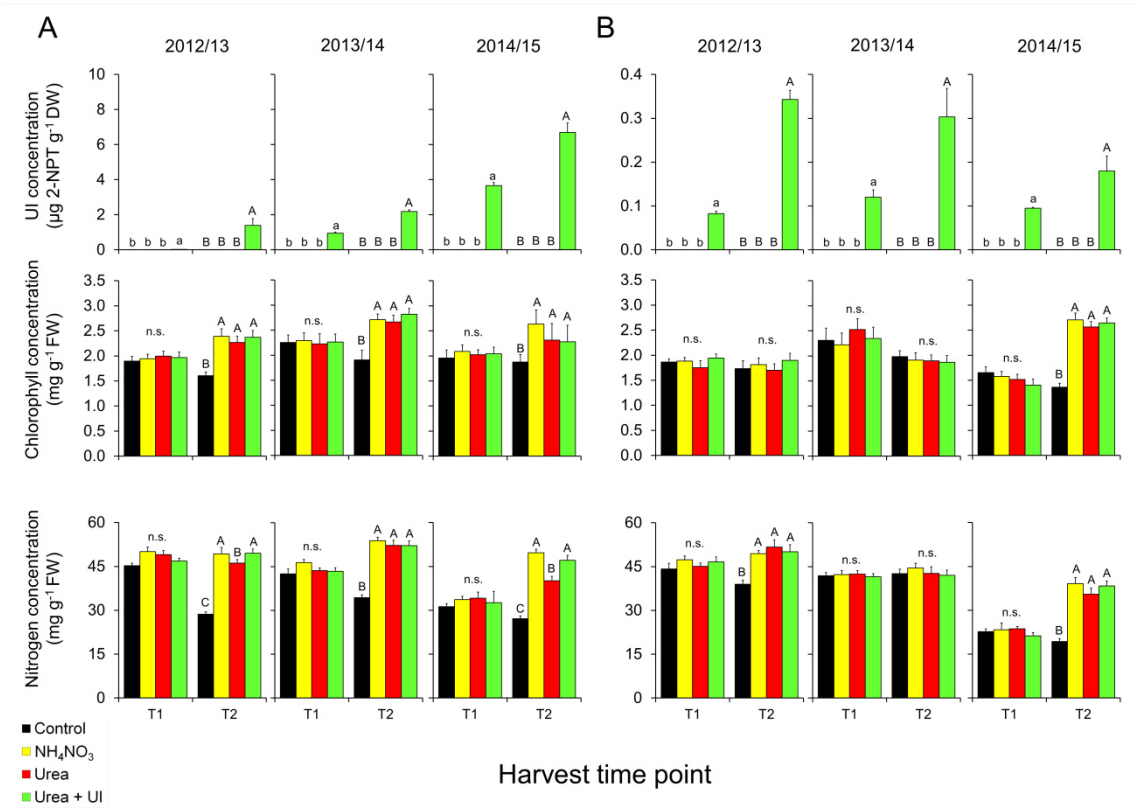


Figure 4-3. Influence of fertilization with different N forms (±UI) on 2-NPT, chlorophyll and N concentrations in leaves. Results are shown for the first (T1) and second (T2) harvest time point in **(A)** Cunnersdorf and **(B)** Gatersleben over the three years 2012/13, 2013/14 and 2014/15. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means ($P<0.05$ by Tukey's test; n.s., not significant).

4.2.3 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on macro- and micronutrient concentrations in leaves

Concentrations of macro- and micronutrients in leaves were measured a few days after each fertilization time point at either location (Figures 4-4 and 4-5) to investigate first whether nickel (Ni) was limiting the urease activity, since this micronutrient is required for the activity of this enzyme in plants (Gerendás & Sattelmacher, 1997b; Gerendás et al., 1999) and second, to check for changes in the concentrations of cationic nutrients, because after urea fertilization, hydrolysis of urea leads to an increase in soil pH in surrounding areas of urea granules (Jones et al., 2007), resulting in transient ammonium accumulation. Owing to nitrification, pH decreases and in consequence protons are released (Marschner, 2012), which can decrease the uptake of other cations (especially K^+, Ca^{2+}, Mg^{2+}), and thus modify the plant's cation-anion balance (Curtin & Wen, 2004).

All essential macroelements were measured, namely nitrogen (N), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg) and sulfur (S) (Figures 4-5A, B). Among the macronutrients, P concentrations in leaves at T1 were similar between non-fertilized plants and N-fertilized treatments at both experimental sites, and concentrations of this nutrient did not differ in response to fertilization with different N forms at this time point. Concentrations of this macronutrient were much higher in Cunnersdorf than in Gatersleben. Only in Cunnersdorf, an effect of N fertilization on P concentrations in leaves could be observed at T2 during 2012/13 and 2014/15, where concentrations were about 14-23% higher than those reached by control plants during the first and third trial years. Interestingly, at this location during the last trial year at T2, P concentrations in leaves were significantly higher after addition of 2-NPT, when compared to urea alone. Nevertheless, no differences were observed in Gatersleben, neither between non-fertilized and N-fertilized plants nor between different N forms. No consistent effect of the UI on P concentrations in leaves was found. In general, all plants were adequately supplied with P.

In Cunnersdorf, leaf K concentrations were generally between 25 and approx. 30 mg g^{-1} DW, but raised to approx. 38 mg g^{-1} DW only at T1 in 2012/13. The reason for this increase is unclear but may be related to the somewhat weaker accumulation of Ca and Mg at this time point. In Gatersleben, K concentrations in leaves tended to be only slightly lower than in Cunnersdorf, with concentrations of 17-33 mg g^{-1} DW. Despite some variation in this range, there was no consistent effect of N fertilizer treatment or of UI addition on leaf K concentrations. Obviously, all plants were adequately supplied with K.

In particular in Cunnersdorf, leaf Ca concentrations tended to be higher at T2 than at T1. To a lower extent, this observation also held true for Mg and S concentrations. In Cunnersdorf, N fertilization at T2 consistently increased leaf accumulation of these three elements, while in Gatersleben, this trend occurred only occasionally. Higher nutrient values at T2 may be related to plant or leaf age, as accumulation of these elements strongly depends on transpiration and thus profits from expanded periods of transpiration in older leaves. On top of that, a positive interaction between N and S nutrition is well documented and caused by their interdependency in amino acid and protein biosynthesis. While Ca and S concentrations indicated an adequate supply level with these elements, Mg concentrations were close to critical deficiency levels of approx. 1.5-3.0% (Bergmann, 1988).

Except for Cl, all essential micronutrients were detected, namely iron (Fe), copper (Cu), manganese (Mn), nickel (Ni), boron (B), molybdenum (Mo) and zinc (Zn). Concentrations of Fe in leaves were lower at Cunnersdorf, where concentrations reached up to 166 µg g^{-1} DW, whereas at Gatersleben they mounted up to 308 µg g^{-1} DW (Figures 4-5A, B). At both locations, concentrations of this micronutrient in leaves were similar at T1 between control and N-fertilized plants and no differences were observed among N treatments. Contrastingly, at T2 an influence was found of N fertilization on Fe concentrations at Cunnersdorf, where values were higher when N fertilization occurred. Fe concentrations at T2 in Cunnersdorf were 24% higher during 2014/15 after fertilization with urea and UI, when compared to urea alone. However, this observation was not consistent over all three cropping years. In Gatersleben, Fe concentrations in leaves at T2 were only different among treatments during the first year, where concentrations were influenced by N fertilization as well as by the N form, but not consistently over years.

Cu concentrations in leaves were similar at the first time point at both sites between control and N-fertilized plants, not revealing differences among N forms, except at Gatersleben during 2012/13, where some differences were detected and where control plants reached higher Cu concentrations in leaves than N-fertilized plants (about 8-20% more). Interestingly, at Cunnersdorf Cu concentrations at T2 followed the same pattern as Fe concentrations, as

there was an influence of N fertilization in all 3 years and higher concentrations when UI was added in 2014/15. At Gatersleben, Cu concentrations at the second harvest time point during the first and second trial years were influenced by N fertilization, but concentrations did not differ significantly between treatments. During the last year this was not observed, thus this trend was not consistent over years.

Regarding Mn, concentrations in leaves were much lower at Cunnersdorf than at Gatersleben, since concentrations mounted only up to 38 µg g^{-1} DW, whereas at Gatersleben they reached up to 65 µg g^{-1} DW. This may be due to the more compact soil structure in Gatersleben, favoring Mn reduction and thus a larger amount of plant-available Mn^{2+}. At both experimental sites, Mn concentrations at T1 were the same between control and N-fertilized plants. At T2, only at Cunnersdorf some differences between N treatments were observed during the first and third year, whereas at Gatersleben no significant differences were registered. In Cunnersdorf, these differences corresponded to an increase of Mn concentrations upon N fertilization by 35-40% in 2012/13 and by > 48% in 2014/15. Anyways, in all treatments Mn concentrations were clearly above critical deficiency levels, which are around 20 µg g^{-1} (Marschner, 2012).

According to Bergmann (1988) and Reuter & Robinson (1997), all measured Ni concentrations in leaves were above the critical deficiency level of 0.1 µg g^{-1} DW, thus being sufficient for urease activity in plants. At Cunnersdorf, differences among treatments were only observed during 2014/15, where Ni concentrations were 28% higher when UI was added. In Gatersleben, Ni concentrations in leaves of control and N-fertilized plants reached similar levels at both time points during 2012/13. Nonetheless, during the second trial year concentrations of this micronutrient increased when UI was added at both time points, but this was not the case in the following year, when at T2 Ni concentrations were about 2-fold higher than in N-fertilized plants.

Concentrations of B and Mo in leaves were approx. the same at both experimental sites and also at both harvest time points. Differences between treatments were observed only at Gatersleben, where at T2 concentrations were up to 17% higher under fertilization with NH_4NO_3 or urea alone, when compared to urea with 2-NPT. Finally, Zn concentrations in leaves were similar between control and N-fertilized plants at T1 in Cunnersdorf, but also in Gatersleben. Differences between treatments were observed only at T2, where N fertilization influenced Zn concentrations in leaves at both locations. At Cunnersdorf, Zn concentrations in leaves at T2 ranged between 12 and 15 µg g^{-1} DW in control plants in all 3 experimental years, where N fertilization led to an increase in these concentrations of up to 20 µg g^{-1} DW, especially under fertilization with ammonium nitrate or urea with UI. This was observed during 2012/13 and 2014/15, similar as for N or S concentrations described above. At Gatersleben, at T2 during the first and second trial years an influence of N fertilization on Zn concentrations was found, where N-fertilized plants reached 59 and 22% higher leaf concentrations than control plants during 2012/13 and 2013/14, respectively. Nevertheless, this was not consistent, since it was not observed during the last trial year.

Taking all observations together, the influence of N fertilization on concentrations of macro- and micronutrients in leaves depended mostly on the experimental site, i.e. the soil type and other properties of the site. Despite some influence of soil type and N fertilization on nutrient concentrations in leaves, all nutrient concentrations remained above their critical deficiency levels and thus indicated that none of the wheat plants suffered from nutrient deficiency.

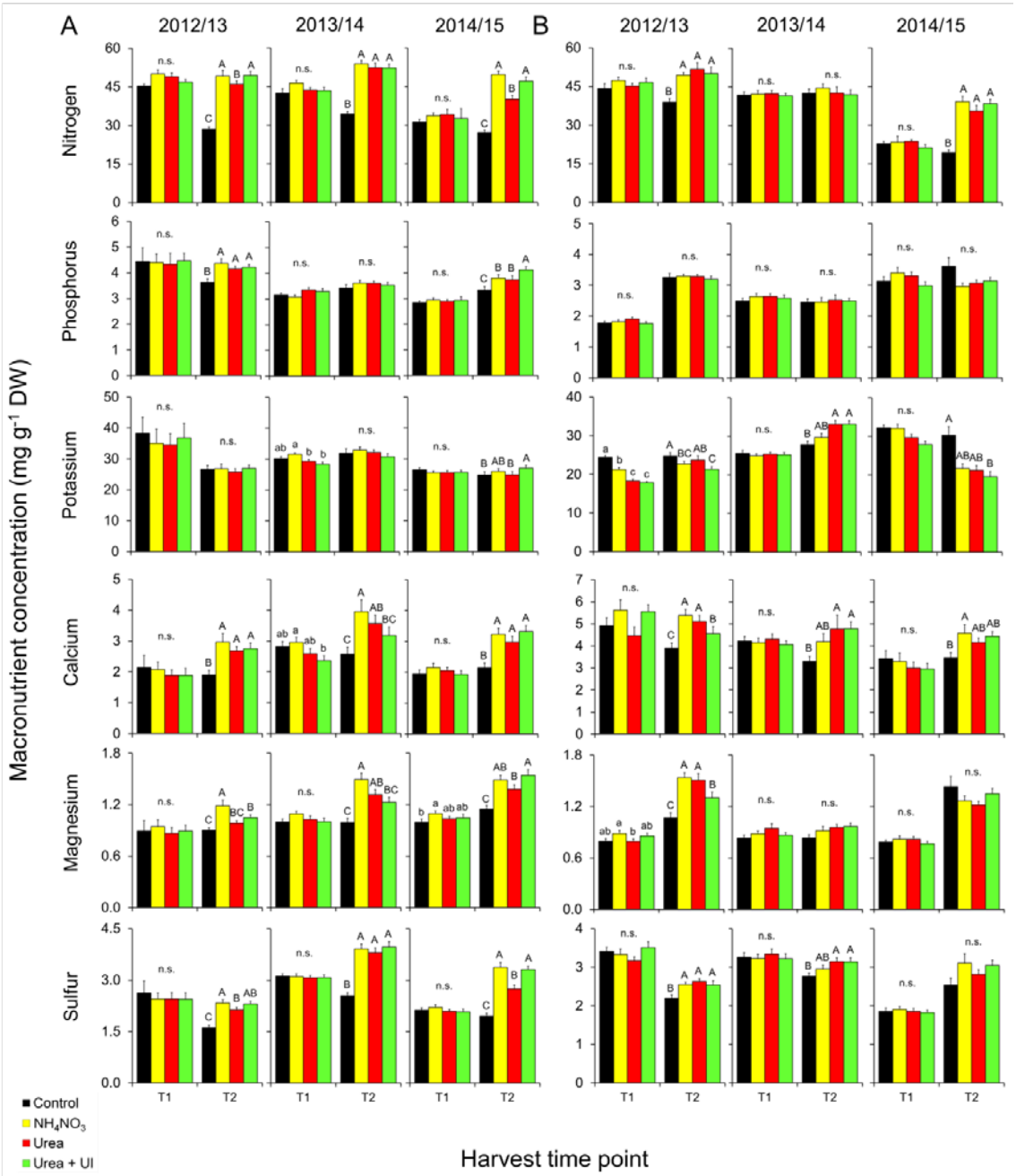


Figure 4-4. Influence of fertilization with different N forms (±UI) on macronutrient concentrations in leaves. Results are shown for the first (T1) and second (T2) harvest time point in **(A)** Cunnersdorf and **(B)** Gatersleben over the three trial years 2012/13, 2013/14 and 2014/15 in winter wheat. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

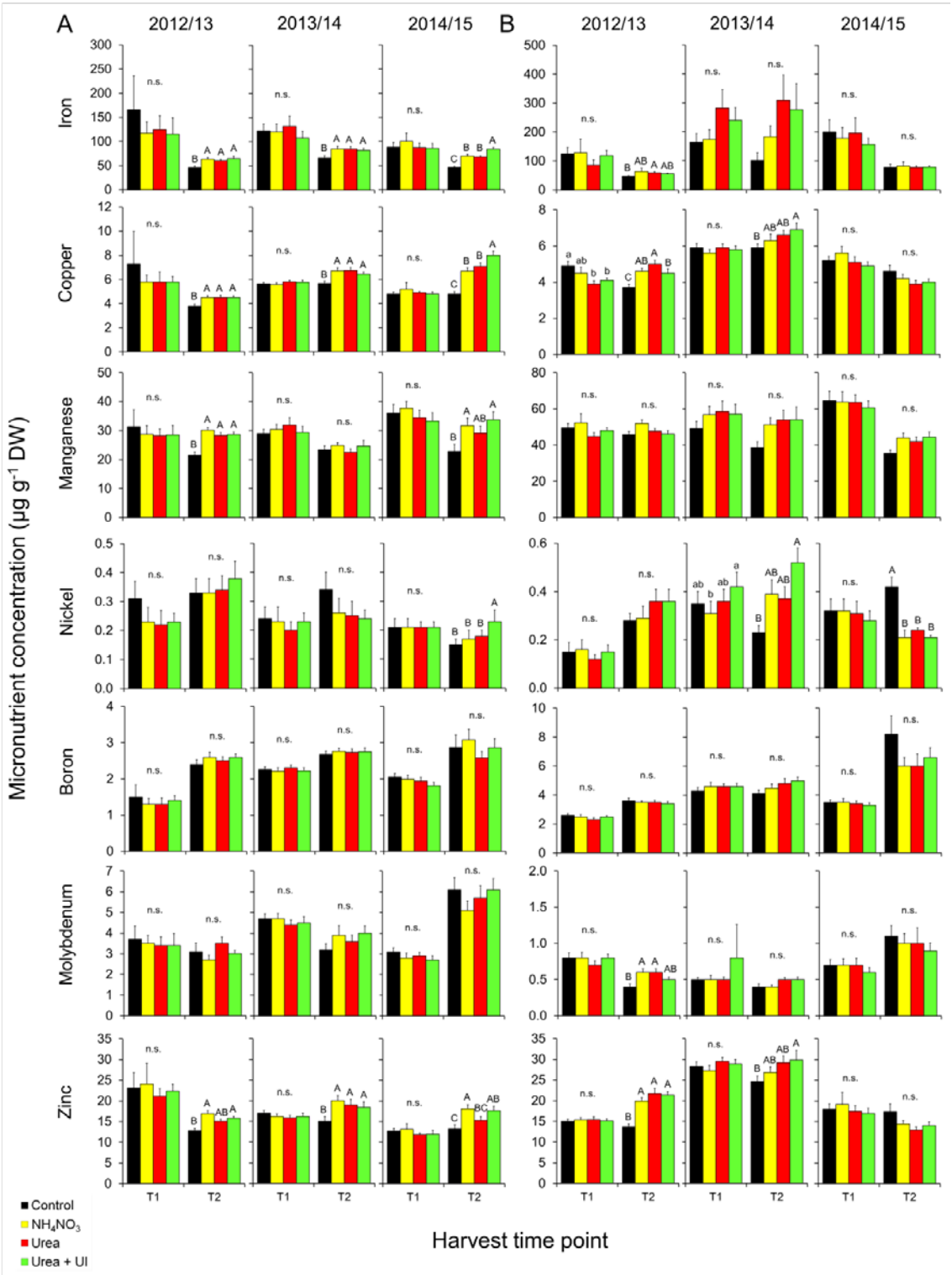


Figure 4-5. Influence of fertilization with different N forms (±UI) on micronutrient concentrations in leaves. Results are shown for the first (T1) and second (T2) harvest time point in **(A)** Cunnersdorf and **(B)** Gatersleben over the three trial years 2012/13, 2013/14 and 2014/15 in winter wheat. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

4.3 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on plant N metabolism in winter wheat

After having learnt that the UI 2-NPT can be taken up by plant roots, it was important to assess whether this compound modified translocation rates of N forms, namely of urea, NH_4^+, NO_3^- or amino acids (AA). Influences of N fertilization and of UI addition on translocation rates of individual AA are presented separately according to the classification of different metabolic pathways, as proposed by Taiz & Zeiger (2010) and Coruzzi (2015). As it was explained previously in section 4.2.1, data of translocation rates at T1 for Cunnersdorf during 2012/13 can not be presented. Considering the presence of 2-NPT in leaves (Figure 4-3), the influence of N fertilization but also of the addition of this UI on soluble sugars in leaves as well as on individual AA and N forms was investigated. Moreover, it was investigated whether plant urease activity in leaves was affected by the addition of the UI.

4.3.1 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on translocation rates of urea, ammonium, nitrate and total amino acids

A few days after each fertilization time point, xylem exudates were collected at both experimental sites, allowing the measurement and calculation of translocation rates of urea, ammonium, nitrate and total amino acids in these samples. These results are shown in Figure 4-6A for Cunnersdorf and Figure 4-6B for Gatersleben.

Urea translocation rates were much higher at Cunnersdorf than at Gatersleben, reaching translocation rates at T2 during 2012/13 of 736 and 234 pmol h^{-1} $plant^{-1}$ in Cunnersdorf and Gatersleben, respectively, and 97 and 41 pmol h^{-1} $plant^{-1}$ in the 2 following years at Cunnersdorf and Gatersleben, respectively. At both experimental locations at the first harvest time point, urea translocation rates were very low in control and NH_4NO_3-fertilized plants, due to the low amount of urea in the soil. After fertilization with urea alone, translocation rates of urea increased significantly, especially at T1. In presence of the UI, urea translocation rates were 5- to 15-fold higher than under urea alone, where the highest values were always observed at T2. These high urea translocation rates correlated very well ($r^2 > 0.99$) with translocation rates of the UI 2-NPT at both experimental sites, as shown in detail in Annex 1. Moreover, strong correlations between urea translocation rates and the content of urea-N in the soil were found especially at Cunnersdorf (Annex 2), which indicated a higher efficacy of this compound over the three experimental years at this location. Thus, these analyses showed that the addition of the UI effectively delayed urea hydrolysis also in Gatersleben.

At Cunnersdorf, translocation of ammonium-N was consistently affected by N fertilization in all years, as ammonium translocation rates of N-fertilized plants exceeded those of control plants. Among N treatments, only a few differences were observed, namely during the first trial year at T2, where ammonium translocation rates were much higher under fertilization with NH_4NO_3, which was not consistent in the following two years and also at T1 during 2013/14, where ammonium translocation rates were higher under fertilization with urea alone, when compared to the other N treatments. In Gatersleben, ammonium translocation rates did not follow any consistent influence neither by N fertilization nor by addition of the UI. Higher translocation rates of ammonium were registered at T1 during 2012/13 under fertilization with ammonium nitrate. A significant increase by N fertilization was observed only at T2 in 2013/14. Furthermore, no significant correlation could be found between ammonium and UI translocation rates at either location (Annex 3) or between ammonium translocation rates and NH_4-N contents in the soil (Annex 4).

In all three years and at both time points, nitrate translocation rates were highest mostly in plants fertilized with NH_4NO_3, while control plants generally showed lowest translocation rates. In a direct comparison, nitrate translocation rates of plants fertilized with NH_4NO_3 at T1 were only slightly higher or tended to be higher than those of plants fertilized with urea. The UI itself had no consistent influence on nitrate translocation rates after urea fertilization, which was verified by correlating translocation rates of the UI and nitrate (Annex 5). On the other hand, nitrate translocation rates in the xylem sap correlated well with soil nitrate contents at most time points, indicating that nitrate availability in the soil rather than plant development determined root-to-shoot transport of nitrate (Annex 6).

The largest fraction of the N pool translocated through the xylem sap corresponded to amino acids. Their concentrations were more than 10 times larger than those of nitrate and even more than 100 times larger than those of ammonium. Regarding the impact of different N fertilizer forms, translocation rates of amino acids mainly reflected those of nitrate and in particular of ammonium. Taken together, this most likely reflected that the majority of the ammonium was assimilated into amino acids before xylem loading. Addition of 2-NPT to urea had no consistent effect on amino acid translocation rates.

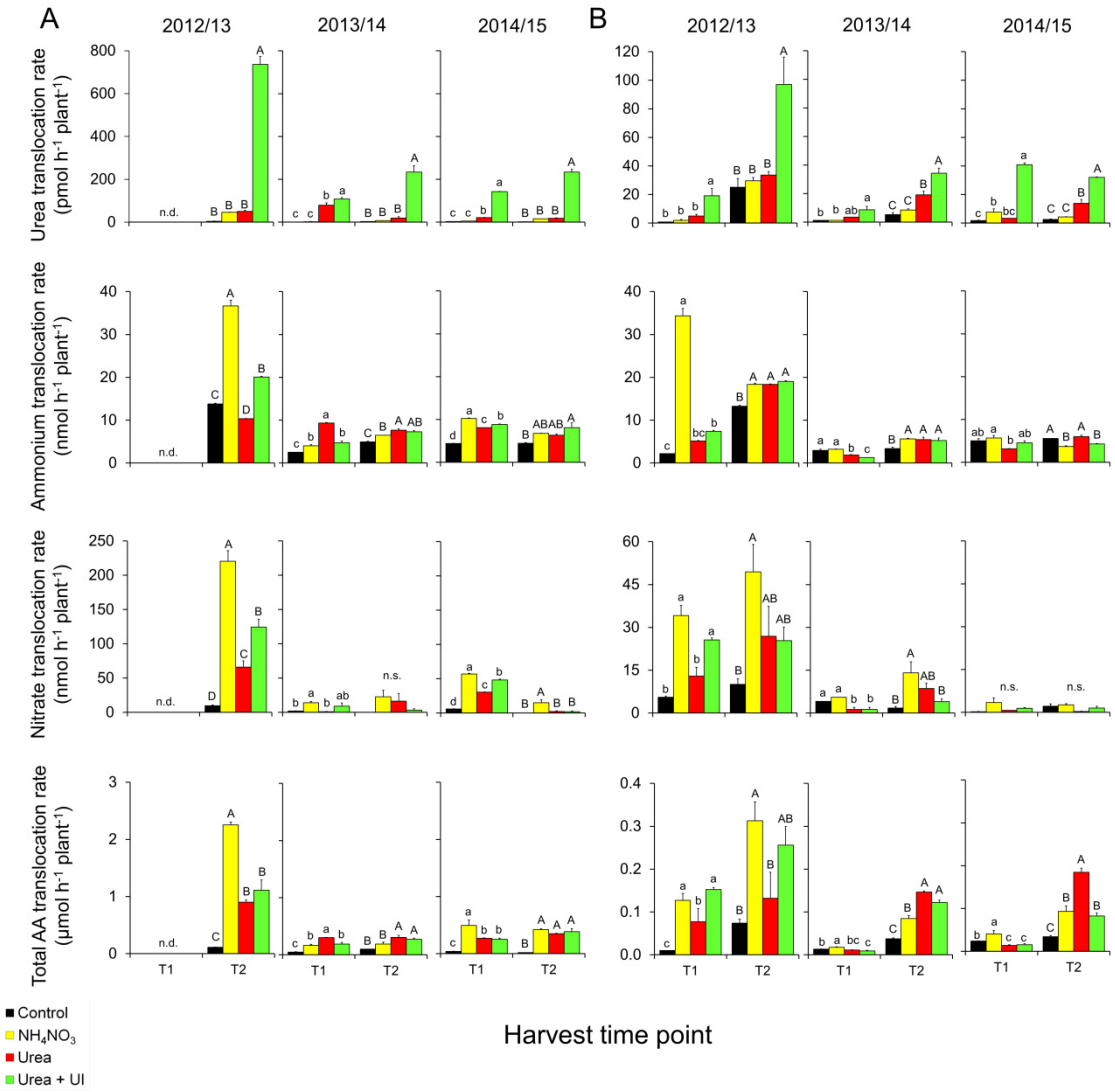


Figure 4-6. Influence of fertilization with different N forms (±UI) on translocation rates of urea, ammonium, nitrate and total amino acids (AA) in the xylem sap. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant n.d.; not determined).

4.3.2 *Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on translocation rates of single amino acids*

As it was shown in the previous section (4.3.1), translocation rates of total amino acids in the xylem sap were altered by N fertilization at both experimental sites and also by the addition of UI, however, not consistently. Additionally, taking all amino acids together did not allow determining to what extent each AA contributed to these effects. In order to determine which amino acids were responsible for the observed effects, translocation rates of 20 single amino acids were analyzed separately. These AA are grouped according to their respective biosynthesis pathways (Figure 4-7). Among them, only translocation rates of the most relevant AA for N assimilation (ref. to section 1.2.3) are presented in this results section (Figures 7D and 7E indicated in blue), whereas the others are available in the annex (Annexes 7 to 11).

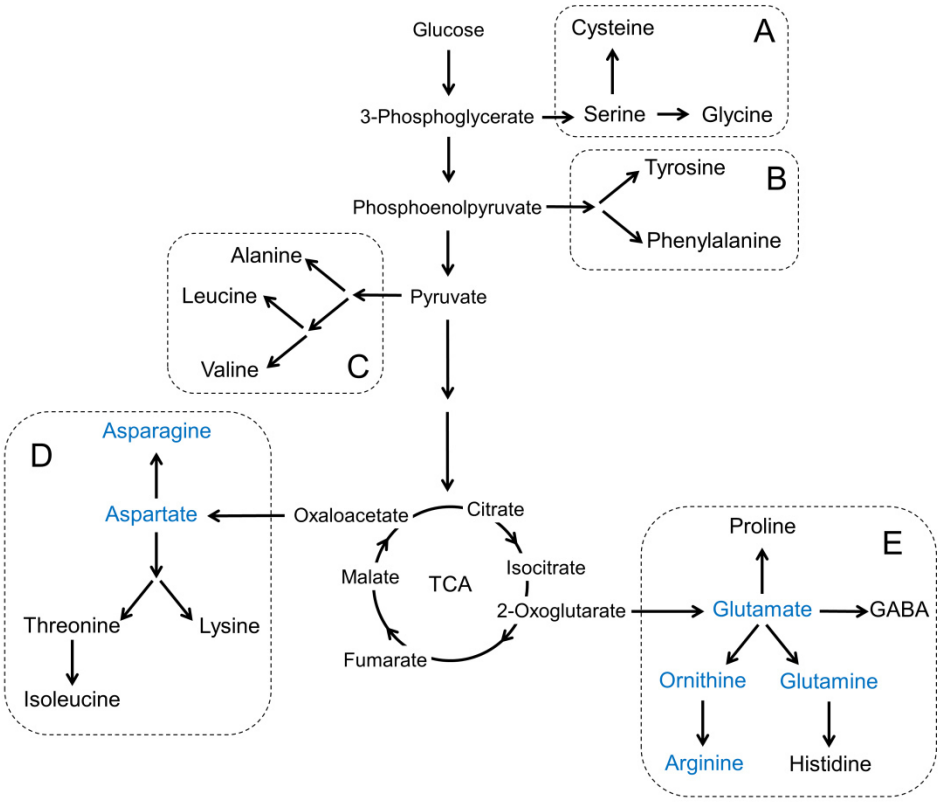


Figure 4-7. Amino acids are synthesized via different pathways in higher plants. Results in the present study for amino acids are shown divided in groups, according to their biosynthesis pathway. Amino acids in **A)** are those synthesized from 3-phosphoglycerate, in **B)** from phosphoenolpyruvate, in **C)** from pyruvate, in **D)** from oxaloacetate and in **E)** from 2-oxoglutarate. Results on translocation rates and leaf concentrations of amino acids from **A**, **B**, **C**, **D** and **E** are available in Annexes 7, 8, 9, 10 and 11 or 13, 14, 15, 16 and 17, respectively. In blue are indicated relevant amino acids (Asn, Asp, Glu, Gln, Orn and Arg) for plant-N assimilation (ref. to section 1.2.3). Dotted lines group amino acids together according to their biosynthesis pathways. TCA indicates tricarboxylic acid cycle. Adapted from Taiz & Zeiger (2010).

According to Stryer et al. (2017), serine (Ser) is an amino acid synthesized from 3-phosphoglycerate (3PGA), which is the precursor to form glycine (Gly) and cysteine (Cys). Translocation rates in xylem exudates of these three amino acids (Figure 4-7A) are presented in Annex 7 for Cunnersdorf and Gatersleben. Control plants had the lowest Ser translocation rates at Cunnersdorf at both harvest time points, except at T2 during the second trial year where rates reached similar levels as after fertilization with ammonium nitrate. After fertilization with N, these translocation rates increased, presenting also variations between N treatments. During the first year at T2, highest Ser translocation rates were found after fertilization with NH_4NO_3 and no differences were observed between plants under urea (±UI) fertilization. This observation was not consistent in the 2 following years, when Ser translocation rates tended to be higher after fertilization with urea during 2013/14 or were similar among N treatments during the last trial year. Interestingly, Gly and Cys translocation rates followed the same pattern as their precursor Ser. However, some differences were apparent between the two sites. For instance, after addition of urea with 2-NPT at T2, translocation rates of Cys were significantly higher than after urea alone. At Cunnersdorf this effect was consistent and might have

contributed to higher S concentrations in leaves (Figure 4-4A), while it was absent in Gatersleben. In contrast, Ser translocation rates in control plants at Gatersleben were similar to those reached by N fertilization, with one exception at T1 during 2012/13, where all treatments significantly differed among each other. On the other hand, Ser translocation rates at T2 were up to 2-fold higher during the second and third trial years after fertilization with urea alone, when compared to urea with UI or ammonium nitrate. The latter was not observed during 2012/13. Gly translocation rates followed a very similar pattern as Ser translocation rates, showing the highest rates after fertilization with urea alone during 2013/14 and 2014/15. Probably, the application of UI to urea decreased translocation rates, so that plants reached similar rates as under fertilization with NH_4NO_3. At Gaterseben, addition of UI to urea significantly decreased Cys translocation rates at T2 during the second and third trial year, which was the opposite at Cunnersdorf. Taking these observations together, not only N fertilization but also addition of UI altered translocation rates of amino acids synthesized from 3PGA.

Tyrosine (Tyr) and phenylalanine (Phe) are synthesized from phosphoenolpyruvate in the shikimate pathway (Figure 4-7B), as described by Tzin & Galili (2010). Tryptophan (Trp), which is also synthesized through this pathway, will be presented in the following section 4.4 together with auxin, as Trp is a precursor for auxin biosynthesis (Bartel, 1997). Translocation rates of theses AA are presented for both locations in Annex 8. At Cunnersdorf, an influence of N fertilization was observed on Tyr translocation rates in the xylem sap at T1, where translocation rates in control plants reached only 0.4-0.5 nmol h^{-1} $plant^{-1}$. Notwithstanding, after fertilization with N, translocation rates of Tyr increased up to 0.9 nmol h^{-1} $plant^{-1}$, especially under fertilization with urea alone in 2013/14 or urea with UI in 2014/15. In the subsequent years, N fertilization led to a slight increase in Tyr levels. At Gatersleben, N fertilization increased Tyr translocation rates only in 2012/2013. Thereafter, there was no consistent effect of N fertilization irrespective of UI supplementation. In general, gross changes in phenylalanine concentrations followed those of Tyr at both experimental sites, and neither N fertilization nor UI supplementation had a consistent impact on Phe translocation rates. Taking together, at both experimental sites neither N fertilization nor addition of UI played an important role in the translocation of Tyr and Phe in the xylem sap.

Alanine (Ala), leucine (Leu) and valine (Val) are derivatives of pyruvate (Figure 4-7C). In Cunnersdorf, Ala translocation rates were markedly influenced by N fertilization at both time points over the three years of field experiments, where control plants presented the lowest translocation rates (Annex 9A). This was not observed in Gatersleben, where N fertilization had no consistent impact on Ala translocation rates (Annex 9B). Regarding the influence of the UI on Ala translocation rates, there was no consistent influence, neither in Cunnersdorf nor in Gatersleben. The latter is important to highlight, since according to Atanasova (2008), increased Ala can be attributed to unbalanced N nutrition. Translocation rates of Leu and Val showed similar patterns to those of Ala. In general, translocation rates of Leu and Val were higher at T2 than at T1, which may reflect the more active amino acid anabolism of wheat leaves at a more advanced developmental stage. An increase of Leu and Val translocation rates by N fertilization was mostly apparent but varied strongly in dependence of site and year. Urea fertilization or the addition of 2-NPT showed no consistent impact.

Originating from the tricarboxylic acid cycle (TCA), oxaloacetate participates in the biosynthesis of aspartate (Asp). Asp serves then as precursor for asparagine (Asn), lysine (Lys) and threonine (Thr) (Bromke, 2013; Figure 4-7D). Finally, isoleucine (Ile) is biosynthesized from Thr (Singh & Shaner, 1995). The influence of N fertilization and addition of UI on translocation rates in xylem sap of Asp and Asn is presented in Figure 4-8A for Cunnersdorf and Figure 4-8B for Gatersleben, and in Annex 10 for Lys, Thr and Ile. Translocation rates of Asp at Cunnersdorf were roughly between 0.3-6.5 nmol h^{-1} $plant^{-1}$, whereas in Gatersleben they varied between 0.1-23 nmol h^{-1} $plant^{-1}$. In Cunnersdorf, Asp translocation rates were neither consistently influenced by N fertilization nor by addition of UI. At both locations highest translocation rates of Asp were observed at T1 after fertilization with NH_4NO_3 during 2012/13. However, the reason for this remains unclear. A consistent positive impact of N fertilization on Asp levels in the xylem sap was only observed at T2 throughout all years, while an impact of UI supplementation to urea fertilization remained inconsistent. Together with Glutamine (Gln), which will be presented in Figure 4-9, Asparagine (Asn) was one of the most abundant AA in the xylem sap, reaching higher rates at Cunnersdorf than at Gatersleben. At Cunnersdorf, an influence of N fertilization on Asn translocation rates was not observed at T1, since during 2013/14 control plants had similar rates as after fertilization with NH_4NO_3 and during 2014/15 similar rates as after fertilization with urea alone. At T2, an influence of N fertilization became apparent, but not consistently, since during the second trial year plants fertilized with ammonium nitrate had similar translocation rates as control plants. As reported by

Zanin et al. (2015), it was expected that urea nutrition leads to an increase in Gln and Asn synthesis in roots, since urea hydrolysis produces NH_4^+, which is subsequently assimilated into these AA. When compared to control plants, higher Asn translocation rates were indeed observed at both time points and at both experimental sites, independently of UI addition. In Gatersleben, a consistent effect of N fertilization on the translocation of Gln as well as of Asn was only observed at T2. The addition of the UI showed no consistent impact. Translocation rates of Lys, Thr and Ile in the xylem sap were very similar, following a comparable pattern at both locations (Annex 10). In general, N fertilization increased translocation of these AA in Cunnersdorf only during 2014/15 and in Gatersleben at most sampling dates, however, not consistently. Again, an impact of urea fertilizer supplementation with 2-NPT remained absent.

Taking together, translocation rates in the xylem sap of Asp, Asn, Lys, Thr and Ile were in general not altered by N fertilization. Moreover, fertilization of urea in combination with 2-NPT had an influence only on translocation rates of Asp and Asn at Gatersleben, specifically at T2, which could be determined only in the last two experimental years.

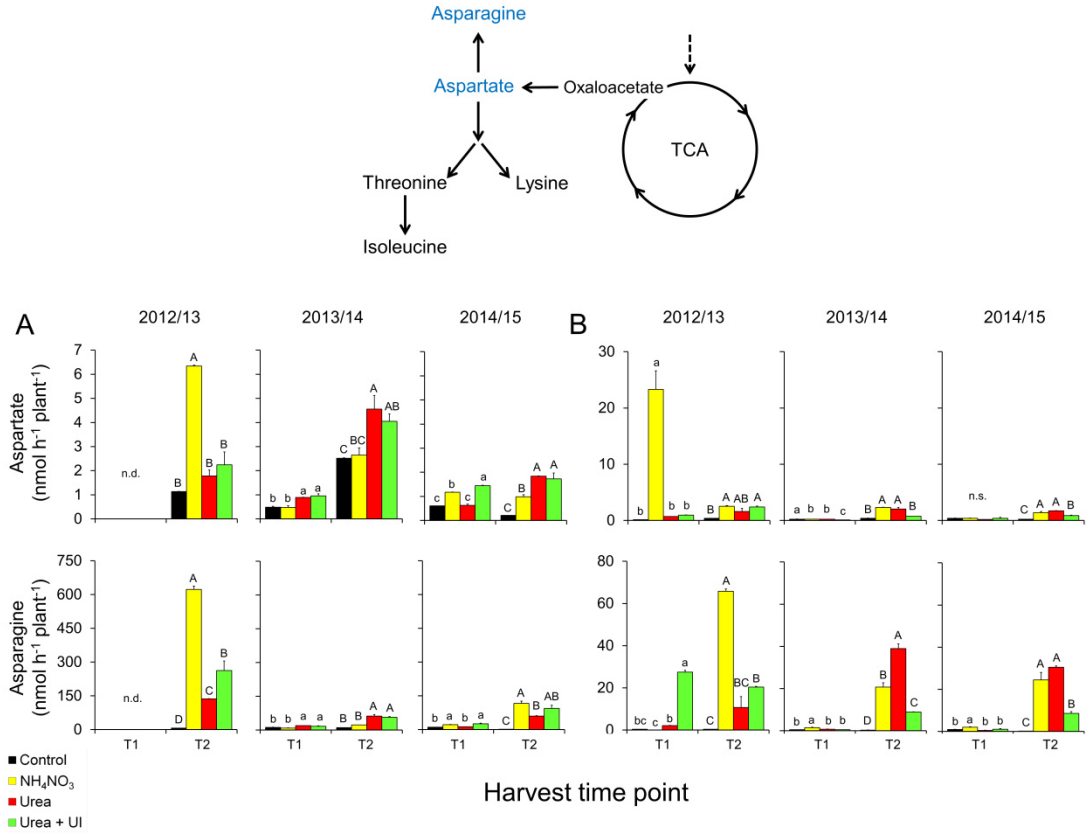


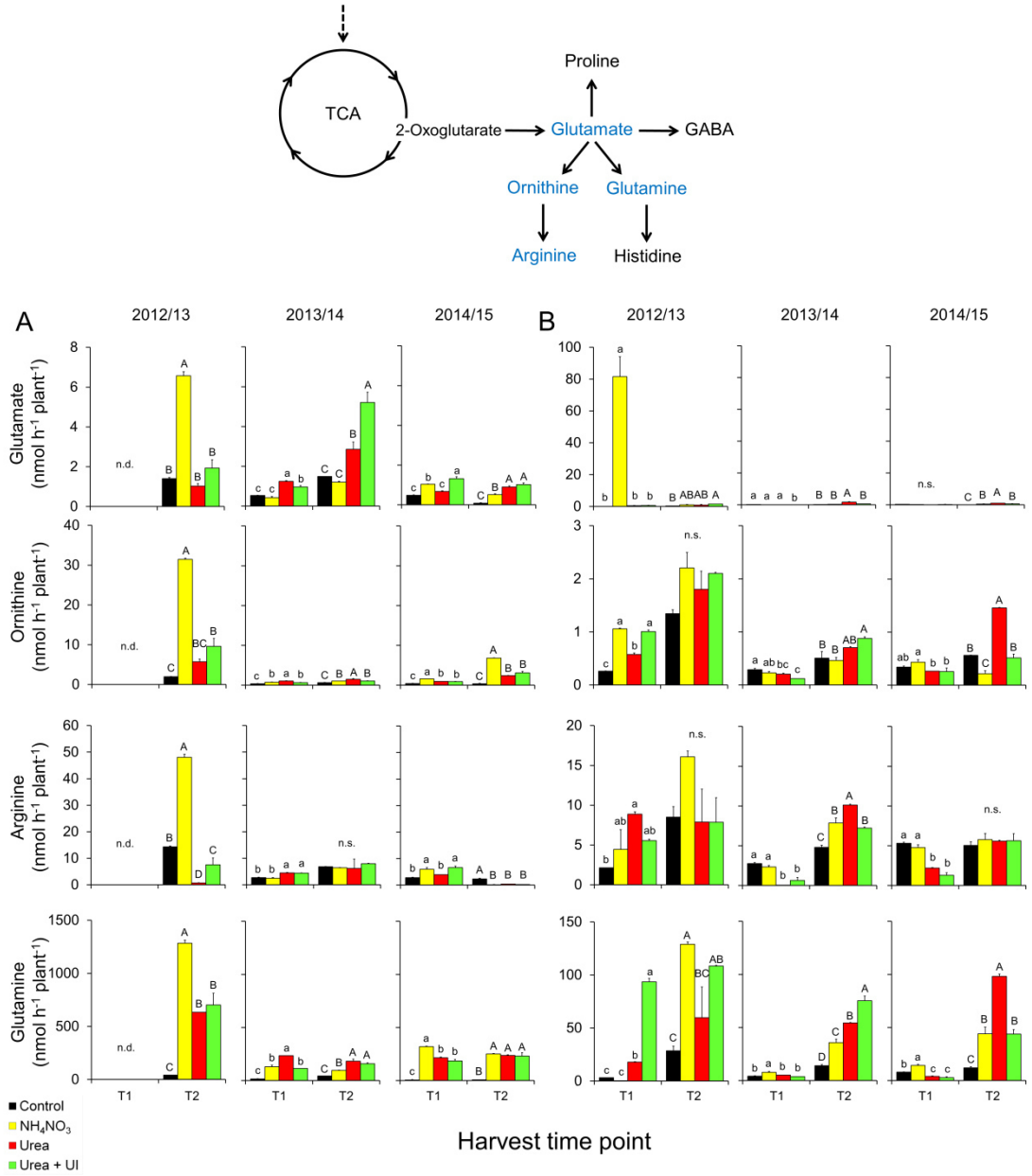
Figure 4-8. Influence of fertilization with different N forms (±UI) on translocation rates of aspartate and asparagine in the xylem sap. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined). TCA indicates tricarboxylic acid cycle. Ref. to Annex 10 for influence on lysine, threonine and isoleucine.

The TCA cycle intermediate 2-oxoglutarate (2-OG) triggers the biosynthesis of glutamate (Glu), which in consequence participates in the synthesis of other AA, among them proline (Pro), gamma-aminobutyric acid (GABA), ornithine (Orn), arginine (Arg), glutamine (Gln) and histidine (His) (Taiz & Zeiger, 2010; Figure 4-7E). The influence of N fertilization with different N forms including the addition of UI on translocation rates in xylem sap of Glu, Orn, Arg and Gln is presented in Figure 4-9A for Cunnersdorf and Figure 4-9B for Gatersleben, whereas in Annex 11 on translocation rates of Pro, GABA and His at both sites.

At Cunnersdorf at T1, translocation rates of Glu in the xylem sap were not consistently influenced neither by N fertilization nor by addition of the UI. At T2, translocation rates were also not affected consistently by N fertilization or presence of UI, finding e.g. the highest rates under fertilization with ammonium nitrate during the first year, under fertilization with urea + UI during the second trial year and similar rates among urea (+UI)-fertilized treatments during the last cropping year. Similarly, in Gatersleben Glu translocation rates were significantly influenced neither by N

fertilization nor by addition of the UI. Interestingly, Pro was affected by N fertilization at both time points in Cunnersdorf, where Pro translocation rates were roughly between 0.1-2.0 nmol h^{-1} $plant^{-1}$ in control plants and between 1.0-6.7 nmol h^{-1} $plant^{-1}$ in N-fertilized plants. Regarding the N forms, there was no consistent influence on Pro translocation rates, also not by addition of the UI. At Gatersleben, no such influence was found. Noteworthy, at both experimental sites, the highest Pro translocation rates were found at T2 in 2012/13. Translocation rates of GABA, Orn and His increased significantly by N fertilization only in 2014/15 in Cunnersdorf and at T1 during 2012/13 in Gatersleben. There was no clear effect of the addition of 2-NPT. Arg is used for N storage and transport in many plants (Hildebrandt et al., 2015; ref. to section 1.2.3). Surprisingly, Arg was among the AA which showed the least changes in response to N fertilization, as translocation rates remained pretty constant among N treatments while they varied considerably among sampling dates.

Taken together, among translocation rates of these AA in xylem sap, only those of Pro, Orn, Gln and His were consistently influenced by N fertilization just at the Cunnersdorf site (mostly at T2), whereas in Gatersleben this was observed only at T2 for Gln translocation rates. There was no consistent significant response to the fertilization with different N forms. Moreover, addition of UI did not alter consistently translocation rates of the investigated AA.

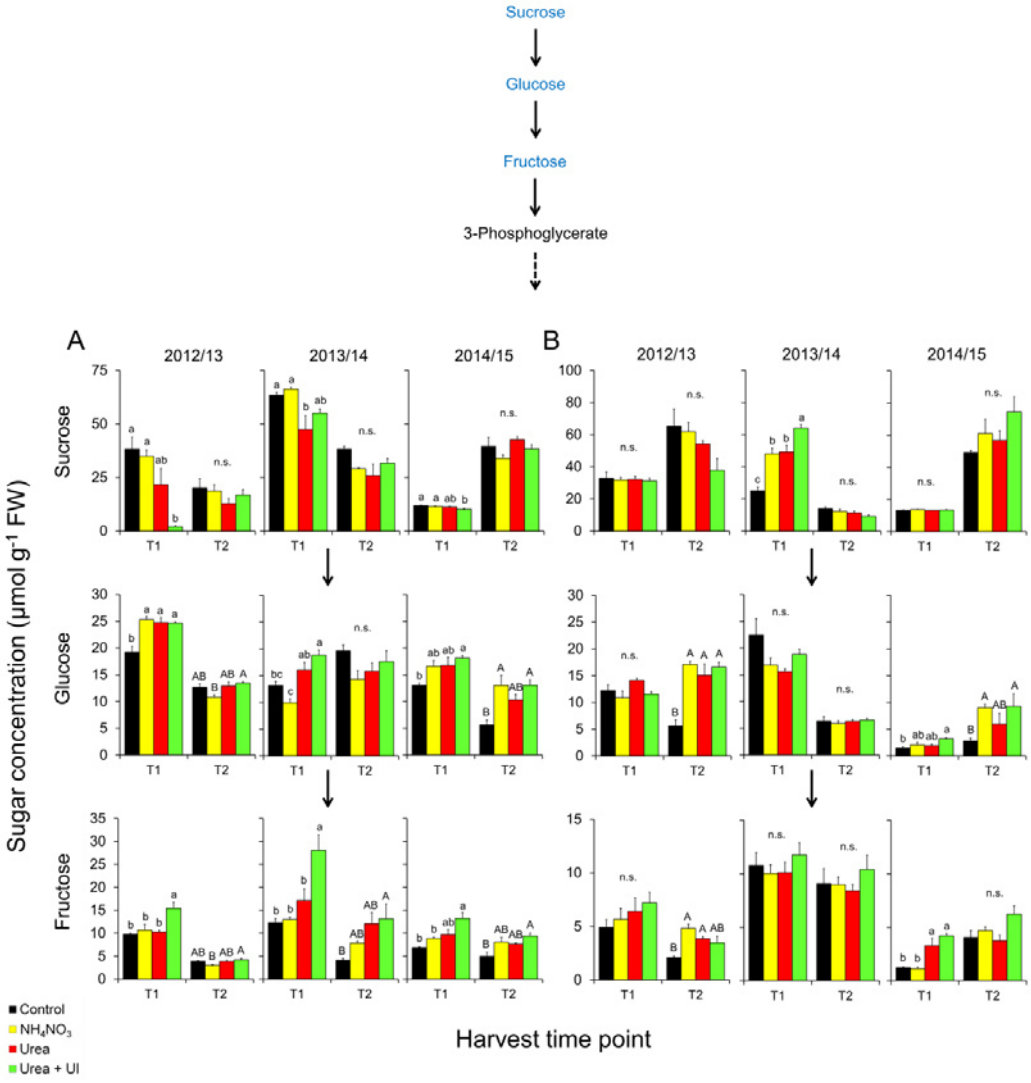


► **Figure 4-9.** (For description of this Figure refer to next page).

◄ **Figure 4-9. Influence of fertilization with different N forms (±UI) on translocation rates of glutamate, ornithine, arginine and glutamine in the xylem sap.** Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined). TCA indicates tricarboxylic acid cycle. Ref. to Annex 11 for influence on proline, GABA and histidine.

4.3.3 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on sugar concentrations in leaves

Soluble sugars are carbohydrates, which represent metabolic precursors for the build-up of starch during grain filling (Pask & Pietragalla, 2011), being directly involved in determining grain yield (Wang et al., 2015). Concentrations of soluble sugars in leaves, namely sucrose (Suc), which is broken down into glucose (Glc) and fructose (Fru) (Rosa et al., 2009), were determined at Cunnersdorf site (Figure 4-10A) and at Gatersleben site (Figure 4-10B). It was assessed whether there was influence of fertilization with different N forms on the concentrations of these individual sugars in leaves. As also reported by Østrem et al. (2011), Suc was found to be the major carbohydrate at both experimental sites, ranging in concentrations that were roughly 2-10-fold higher than those of Glc or Frc. Apart from T1 during 2013/14 when there was a clearly positive influence of N fertilization on Suc concentrations, there was no further consistent impact of N fertilization or UI addition on Suc levels in leaves. Instead, Suc concentrations varied strongly in dependence of year, harvest date and site. Concentrations of Glc and Frc in leaves roughly showed a similar pattern as those of Suc with mostly parallel changes for Glc and Frc upon sampling date and site. A significant increase of Glc and Frc concentrations by N fertilization appeared in approx. half of the harvesting dates. However, an impact of UI supplementation to urea fertilization remained absent.



► **Figure 4-10.** (For description of this Figure refer to next page).

◄ Figure 4-10. Influence of fertilization with different N forms (±UI) on concentrations of sucrose, glucose and fructose in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

4.3.4 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on single amino acids and organic acids concentrations in leaves

To assess, whether the N fertilization or the addition of the UI 2-NPT had an influence on concentrations of amino acids (AA) as well as organic acids in leaves, leaf samples were collected a few days after each fertilization time point at each experimental location. In the same way as for translocation rates of AA shown in the previous section 4.3.2, concentrations of the most relevant AA for N assimilation (ref. to section 1.2.3; Figure 4-7) are presented in this results section (Figures 4-12 and 4-13) and the remaining AA are available in the annex section (Annexes 13 to 17).

As reported previously by Robinson & Hodges (1977), concentrations of individual AA in leaves can increase or decrease after fertilization with different N sources. These authors observed e.g. that concentrations of Ser, Tyr, Thr, Lys, Ile, Glu and Pro in leaves of *Poa pratensis* significantly increased under fertilization with NH_4NO_3, when compared to control plants. Correlations between AA translocation rates in xylem exudates and AA concentrations measured in leaves were calculated for each AA (Annex 12), where Asp, Gln, and His correlated well at Cunnersdorf (T1), Gly at Gatersleben (T1) and also Gln at Cunnersdorf at T2. Interestingly, close correlations were found for Asp and Glu in Gatersleben at T2. Nevertheless, during 2013/14 correlations were also strong but negative.

Concentrations of Ser, Cys and Gly in leaves are shown in Annex 13 for both experimental locations. At Cunnersdorf, Ser concentrations in leaves were not consistently influenced by N fertilization at T1. However, at T2 a strong influence was observed of N fertilization on leaf concentrations of Ser, where concentrations in control plants ranged between 5.2-11.5 µmol g^{-1} FW and N-fertilized plants reached up to 28.3 µmol g^{-1} FW. Nonetheless, among the N-fertilized treatments no consistent differences were observed. At both time points in Gatersleben, neither an influence of N fertilization nor of the addition of 2-NPT was found. Leaf concentrations of Cys responded strongly even though inconsistently to N fertilization at Cunnersdorf without showing a particular response to UI supplementation. In Gatersleben, however, Cys concentrations during 2013/14 and 2014/15 were down, irrespective of N fertilizer forms. The reason for this severe drop remained unclear. Concentrations of Tyr and Phe in leaves were also determined over the three years of experiments and are shown in Annex 14. Significant differences in concentrations of these two AA in leaves among treatments at both experimental sites were found occasionally. Concentrations of Ala, Leu and Val in leaves are shown in Annex 15. At both sites, quite similar concentrations of Ala, Leu and Val were found for control and N-fertilized plants at T1. An effect of N fertilization was more apparent at T2, when N-fertilized plants mostly increased leaf concentrations of Ala, but less consistently of Leu and Val. The addition of the UI to urea fertilization did not alter concentrations of these AA in leaves at any experimental site.

To further investigate the influence of fertilization with different N forms and addition of UI to urea fertilizer, leaf samples were harvested after each fertilization time point to determine the concentration in leaves of four organic acids, which play a role within the TCA cycle. As a result of incomplete oxidation of photosynthetic assimilates, organic acids can accumulate in leaves for subsequent AA biosynthesis (Igamberdiev & Eprintsev, 2016). Measured organic acids were malate (Mal), citrate (Cit), fumarate (Fum) and isocitrate (Isocit). Results after measurements are shown in Figure 4-11A for Cunnersdorf and in Figure 4-11B for Gatersleben. Among the organic acids, Isocit was the most abundant at both experimental sites, reaching concentrations up to 295 µmol g^{-1} DW. At Cunnersdorf, concentrations of Mal, Cit, Fum and Isocit were similar between control and N-fertilized plants over the three years of experiments. Some differences among treatments were observed only during the second trial year, when N-fertilized plants, esp. ammonium nitrate-fertilized plants, tended to show higher Mal, Cit and Isocit concentrations, but these effects were not seen in the other years. Similarly, at Gatersleben no consistent influence of N fertilization or addition of UI was observed on the concentration of any of the 4 organic acids analyzed. There was a trend for higher organic acid concentrations in control and NH_4NO_3-fertilized than in urea-fertilized plants.

Taking together, concentrations of organic acids were not altered consistently by fertilized N forms at any of the two experimental locations. Nonetheless, it remains to be noted that at Gatersleben urea-fertilized plants accumulated eventually less organic acids in leaves, irrespectively of UI addition.

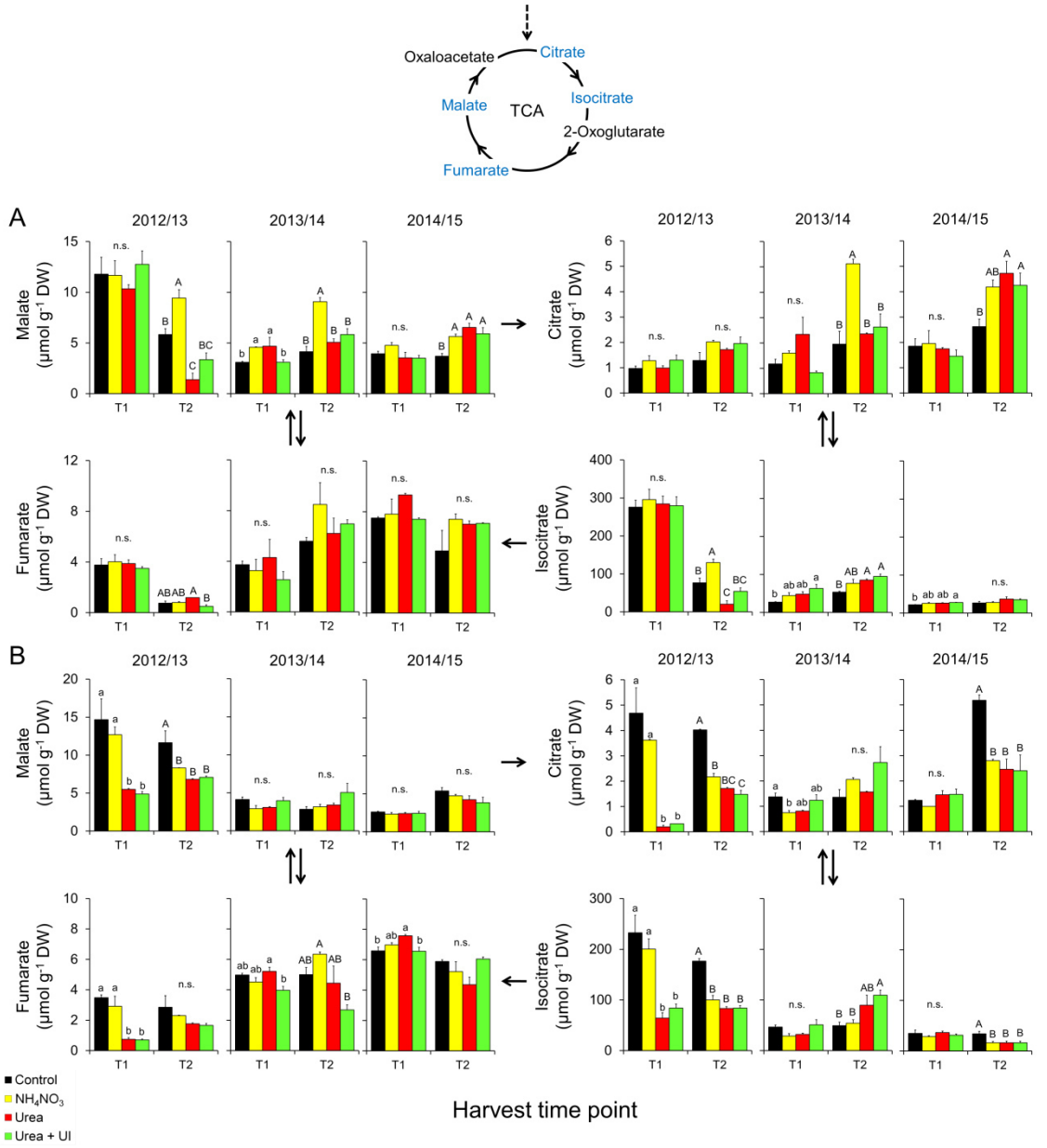


Figure 4-11. Influence of fertilization with different N forms (±UI) on concentrations of malate, citrate, fumarate and isocitrate in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant). TCA indicates tricarboxylic acid cycle.

Concentrations of AA synthesized from oxaloacetate within the TCA cycle, i.e. Asp, Asn, Lys, Thr and Ile, were determined in leaves and these results are shown in Figure 4-12 for Asp and Asn and in Annex 16 for Lys, Thr and Ile. At Cunnersdorf, similar Asp concentrations in leaves were found at T1 between control and N-fertilized plants. However, a significant influence of N fertilization was found at T2, which was consistent over the three trial years. At T2, control plants reached up to 12.9 µmol g^{-1} FW, whereas N fertilization significantly enhanced this concentration. Among the N treatments, no consistent effect on the fertilized N form was found. At Gatersleben, leaf concentrations of Asp were higher during the second trial year. However, there was no consistent effect of the addition of UI on Asp concentrations in leaves at any location. Although NH_4NO_3-fertilized plants tended mostly to lowest Asn accumulation in leaves at either site, absolute concentrations varied little, commonly by less than 20%. Apart from highly variable Lys concentrations in control plants at Cunnersdorf in 2014/15, Lys was not affected by N fertilization at all. Similarly, at Gatersleben, leaf Lys accumulation also responded poorly to N fertilizer forms. By contrast, the accumulation of Thr, which was among the more abundant AA, responded mostly to N fertilization with a two- to fourfold increase throughout all trial years. Such an effect of N fertilization was not observed for leaf concentrations of Ile.

Taking together, among all these described concentrations of AA in leaves synthesized from oxaloacetate, concentrations of Asp and Thr were most consistently influenced by N fertilization, esp. at T2, i.e. when leaf N levels were generally higher. However, no differences were noted among N forms or by addition of UI to the urea fertilizer.

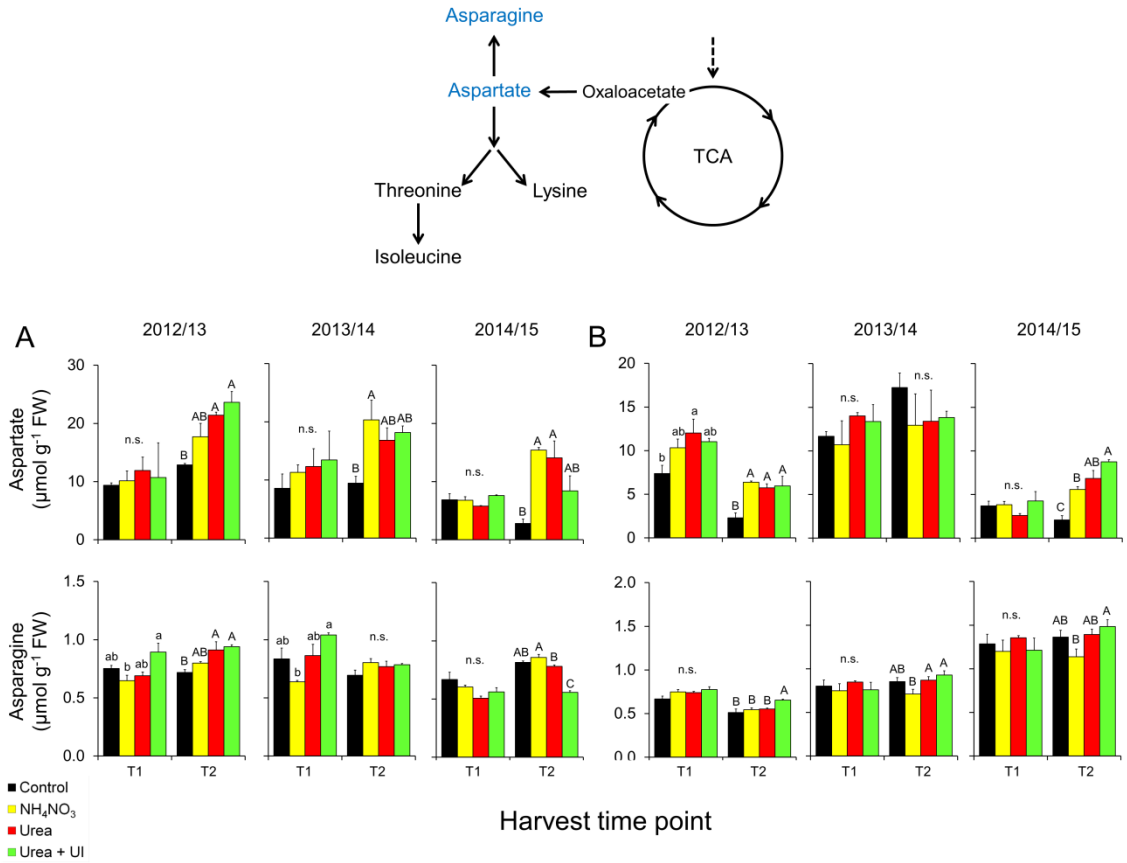


Figure 4-12. Influence of fertilization with different N forms (±UI) on concentrations of aspartate and asparagine in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant). TCA indicates tricarboxylic acid cycle. Ref. to Annex 16 for influence on lysine, threonine and isoleucine.

Concentrations of AA synthesized from 2-OG were also determined to investigate whether N fertilization or a specific N form influenced their concentrations in wheat leaves. Concentrations of Glu, Orn, Arg and Gln are shown in Figure 4-13A for Cunnersdorf and Figure 4-13B for Gatersleben. This influence on leaf concentrations of Pro, GABA and His is presented in Annex 17 for both locations. As observed previously for translocation rates of these AA, only those of Pro, Orn, Gln and His were consistently altered by N fertilization at Cunnersdorf site, while at Gatersleben this held true only for Gln. It was therefore expected to observe similar effects also for leaf concentrations of these AA. Only at T2 in 2012/13 and 2014/15, there was a significant effect of N fertilization on Glu concentrations in leaves. In the other harvests, such an impact was only observed in tendency. Pro concentrations in leaves at Cunnersdorf site were influenced by N fertilization at both times points, except at T1 during the second trial year, showing highest concentrations after N fertilization. At Gatersleben, significant differences among treatments were observed only during the first trial year, when mainly plants fertilized with urea + UI reached the highest Pro concentrations, especially at T2.

No consistent influence of N fertilization on GABA accumulation was found at Cunnersdorf or Gatersleben, where plants reached similar GABA concentrations irrespective of N fertilization. Orn concentrations in leaves were consistently influenced by N fertilization at T2 in Cunnersdorf. Nonetheless, such an effect was observed in Gatersleben only in tendency, especially at T1. Arg, Gln and His showed a rather comparable response to N fertilization. At Cunnersdorf, leaf concentrations of these three AA increased upon N fertilization in 2012/13 and 2013/14, while this effect got lost in the third year. The same response was observed in Gatersleben at both harvests in 2012/13 but was lost in the subsequent two years. Whenever such a N fertilization effect was observed, there was no further influence of the provided N form nor of the application of 2-NPT.

Taking together, among those AA synthesized from 2-OG, N fertilization raised most consistently leaf concentrations of Pro, Orn, Gln and His at Cunnersdorf. This was in agreement with translocation rates of the same AA in the xylem sap (Figure 4-9; Annex 11). As this effect occurred in Gatersleben only in 2012/13, N fertilization worked differently, most likely according to the N reserves at the respective experimental location. However, a consistent effect of the UI on leaf AA concentrations could not be observed.

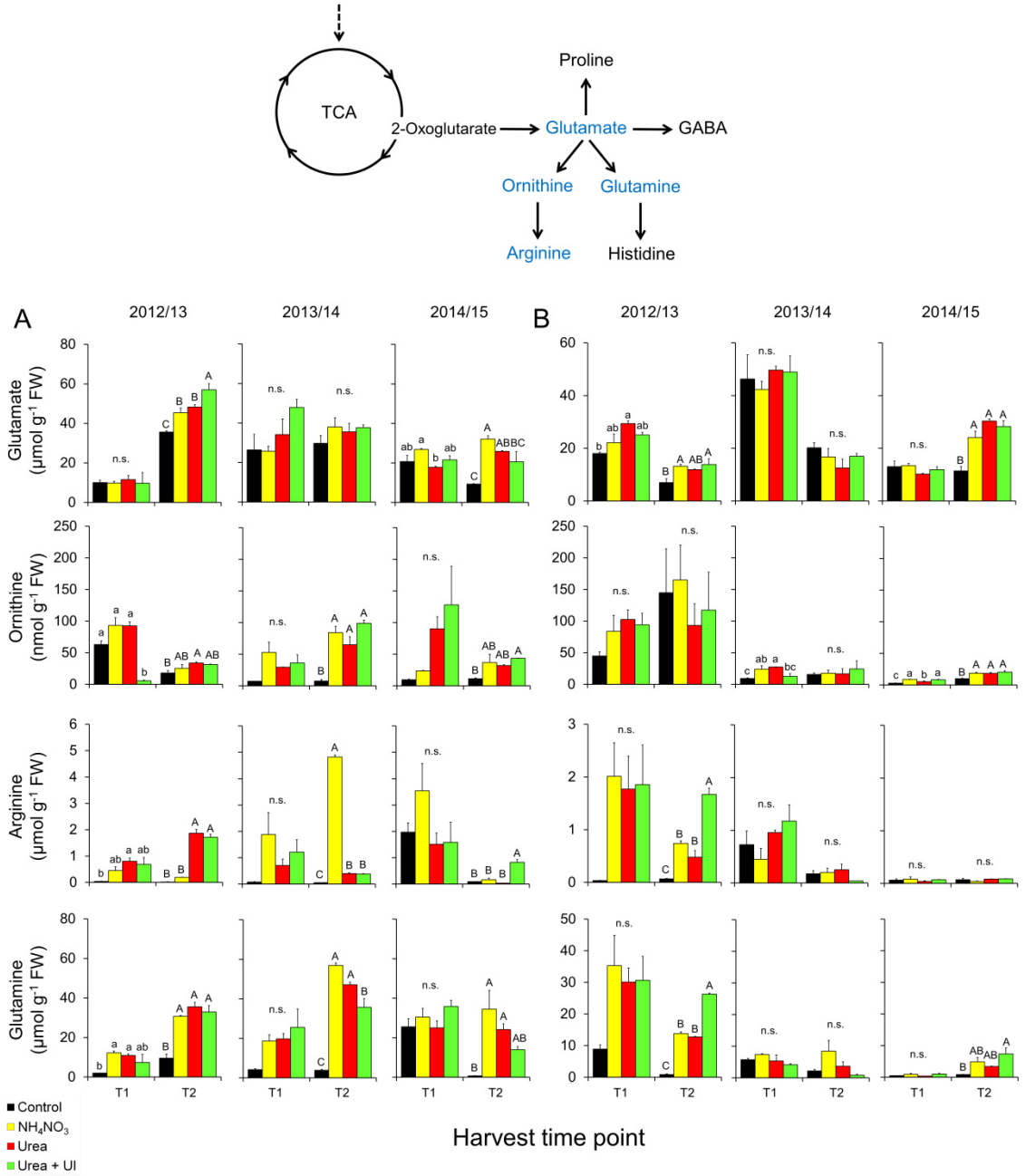


Figure 4-13. Influence of fertilization with different N forms (±UI) on concentrations of glutamate, ornithine, arginine and glutamine in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant). TCA indicates tricarboxylic acid cycle. Ref. to Annex 17 for influence on proline, GABA and histidine.

4.3.5 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on leaf urea, ammonium, nitrate and total amino acids concentrations and urease activity in leaves

In addition, it was investigated whether N fertilization and in consequence fertilization with different N forms altered leaf concentrations of urea, ammonium, nitrate or total amino acids. Results obtained after analyzing translocation rates in xylem sap of these N forms as well as translocation rates of total AA (section 4.3.1) raised the expectation that urea should also accumulate in leaves. Leaf concentrations of urea, NH_4^+, NO_3^- and total AA are presented in Figure 4-14A for Cunnersdorf and Figure 4-14B for Gatersleben.

In all three experimental years at Cunnersdorf, urea concentrations in leaves at both harvests were around or below 10 µmol g^{-1} DW, and there were no significant differences among control plants and those fertilized with ammonium nitrate or urea, except for the treatment with application of UI. Notably, addition of the UI triggered a strong increase in urea accumulation in leaves, which during all three trial years reached up to 11-fold higher levels when compared to urea alone. Such high urea concentrations in leaves (of about 100 µmol g^{-1} DW) could be toxic to plants, as reported by Krogmeier et al. (1991), who observed that similar urea concentrations in soybean leaves triggered leaf necrosis. With respect to this observation, it was observed that some tips of UI-supplemented plants were yellowing at T2 in Cunnersdorf during the last experimental year. However, it was not possible to clarify whether these symptoms were due to urea toxicity. Such elevated urea concentrations in leaves after addition of the UI confirmed the effectiveness of the UI in the soil at Cunnersdorf, which promoted urea uptake from the soil and translocation to the leaves.

At Gatersleben, urea concentrations in leaves were lower than those measured in Cunnersdorf. During the first trial year, urea concentrations in leaves were similar between control and N-fertilized plants, probably as a consequence of the low soil urea-N contents and the high rainfalls at this site between fertilization and harvest time points. Rainfalls could have translocated the urea and the UI to deeper soil layers, making UI-amended urea fertilization ineffective. Nonetheless, there were lower rainfalls during 2013/14 and 2014/15 and thus a lower associated leaching risk. Then, urea concentrations in leaves were higher under fertilization with urea and especially with UI, especially at T2. Similar as in Cunnersdorf, fertilization with urea + UI in Gatersleben tended to lead to higher urea concentrations in leaves than under urea alone, which indicates that the UI was also effective at this location. This was also supported by the urea-N content in the soil and eventually by the urea translocation rates in the xylem sap.

Correlations between urea and UI concentrations in leaves as well as between translocation rates in xylem sap of different N forms and their corresponding concentrations in leaves were carried out to check whether these parameters were related. Interestingly, at Cunnersdorf positive correlations of $r^2 > 0.93$ were found between urea and UI concentrations in leaves at both time points (Annex 18). At Gatersleben, these two parameters correlated also strongly at both time points. Similarly, a correlation between urea translocation rates in the xylem sap with their corresponding urea concentrations in leaves (Annex 19) showed that both parameters correlated very well at both experimental sites and also at both time points over the three years of field experiments.

Regarding ammonium concentrations in leaves at Cunnersdorf, at T1 no fertilization-dependent differences were observed throughout all three years. However, at T2 there was a trend that plants fertilized with NH_4NO_3 or urea + UI had higher ammonium concentrations than plants fertilized with urea alone. In the latter case, these values mounted not substantially above the levels found in control plants, indicating that leaf ammonium concentrations were not substantially influenced by urea fertilization but required either ammonium supply or retarded urea hydrolysis to increase. In Gatersleben, such trends were not apparent. Leaf ammonium concentrations in N-fertilized plants were mostly similar or even slightly below those of control plants. Hence, correlations between leaf ammonium concentrations and ammonium translocation rates were mostly weak (Annex 20).

As expected, in Cunnersdorf leaf nitrate concentrations were highest after fertilization with NH_4NO_3 at both time points in all three years of field experiments. Contrastingly, this was not observed at Gatersleben, where any consistent influence on nitrate concentrations, either by N fertilization or by a specific N form including addition of UI, was lacking. Correlations between nitrate translocation rates in the xylem sap and leaf nitrate concentrations were calculated for both locations. For Cunnersdorf, these two parameters correlated very well, except at T1 during 2014/15. For Gatersleben, good correlations were found only during the second trial year (Annex 21).

Concentrations of total amino acids in leaves were also calculated, yielding at both experimental sites similar concentrations between control and N-fertilized plants at T1. At T2, N fertilization increased leaf AA concentrations by 2-3-fold at both sites, except for the year 2013/14 in Gatersleben. At both locations, close correlations were found between AA translocation rates in the xylem sap and their corresponding concentrations in leaves only in some years (Annex 22). With regard to the addition of UI to urea fertilizers, no differences on leaf AA levels were observed.

Considering the elevated urea concentrations in leaves determined especially at Cunnersdorf, it was important to assess whether the accumulation of the UI in leaves had an effect on the urease activity in these tissues. For this purpose, the urease activity in leaves was determined at both time points in Cunnersdorf and in Gatersleben, by harvesting leaf samples a few days after each fertilization time point. At Cunnersdorf, urease activity in leaves was higher at T2 rather than at T1 over all three years of experiments. This may be explained by a higher uptake and accumulation of urea in the plants after the second fertilization time point or due to the more advanced leaf age, which is usually associated with an increase in protein degradation processes and subsequent urea release. No important differences in urease activity were found between control and N-fertilized plants, except for T2 during 2014/15 in Gatersleben, when urease activity in N-fertilized leaves was approx. twofold higher than in control leaves. Important to note, the application of the UI did not lead to a reduction in the urease activity in the leaves at any of the two experimental sites.

In order to clarify a relation between urease activity and urea concentrations, correlations were established with the obtained values over all three trial years for Cunnersdorf and Gatersleben at both time points (Annex 23). In the non-fertilized treatment only a negative correlation was found in Gatersleben, probably because in these plants the level of leaf urea was lower and thus urea concentrations became a consequence of increased urease activity. When urea concentrations in leaves were higher, a positive correlation could be observed with the urease activity in the variant urea + UI. This indicated an induction of urease activity by the substrate urea. Nevertheless, under most conditions there was no relation between urea concentration and urease activity.

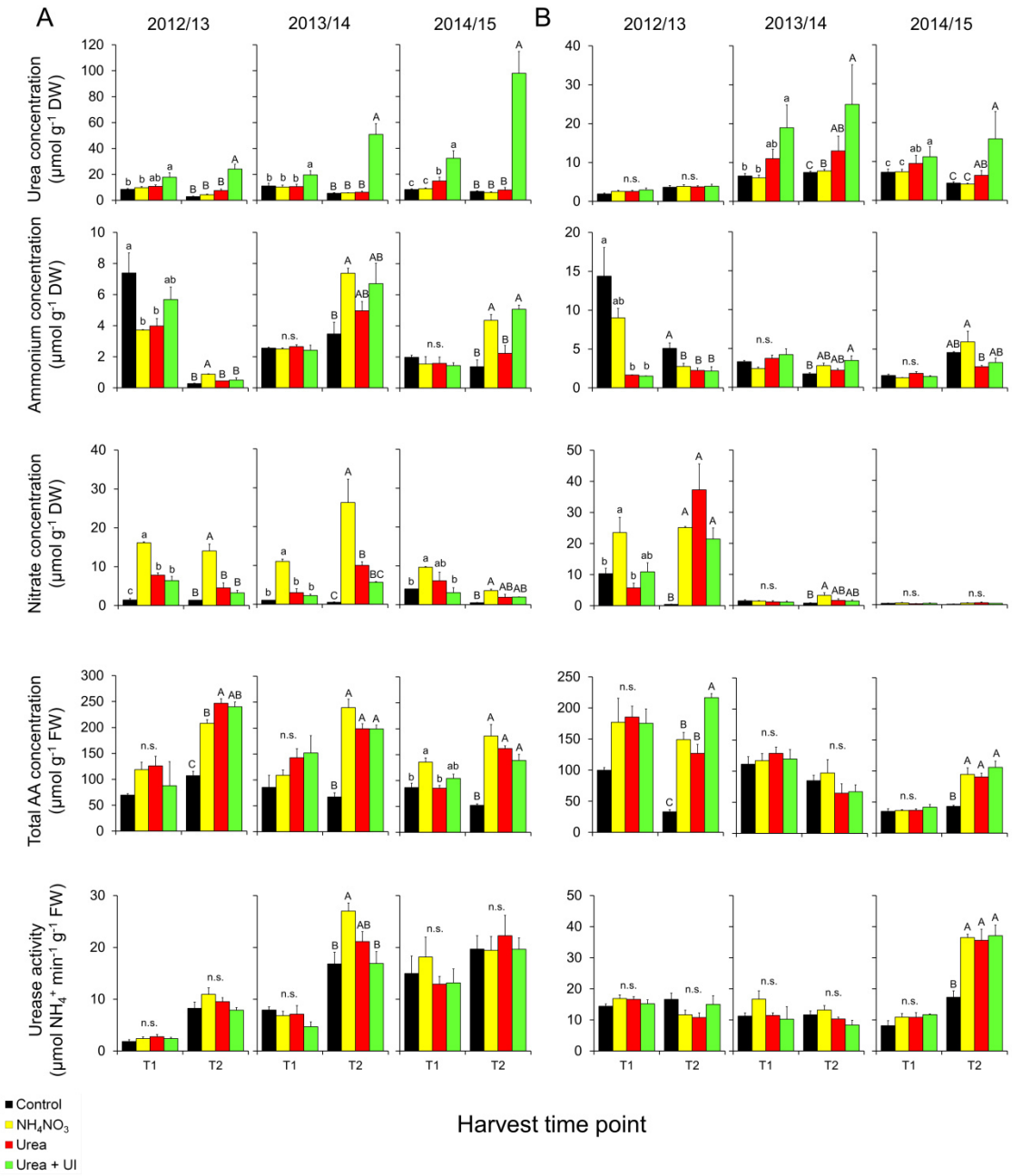


Figure 4-14. Influence of fertilization with different N forms (±UI) on leaf urea, ammonium, nitrate and total amino acids (AA) concentrations as well as on urease activity in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means ($P<0.05$ by Tukey's test; n.s., not significant).

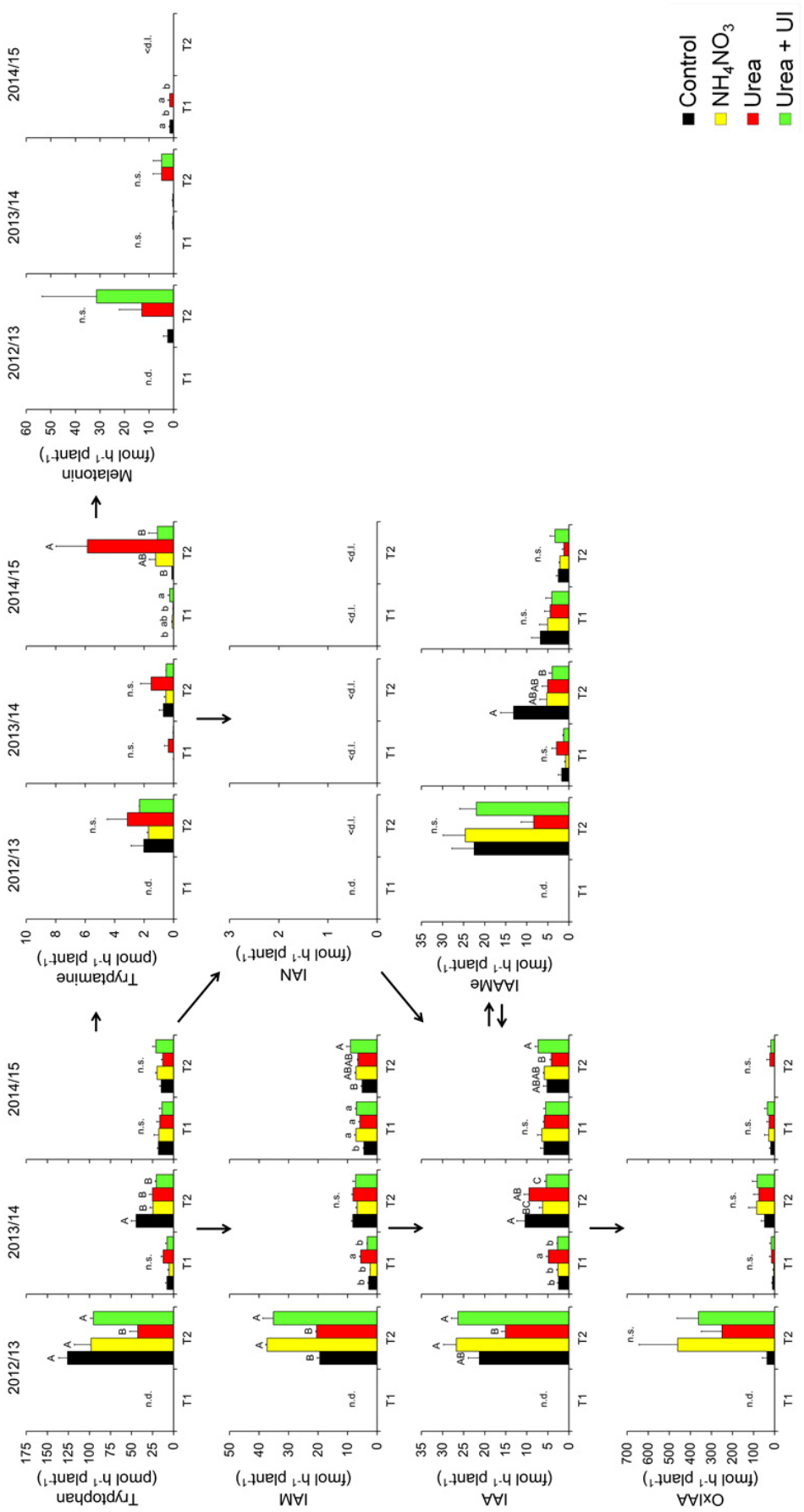
4.4 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on the phytohormonal balance of winter wheat

It has been already well documented for different plant species that phytohormones respond to N fertilization and special attention has been paied to changes in auxins (AUXs), cytokinins (CKs) or abscisic acid (ABA). To assess whether translocation rates of phytohormones in the xylem sap as well as concentrations of these metabolites in leaves were influenced by fertilization with different N forms in winter wheat plants grown under field conditions, xylem sap and leaf samples were collected after each fertilization time point at both experimental locations over the three years of field experiments and levels of AUXs, CKs, ABA, phaseic acid (PA), salicylic acid (SA) and some of their conjugates were determined.

4.4.1 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on translocation rates of auxins in the xylem sap and auxin concentrations in leaves

A total of 5 different AUXs including their precursor Trp were detected in xylem exudates of winter wheat, finding among them indole-3-acetonitrile (IAN), indole-3-acetamide (IAM), indole-3-acetic acid (IAA), indole-3-acetic acid methyl ester (IAAMe) and 2-oxindole-3-acetic acid (OxIAA). In addition, two further Trp-derivatives were analyzed, tryptamine (Tryp) and melatonin (Mel) that play a role in leaf senescence (Liang et al., 2015). Translocation rates in the xylem sap of these AUXs are shown in Figure 4-15 for Cunnersdorf and in Figure 4-16 for Gatersleben.

The amino acid Trp is the starting point for auxin biosynthesis and its concentration can be limiting for the formation of AUXs. At Cunnersdorf, xylem translocation rates of Trp (Figure 4-15) were similar between control and N-fertilized plants at T1, whereas at T2 some differences were found during the first and second trial years, namely lower translocation rates under fertilization with urea alone during 2012/13. However, these differences were not consistent over the three years of field experiments. Only few differences, which were not consistent over the three trial years, were found for Tryp and Mel translocation rates at this location. At Cunnersdorf, translocation rates of IAA derivatives followed a similar pattern, except for IAN, whose rates were below detection limit. Apart from elevated translocation rates for all IAAs at T2 in the first year, which were still due to stronger xylem sap exudation after abundant watering of the plants, there were no consistent changes in any of the IAA species, neither in response to the fertilized N form nor in response to UI application.

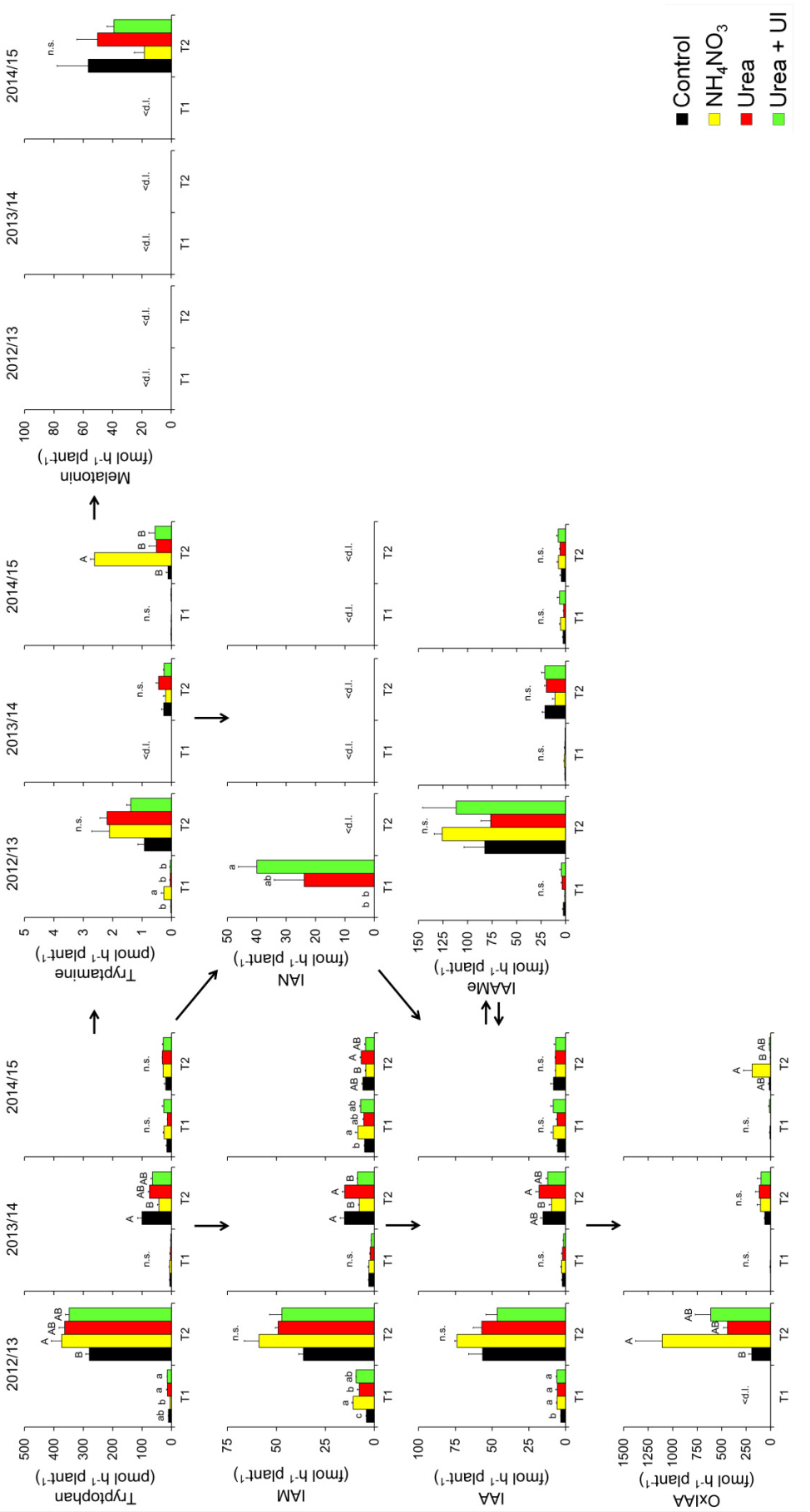


► **Figure 4-15.** (For description of this Figure refer to next page).

◄ **Figure 4-15. Influence of fertilization with different N forms (±UI) on translocation rates of auxins.** Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 at Cunnersdorf. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined; <d.l., below limit of detection).

Also at Gatersleben (Figure 4-16), translocation rates of IAA precursors, active forms and inactivated metabolites showed no consistent changes in response to N fertilization or UI addition. In this regard, even the changes from year to year were comparable between Gatersleben and Cunnersdorf, clearly indicating that AUX homeostasis was not considerably affected by the imposed fertilizer treatments.

Correlations between AUXs translocation rates (only for IAA derivatives) in the xylem sap and the content of major plant-available N forms in soil were established to check whether there was also a dependence amongst these two parameters. Furthermore, correlations were calculated between AUXs translocation rates in the xylem sap and translocation rates of major plant-available N forms. These correlations are shown in Annex 24 for Cunnersdorf and Gatersleben. Quite close correlations with r^2 = 0.96 and 0.84 were found between translocation rates of IAN and urea-N content in the soil as well as between IAN and urea translocation rates, respectively. Nevertheless, this could not be confirmed in the other years when IAN was below detection limit. Under urea fertilization, OxIAA translocation rates correlated well with urea-N content in soil (r^2 > 0.74) and with urea translocation rates (r^2 > 0.63) at Cunnersdorf (T1), which was consistent over years. IAM translocation rates were found to correlate also well with NH_4^+ translocation rates at T1 in Cunnersdorf (r^2 > 0.87). Finally, OxIAA translocation rates at T2 tended to correlate well with nitrate translocation rates in Gatersleben with correlation coefficiens of r^2 = 0.95, r^2 = 0.46 and r^2 = 0.91 during the first, second and third trial years, respectively.

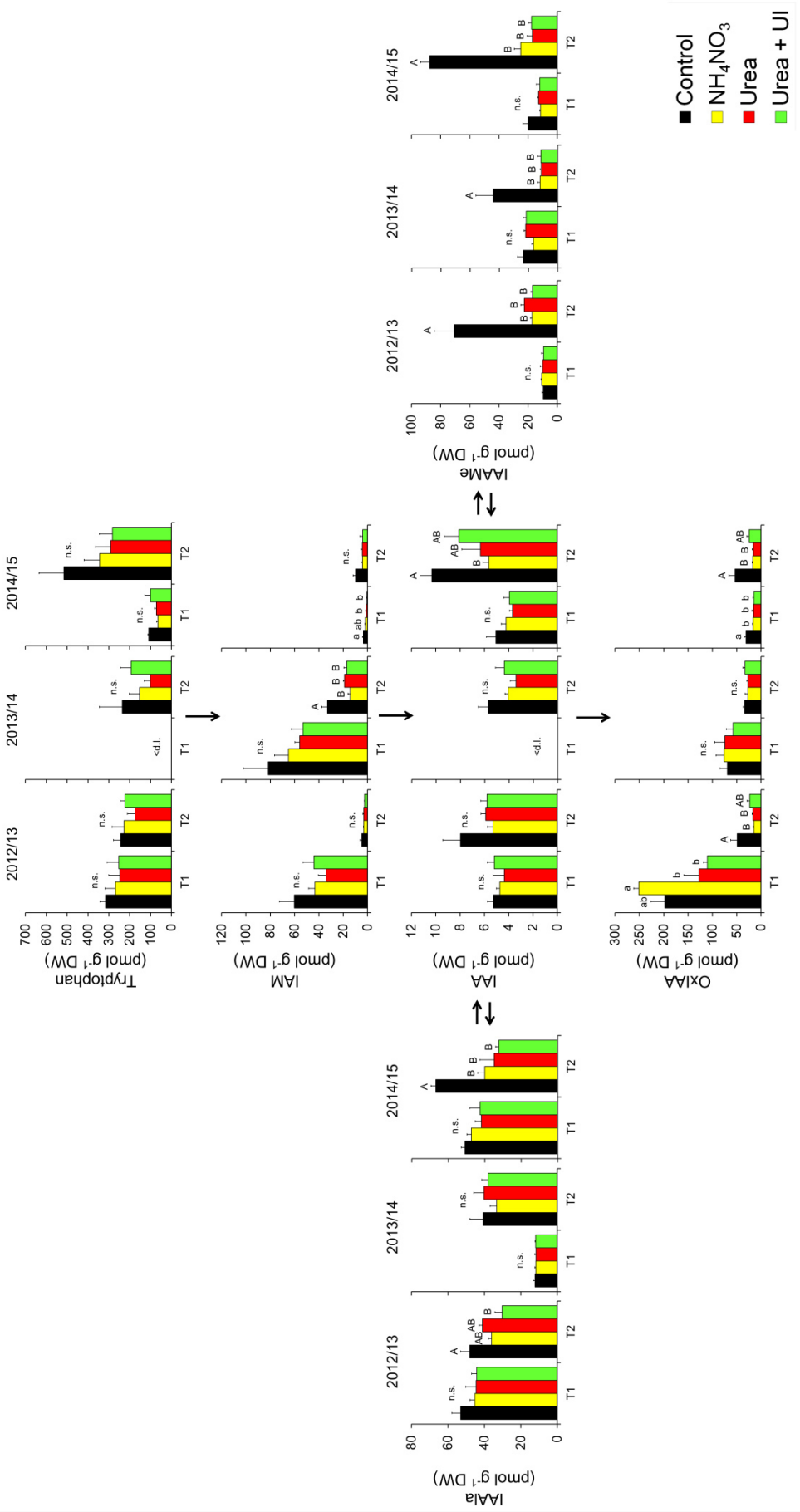


► **Figure 4-16.** (For description of this Figure refer to next page).

◄ **Figure 4-16. Influence of fertilization with different N forms (±UI) on translocation rates of auxins.** Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 at Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined; <d.l., below limit of detection).

Concentrations of AUXs in leaves were also determined at both time points and in total six AUXs including Trp could be detected, i.e. IAA, IAM, IAAla, IAAMe and OxIAA. Results of these analyses are shown in Figure 4-17 for Cunnersdorf and Figure 4-18 for Gatersleben.

At Cunnersdorf, there were remarkably little differences in the leaf concentrations of different IAA forms. Maybe most outstanding was the observation that the physiologically active form IAA tended to be higher in control plants than in N-fertilized plants at T2, which partly held true for the inactivated form IAAla, but fully held true for the inactivated form IAAMe. This may indicate that at T2 leaves of control plants inactivated more IAA to IAAMe. Correlations between AUXs concentrations in leaves and concentrations of major plant-available N forms in leaves were established at Cunnersdorf site (Annex 25), yielding close correlations only among IAM ($r^2 > 0.73$), IAA ($r^2 > 0.70$) or IAAla ($r^2 > 0.63$) and NH_4^+ concentrations in leaves. However, these correlations were not consistent over the three years of field experiments and were found mainly during the first and third trial years.



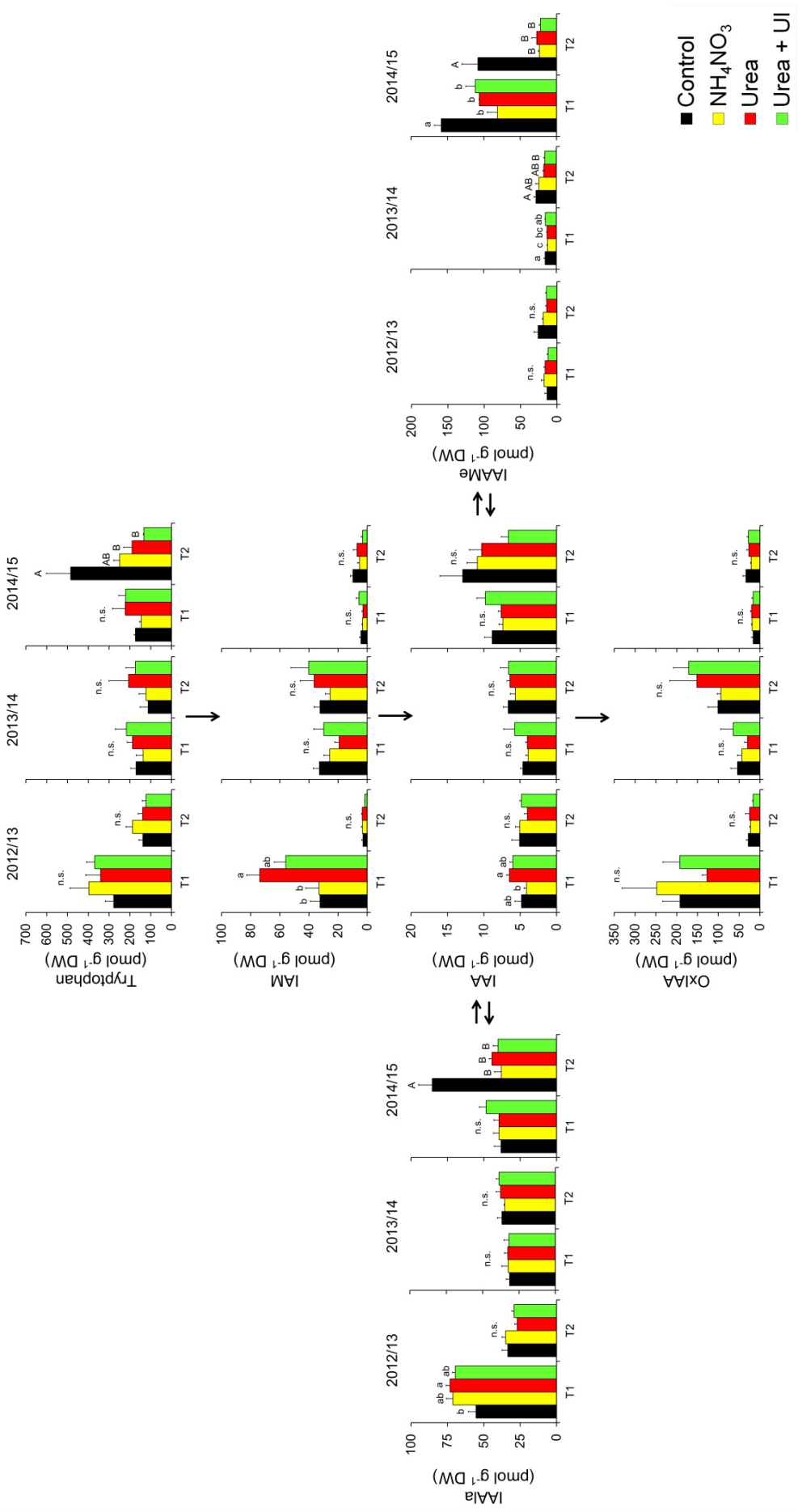
► **Figure 4-17.** (For description of this Figure refer to next page).

◄ **Figure 4-17. Influence of fertilization with different N forms (±UI) on concentrations of auxins in leaves.** Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 at Cunnersdorf. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; <d.l., below limit of detection).

At Gatersleben, concentrations of the physiologically active form IAA were quite stable (Figure 4-18) with up to twofold higher levels in the third trial year. This was unexpected regarding the different levels esp. of IAM between T1 and T2 in the first year or the considerably lower absolute levels of IAM in the third year. Thus, IAA levels were most likely uncoupled from IAM synthesis and steady-state levels, suggesting that IAA was synthesized via another route such as via IAN. Regarding the inactivated forms, N treatments had no considerable impact except for T2 in 2014/15 when control plants showed 2-3-fold higher levels of IAAMe and IAAla than N-fertilized plants.

Correlations between AUXs concentrations in leaves and concentrations of major plant-available N forms in leaves were established also for Gatersleben (Annex 25). Both parameters did not correlate well over the three years of field experiments. Interestingly, among IAAla and urea concentrations in leaves, correlations of $r^2 > 0.69$ were found, but only at T1 during the first and third trial years and not during 2013/14.

Taken together, neither N fertilization nor the application of a specific N form influenced consistently leaf concentrations of different auxin forms at Gatersleben.



► **Figure 4-18.** (For description of this Figure refer to next page).

◄ **Figure 4-18. Influence of fertilization with different N forms (±UI) on concentrations of auxins in leaves.** Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 at Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; <d.l., below limit of detection).

4.4.2 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on translocation rates of cytokinins in the xylem sap and cytokinins concentrations in leaves

A total of 10 different CKs were detected in xylem exudates of winter wheat, finding among them the bioactive forms and their ribosides isopentenyl adenine (iP), isopentenyl adenosine (iPR), trans-zeatin (tZ), trans-zeatin riboside (tZR), cis-zeatin (cZ) and cis-zeatin riboside (cZR) as well as the storage forms trans-zeatin riboside-O-glucoside (tZROG), cis-zeatin riboside-O-glucoside (cZROG) and cis-zeatin-O-glucoside (cZOG). Also, dihydrozeatin (dhZ) was detected, which typically accumulated in storage organs (Frébort et al., 2011). Translocation rates in the xylem sap of these CKs are shown in Figure 4-19 for Cunnersdorf and in Figure 4-20 for Gatersleben.

Nitrogen fertilization induces translocation from roots to shoots of the active CK form tZ and in particular of its transport form tZR (Rahayu et al., 2005). In fact, at Cunnersdorf, translocation rates of the most physiologically active CK form tZ as well as its transport form and direct precursor tZR were strongly increased after N fertilization in all experimental years and at both harvest time points (Figure 4-19), with some exceptions i.e. at T1 during the last trial year. During 2012/13 at T2, translocation rates of tZ and tZR were in tendency higher under fertilization with NH_4NO_3 and urea + UI, when compared to fertilization with urea but without UI. However, this trend did not prevail in the following years, as a significant influence of fertilized N forms or UI addition on translocation rates of these active CK forms could not be observed. Translocation rates of cZ and iPR tended also to be higher in N-fertilized plants than in control plants, in particular after fertilization with urea (±UI). Hence, they followed a similar stimulation of biosynthesis by N as the trans-zeatins. On the other hand, translocation rates of dhZ were mostly highest in control plants in all three experimental years at both time points. This suggested that the storage of CKs was also influenced by N and apparently accelerated in non-fertilized plants.

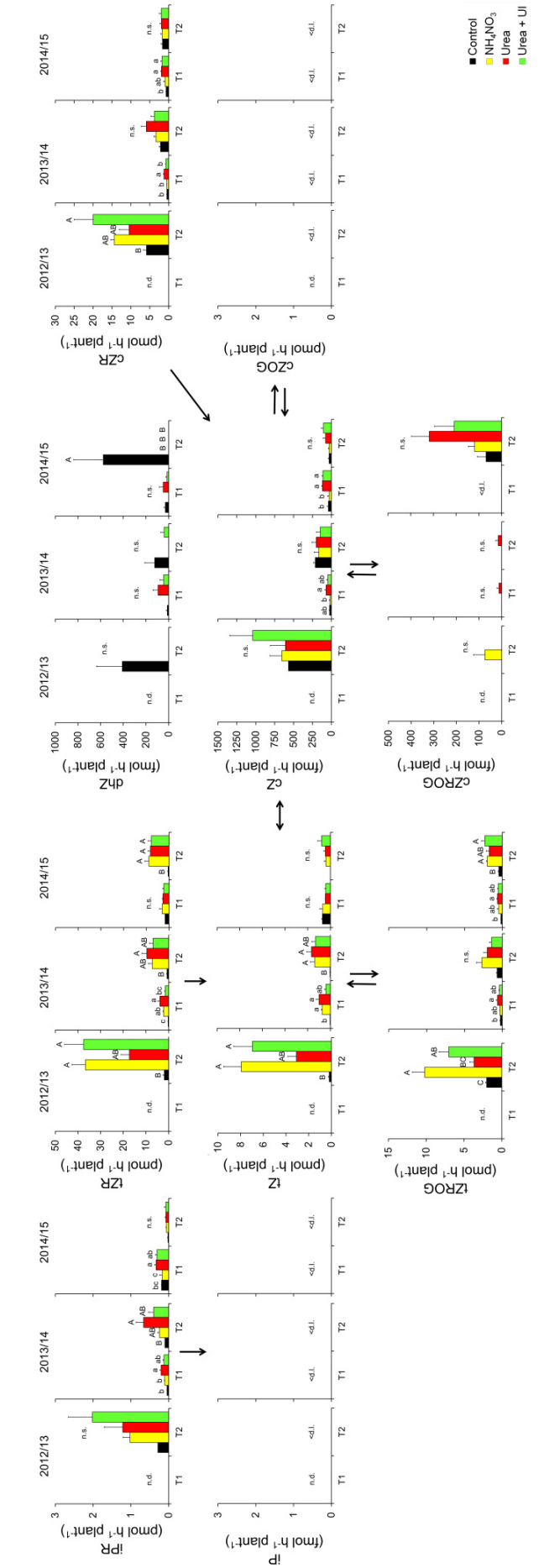
At Gatersleben (Figure 4-20), translocation rates of tZ and tZR were also influenced by N fertilization, mainly at T2, where also significant differences were found among N treatments. Regarding fertilization with urea + UI, translocation rates of these active CK forms were repressed or tended to be lower, when compared to urea. The same was also observed for the storage form tZROG, especially at T2. On the other hand, similar translocation rates were found between control and N-fertilized plants, with only a few differences among treatments i.e. during the first trial year at T1, when the highest cZ translocation rates were found after fertilization with NH_4NO_3. No consistent differences were observed among treatments for translocation rates of iPR at this location.

Correlations were established among translocation rates in the xylem sap of CKs and soil content of major plant-available N forms (Annex 26). In Cunnersdorf, very close correlations (up to $r^2 = 0.98$) were found at T2 between iPR, cZ or cZR and soil urea-N content during the first and third trial years, but not during 2013/14. Regarding soil NH_4-N content, close correlations were observed with tZ, tZR or iPR, but only during the second year. Contrastingly, with soil NO_3-N content no clear correlations were found in all three years. On the other hand, correlations between these CKs and translocation rates in the xylem sap of major plant-available N forms were also established (Annex 26). At T2 during 2012/13, iPR, cZ and cZR correlated well ($r^2 = 0.73$, 0.98 and 0.71, respectively) with urea translocation rates. Nevertheless, this was not consistent over all three years. On the other hand, tZ and its transport form tZR correlated very well (up to $r^2 = 0.99$) with NH_4^+ translocation rates, mainly at T2. Interestingly, most of these CKs tended to correlate just during 2013/14 at both time points. Finally, tZ and tZR correlated well with NO_3^- ($r^2 = 0.88$ and 0.78, respectively) mainly at T2 during the first trial year, considering close correlations at both time points just for tZR during the last trial year. The influence of N fertilization or of a specific N form on cytokinin translocation rates was not consistent but occurred only under certain conditions.

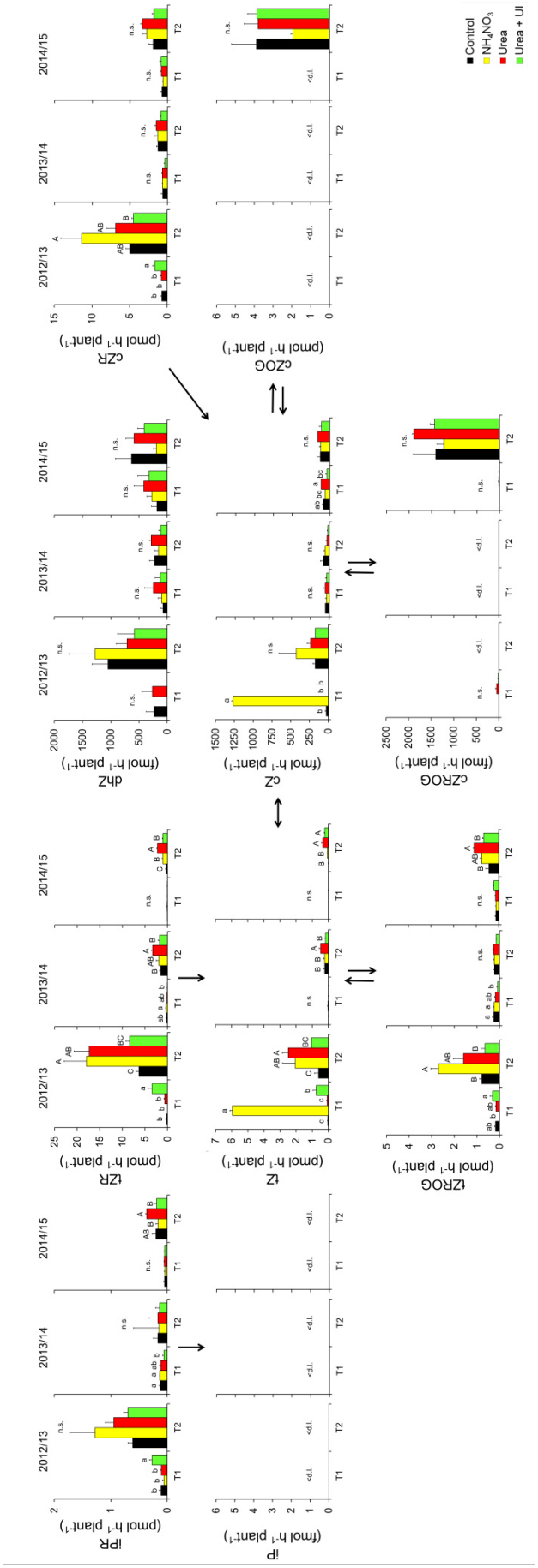
Correlations were also carried out at Gatersleben (Annex 26), with correlations of $r^2 = 0.61$ and 0.65, respectively at T1 between tZR and urea-N content in the soil during 2012/13 and 2014/15. In the same way, tZ and soil NO_3-N content correlated with $r^2 = 0.97$ and 0.98 during the first and second trial years at T1, respectively. In addition, tZ and nitrate translocation rates in xylem sap correlated at T1 ($r^2 = 0.63$ and 0.85) during the first 2 experimental years. Although some close correlations were found between translocation rates of active CK forms and N forms either in soil or in xylem exudates also at this location, these observations lacked of consistency among trial years.

Taken together, translocation rates of CKs at Cunnersdorf generally increased under fertilization with N. This held especially true for tZ, tZR, tZROG, iPR, cZ and cZR. Additionally, for the CK form dhZ, consistently higher translocation rates were found at T2 in control plants without N fertilization. Considering all CKs, no effects of fertilization with urea + UI were observed on translocation rates of individual CKs in winter wheat at any time point. On the other hand, at Gatersleben translocation rates of tZR were increased consistently at T2 under N fertilization. It is noteworthy that a consistent influence of UI addition was observed at both time points, when translocation rates of active CKs (tZ and tZR) were repressed or tended to be lower.

► **Figure 4-19. Influence of fertilization with different N forms (±UI) on translocation rates of cytokinins.** Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in Cunnersdorf. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means ($P<0.05$ by Tukey's test; n.s., not significant; n.d., not determined; <d.l., below limit of detection).



◄ **Figure 4-19.** (For description of this Figure refer to previous page).



► **Figure 4-20.** (For description of this Figure refer to next page).

◄ **Figure 4-20. Influence of fertilization with different N forms (±UI) on translocation rates of cytokinins.** Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined; <d.l., below limit of detection).

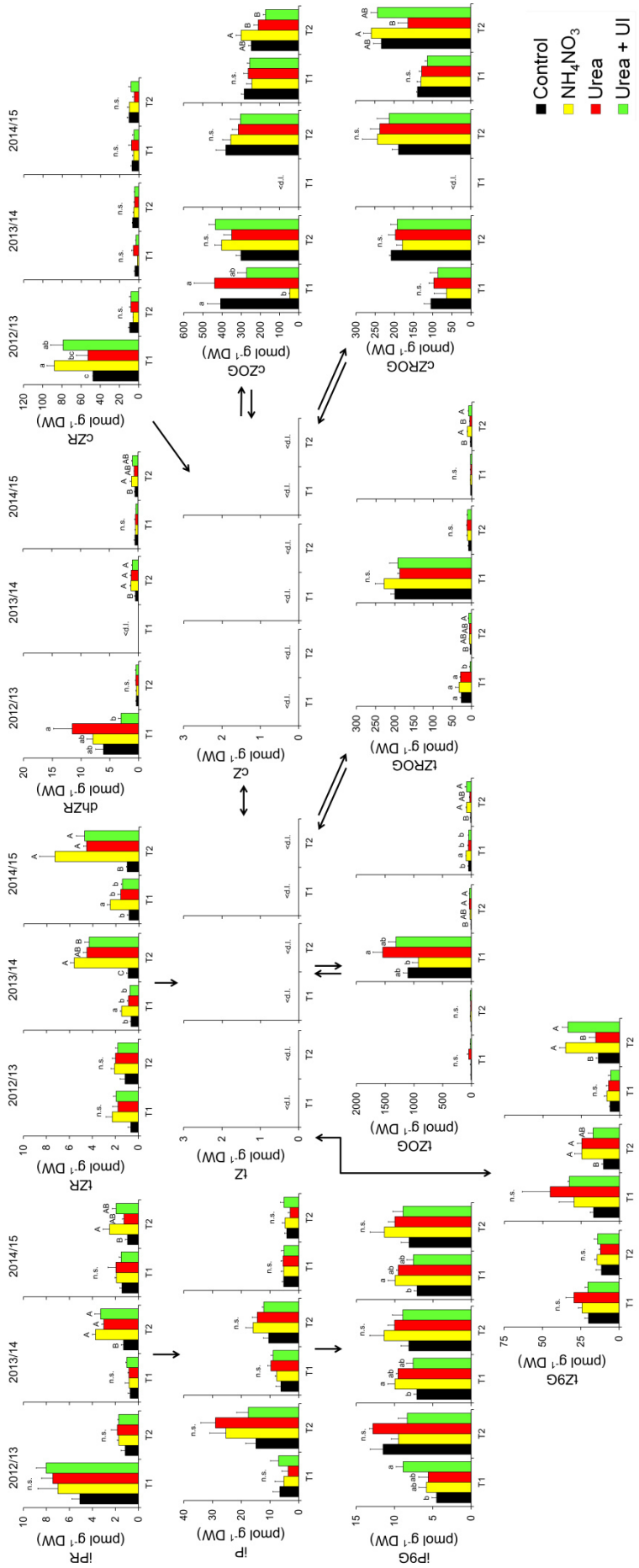
To assess, whether N fertilization or addition of UI influenced CK concentrations in leaves, leaf samples were collected a few days after each fertilization time point over the three years of experiments at both locations and analyzed to determine concentrations of CKs. A total of 12 different CKs were detected in leaf samples of winter wheat, finding among them iP, iPR, isopentenyl adenine-9-glucoside (iP9G), tZR, trans-zeatin-O-glucoside (tZOG), tZROG, trans-zeatin-9-glucoside (tZ9G), dehydrozeatin riboside (dhZR), cZ, cZR, cZOG and cZROG. The active CK form tZ remained below the limit of detection, whereas cZ could only be detected at Gatersleben during the first trial year at T1. Results for concentrations in leaves of CKs are shown for Cunnersdorf in Figure 4-21 and for Gatersleben in Figure 4-22.

During vegetative growth, processes like cell division and cell elongation in leaves are supported by the synthesis of CKs in roots and their translocation to the shoots. Since formation of tZ and tZR in the roots is increased under nutrition with nitrate (Rahayu et al., 2005), CK concentrations in leaves were expected to respond to fertilizer N forms. In fact, at Cunnersdorf leaf concentrations of tZR increased in tendency after N fertilization during the first trial year at both time points, as well as during the following years, especially at T2 (Figure 4-21). The same was observed for the glucoside forms tZOG, tZROG and tZ9G, and this occurred especially at T2 during the second and third year. Nevertheless, significant changes were not consistent over the three years of field experiments.

In the case of cZ, a physiologically less active CK form, the precursor and transport form cZR was not substantially different in control compared to N-fertilized plants. Furthermore, no differences were observed among N-fertilization treatments. On the other hand, leaf concentrations of iP and its derivatives did not respond to N fertilization, but showed only developmental stage-dependent differences, in which they generally increased at T2. This development was reflected in the concentrations of dhZR, a putative storage metabolite in CK metabolism, which decreased significantly at T2.

Correlations between concentrations of CKs in leaves and concentrations of major plant-available N forms in these tissues were calculated for this location (Annex 27). Among these different N forms, close and consistent correlations (up to $r^2 = 0.99$) were found only between leaf concentrations of nitrate and of the active CK form tZR, which was observed at both time points over all three years of field experiments.

► **Figure 4-21. Influence of fertilization with different N forms (±UI) on concentrations of cytokinins in leaves at Cunnersdorf.** Results are shown for the first (T1) and second (T2) time point over the three cropping years 2012/13, 2013/14 and 2014/15 . Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; <d.l., below limit of detection).



◄ **Figure 4-21.** (For description of this Figure refer to previous page).

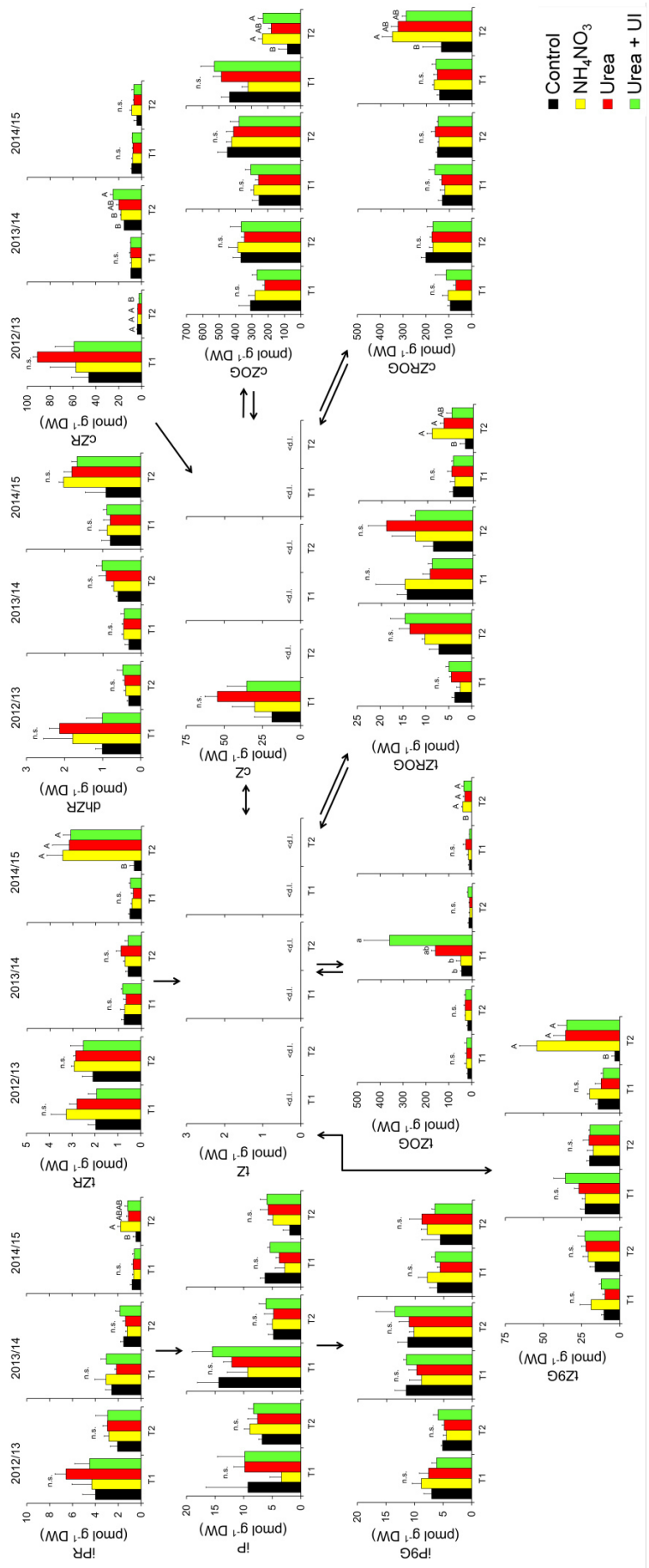
At Gatersleben (Figure 4-22), leaf concentrations of the active CK form tZR were similar between control and N-fertilized plants. An effect of N fertilization could be observed only at T2 during the last trial year. This was reflected in all its derivatives, i.e. the concentrations of tZOG, tZROG and tZ9G. After fertilization with urea (±UI), concentrations of tZOG increased significantly, when compared to control or NH_4NO_3-fertilized plants. However, this response occurred only at T1 during the second trial year and therefore, it was not consistent over years.

Among the less active CK forms, leaf concentrations of cZ, which was only detected at T1 during the first trial year, were similar among control and N-fertilized treatments. Regarding the cZ derivatives, cZOG and cZROG concentrations in leaves were similar in all treatments, finding some significant differences triggered by N fertilization just at T2 during the last trial year, where control plants reached the lowest concentrations. At T2, during the first and second trial years, concentrations of the cZ precursor cZR were oppositely affected by UI addition, as these concentrations decreased in 2012/13 and increased in 2013/14, when compared to urea-fertilized plants without UI.

In a different way from what was observed at Cunnersdorf, leaf concentrations of dhZR were not influenced by N fertilization at any time point, with the exception of a trend at T2 during the last trial year, when N fertilization tended to increase concentrations of this CK form. For iP, its precursor iPR and its storage form iP9G, there was just a developmental stage-dependent response, in which younger plants tended to show higher concentrations than during a later developmental stage. No significant differences were observed between control and N-fertilized plants, as well as among N treatments.

Correlations between different CK concentrations in leaves and major plant-available N forms in these tissues were also established for this location (Annex 27). Although there were some very close correlations, raising even up to $r^2 = 1.00$ among these parameters, these were not consistent over years, indicating that CKs responded in a different manner according to seasonal variables conditioned by i.a. by the weather or by the plant development during each individual cropping year. Taken together, CK concentrations in leaves were not considerably affected, neither by N fertilization nor by UI addition over the three years of experiments.

► **Figure 4-22. Influence of fertilization with different N forms (±UI) on concentrations of cytokinins in leaves at Gatersleben.** Results are shown for the first (T1) and second (T2) time point over the three cropping years 2012/13, 2013/14 and 2014/15 . Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; <d.l., below limit of detection).



◄ **Figure 4-22.** (For description of this Figure refer to previous page).

4.4.3 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on translocation rates in xylem sap and concentrations in leaves of abscisic and phenol carbon acids

Translocation rates in the xylem sap as well as leaf concentrations of abscisic acid (ABA) and its oxidative catabolite phaseic acid (PA) were determined at both locations to check whether these were influenced by N fertilization or by a specific N form, including the addition of UI. Translocation rates of these two phytohormone species are shown in Figure 4-23A for Cunnersdorf and in Figure 4-23B for Gatersleben.

At Cunnersdorf (Figure 4-23A), ABA and PA translocation rates were or at least tended to be higher at T2 than at T1, thus showing developmental stage-dependent differences. ABA translocation rates at T2 did not differ considerably among treatments. However, during the last trial year, translocation rates of ABA were higher in control plants, when compared to all N-fertilized plants. Among N forms, a tendency was observed with similar translocation rates in plants after fertilization with NH_4NO_3 or urea + UI during 2012/13 and 2014/15. Similarly, translocation rates of PA at T1, were similar between control and N-fertilized plants, while just during 2013/14 higher rates were observed after fertilization with urea alone, but this was not consistent over the three years of experiments. At T2, the influence of the N form observed for ABA translocation rates was stronger for PA translocation rates, which were significantly higher after fertilization with urea + UI, when compared to urea alone, reaching similar rates as under NH_4NO_3. Nevertheless, this effect could not be observed during the second trial year.

At Gatersleben (Figure 4-23B), translocation rates of ABA were much lower than those observed at Cunnersdorf. In turn, especially during 2012/13 PA translocation rates were higher at this location. Similarly as observed at Cunnersdorf, translocation rates of both ABA and PA were higher at T2 than at T1, indicating that levels of these phytohormones are strongly influenced by plant age. During the first trial year at T1, the highest translocation rates of ABA were observed after fertilization with urea alone, however, this observation was not consistent over the other years. Translocation rates of PA were also not consistently influenced by N treatments.

Correlations were calculated for both locations between translocation rates in the xylem sap of ABA or PA and soil contents of major plant-available N forms as well as between translocation rates of these phytohormones and translocation rates of the major plant-available N forms (Annex 28). Close correlations were found especially for Cunnersdorf. ABA translocation rates correlated well with translocation rates of NH_4^+ at T2 during all three trial years, with $r^2 = 0.68$ and 0.56 in the first and second years of experiments, respectively. Interestingly, the correlation at T2 observed during the last trial year was negative with $r^2 = -0.99$, suggesting that there was another factor driving ABA that dominated over the NH_4^+ effect. Also with translocation rates of ammonium, close correlations with $r^2 = 0.68$ and 0.94 were observed in 2013/14 and 2014/15, respectively, taking into account, that during the first trial year no xylem sap could be collected at Cunnersdorf. Alltogether, in none of the three trial years a consistent influence of N-forms on translocation rates of ABA or PA was observed, neither at Cunnersdorf nor at Gatersleben.

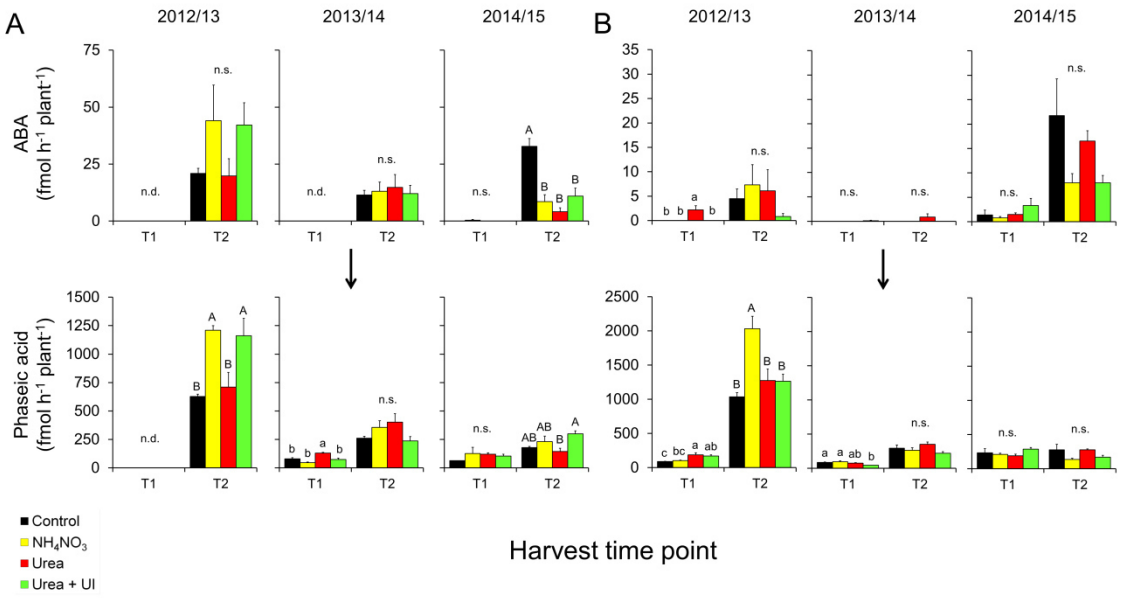


Figure 4-23. Influence of fertilization with different N forms (±UI) on translocation rates of abscisic acid and its degradation form phaseic acid. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined).

Also of interest was the analysis of the stress hormone abscisic acid (ABA) in leaves. These results, together with leaf concentrations of its derivatives abscisic acid-glucosyl ester (ABAGlu) and phaseic acid (PA) are shown in Figure 4-24A for Cunnersdorf and in Figure 4-24B for Gatersleben.

At Cunnersdorf, ABA concentrations were mostly similar in control and N-fertilized plants during all three years of experiments (Figure 4-24A). Only at T2 during the first year, there was an influence of N fertilization on ABA concentrations in leaves, where concentrations increased significantly after fertilization with N. But this effect was not consistent over years. ABAGlu, a hydrolysable ABA conjugate (Burla et al., 2013), showed a similar response to N fertilization with different N forms as observed for its precursor ABA, where leaf concentrations did not differ between control and N-fertilized plants at any time point. Nonetheless, at T2 during the first trial year, control plants reached significantly higher concentrations than N-fertilized treatments. This response was also reflected in PA concentrations.

Leaf concentrations of PA in Cunnersdorf were similar between control and N-fertilized plants at T1, except during 2014/15, when concentrations in control plants were higher than in N-fertilized plants. Noteworthy, a consistent effect of N fertilization on PA concentrations in leaves was observed at T2 in all three years of field experiments, when N fertilization led to lower PA concentrations, namely up to 40% less than in control plants. Interestingly, this was reflected in chlorophyll concentrations in leaves (Figure 4-3, section 4.2.2), which were significantly higher in all fertilized plants at T2 than in control plants. This observation was verified by close negative correlations between these two parameters (Annex 29) with r^2 = -0.96, -0.91 and -0.75 at T2 during 2012/13, 2013/14 and 2014/15, respectively.

At Gatersleben, concentrations of ABA and its derivatives were higher at T2 than at T1, indicating a developmental stage-dependent effect, as higher concentrations of this stress hormone were present in older leaves (Figure 4-24B). No consistent influence of N fertilization on ABA concentrations in leaves was observed at any time point, except for an effect of N fertilization at T2 in the last trial year, when concentrations increased with N fertilization. In the same way, concentrations of ABAGlu and PA were not consistently affected by N fertilization. No significant correlations were observed between PA and chlorophyll concentrations in leaves (Annex 29).

To verify a relation between concentrations of ABA or PA and concentrations of major plant-available N forms in leaves, correlations were established among these parameters (Annex 30). Close correlations (r^2 > 0.60) were found only at Cunnersdorf between ABA and nitrate concentrations in leaves at T1 and T2 during the first trial year.

However, this relation was not consistent. Contrastingly, at Gatersleben PA and nitrate concentrations in leaves correlated negatively during 2012/13, 2013/14 and 2014/15, with r^2 = -0.96, -0.74 and -0.73 respectively.

Taken together, only at Cunnersdorf a consistent influence of N fertilization on leaf concentrations of ABA and its derivative PA was found at T2 in all three years of field experiments. Moreover, ABA and PA concentrations also correlated inversely with chlorophyll concentrations in leaves at this location. Obviously, the addition of the UI 2-NPT did not alter leaf concentrations of ABA and its derivatives.

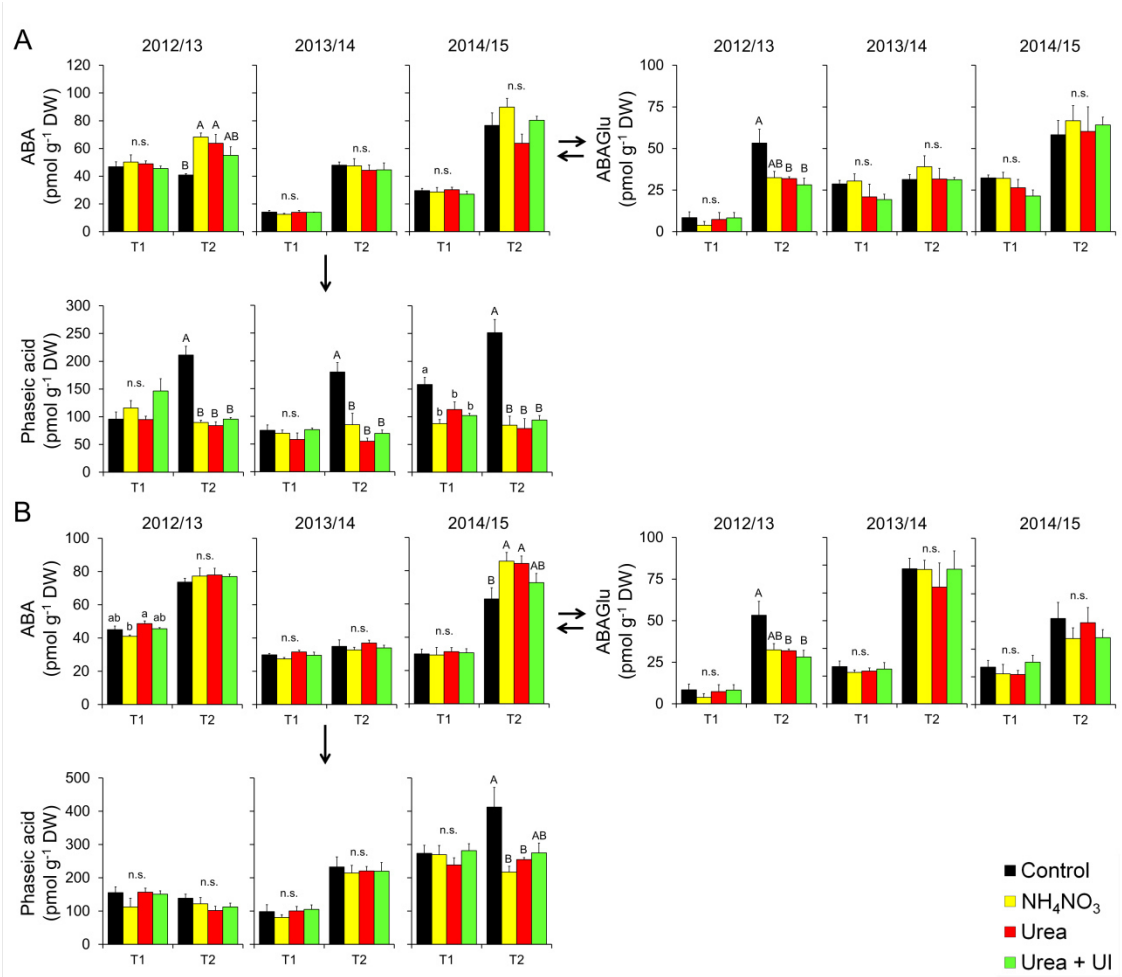


Figure 4-24. Influence of fertilization with different N forms (±UI) on concentrations of abscisic acid and its derivatives phaseic acid and ABA-glucosyl ester in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

Finally it was verified whether the addition of UI triggered effects on the regulation of phenol carbon acids, namely salicylic acid (SA) and salicin (Sal) in xylem exudates or only on SA concentrations in leaves of winter wheat. Translocation rates of SA and Sal in the xylem sap are shown in Figure 4-25A for Cunnersdorf and in Figure 4-25B for Gatersleben.

In general, translocation rates of SA and Sal were higher at the T2 than at T1 at both sites. This was expected and in agreement with previous studies showing that SA accumulates during plant development (Gutiérrez-Coronado et al., 1998; Rivas-San Vicente & Plasencia, 2011). Only in 2012/13, fertilization with NH_4NO_3 yielded higher SA translocation rates than fertilization with urea or no fertilization. For Sal, higher translocation rates were found in control plants at both sites during 2012/13 at T2. Thus, translocation rates of SA and Sal in the xylem sap remained not consistently influenced by N fertilization or by application of a specific N form.

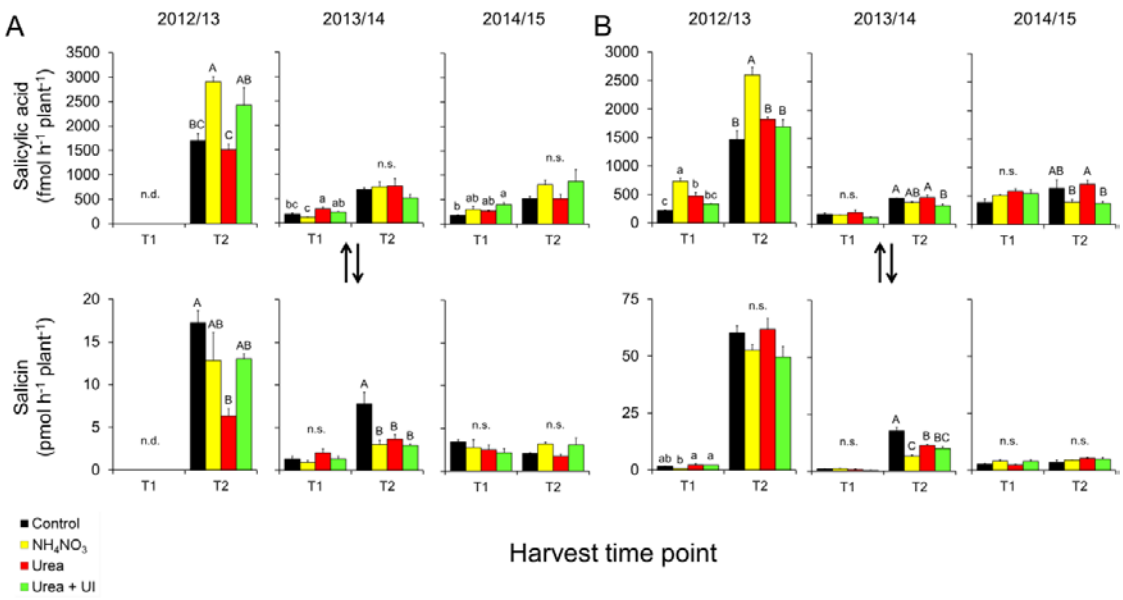


Figure 4-25. Influence of fertilization with different N forms (±UI) on translocation rates of phenol carbon acids. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined).

Concentrations of SA in leaves are shown in Figure 4-26A for Cunnersdorf and in Figure 4-26B for Gatersleben. Fertilization with N or UI supplementation did not influence SA concentrations in leaves at any time point or any location, since leaf concentrations of this phytohormone were always highly similar between control and N-fertilized plants. Noteworthy, only during 2012/13 leaf concentrations of SA were much higher at T1 than at T2, which may have been due to the advanced developmental stage, although this effect was not observed in the following two years. Correlations between leaf concentrations of SA and of major plant-available N forms in leaves did not point to any significant relation (Annex 30).

According to Chen et al. (2009), biochemical studies suggested that the synthesis of the phytohormone SA depends on the levels of the amino acid Phe in plant. To verify this, correlations were established between translocation rates of SA and Phe in the xylem sap (Annex 31) or in leaves (Annex 32). Close correlations (with r^2 up to 0.99) were found only for the xylem sap (Annex 31) between translocation rates of SA and Phe, mainly at T1, which were consistent at Cunnersdorf, indicating that SA biosynthesis depends on Phe levels rather during early vegetative development.

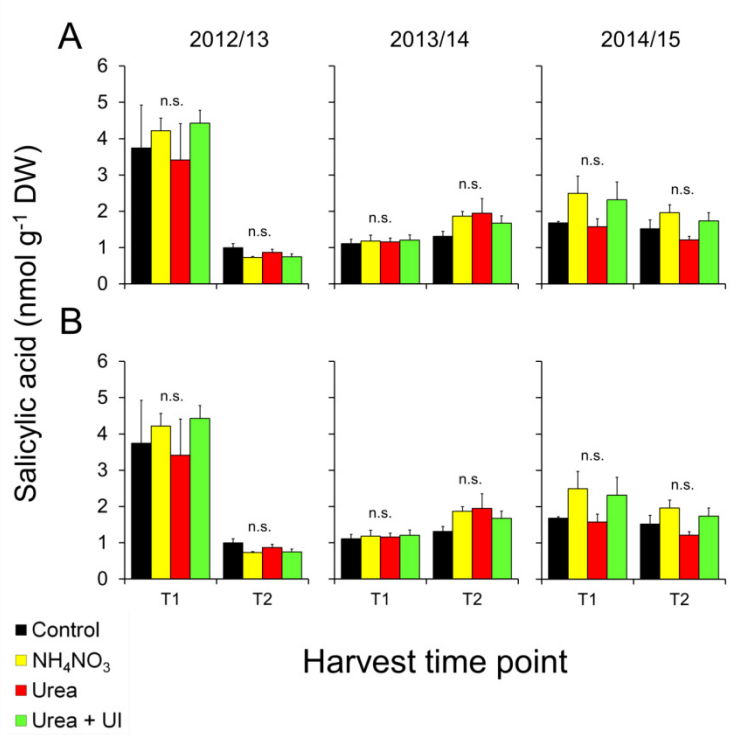


Figure 4-26. Influence of fertilization with different N forms (±UI) on salicylic acid concentrations in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

4.5 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on yield formation and quality parameters of winter wheat

During the whole growth cycle, starting from germination until the ripening stage, winter wheat underwent the influence of several factors, which were governed on one hand by N fertilization with different N forms, but also by interactions with soil and weather or other environmental conditions, thus interfering with crop physiology and yield formation. This section investigates the influence of fertilization with different N forms, including the addition of the UI 2-NPT, on different agronomic parameters, which were determined when the crop was harvested at the end of the ripening stage.

4.5.1 Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on yield formation

In order to answer the question how enhanced urea uptake affects yield formation of winter wheat, different yield parameters were determined, which included the grain yield, the number of spiked tillers per square meter, the number of grains per spike, the thousand kernel weight (TKW) and straw yield. These results are shown in Figure 4-27A for Cunnersdorf and in Figure 4-27B for Gatersleben.

At Cunnersdorf, grain yield during 2012/13 in N-fertilized treatments reached about 100 dt ha^{-1}, whereas the control treatment reached only half of it (Figure 4-27A). The grain yield of plants fertilized with NH_4NO_3 was higher than that of plants fertilized with urea alone.In the case of plants fertilized with urea + UI, grain yield was the same as for plants fertilized with NH_4NO_3, which may point to N losses in the absence of the UI. Interestingly, this observation was reflected only in one other yield component, namely the number of spiked tillers per square meter. Nevertheless, a superior effect of urea fertilization with UI on grain yield and tillers per square meter was not or only in tendency observed in the following two years.

Among the other yield parameters, the influence of N fertilization observed during 2012/13 on the number of spiked tillers per square meter was also observed during the second trial year, but not in the last one, where similar numbers were found in control and N-fertilized treatments. Moreover, an effect of N fertilization was observed on the

number of grains per spike during 2012/13 and 2014/15, but not during the second trial year. TKW was similar in control and N-fertilized plants only in 2012/13 but lower in the following two years. Contrastingly, straw yields were consistently influenced by application of N, with approx. 2-fold higher yields when N fertilization took place. However, an influence by the addition of UI could not be observed.

In 2012/13, grain yield in Gatersleben was lower than in Cunnersdorf, probably because plants were not treated with CCC (2-chloroethyl trimethylammonium chloride). This was in agreement with higher straw yields in Gatersleben (up to 85 dt ha^{-1}) than in Cunnersdorf (up to 65 dt ha-^{1}) (Figure 4-27B). Over the three years of field experiments, grain yield at Gatersleben was consistently influenced by N fertilization, where higher yields were reached after application of N fertilizer to plants. Regarding the different N forms, no significant differences were observed. The increase in grain yield by N fertilization went back to enhanced tiller formation in the first two years but was rather based on an enhanced number of spikes per plant in the third year. The latter was consistent with the yield formation observed in Cunnersdorf, indicating that in 2014/15 N fertilization was not yet effective during tillering but instead triggered grain development during generative growth. At this location there was no significant effect of UI addition on any of the yield parameters.

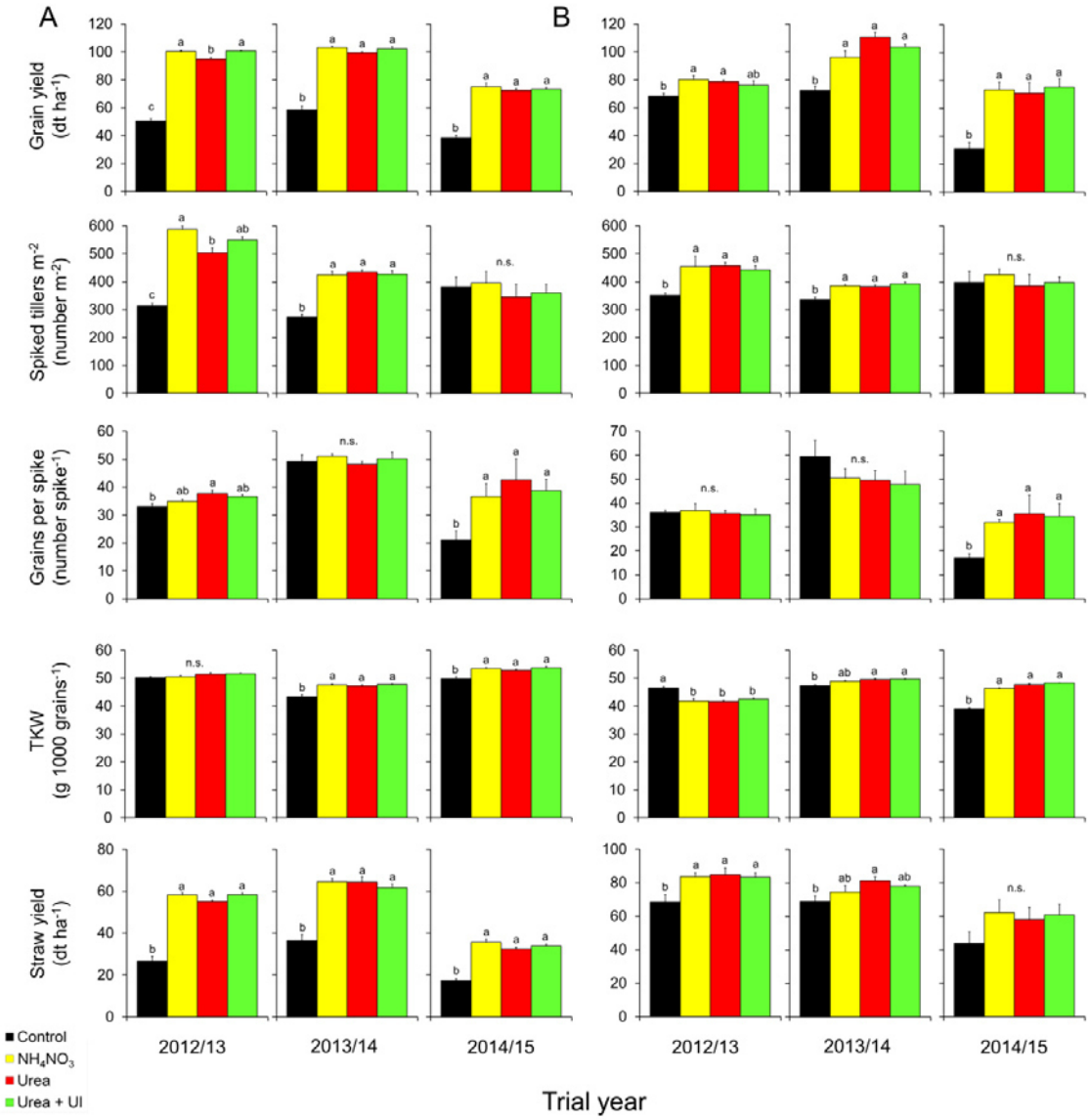


Figure 4-27. Influence of fertilization with different N forms (±UI) on grain yield, spiked tillers per m-2, grains per spike, thousand kernel weight (TKW) and straw yield in winter wheat. Results are shown for **(A)** Cunnersdorf and **(B)** Gatersleben over the three cropping years 2012/13, 2013/14 and 2014/15. Bars indicate means ± SE, n=4. Different letters indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

4.5.2 *Influence of fertilization with different nitrogen forms and addition of the urease inhibitor 2-NPT on grain quality parameters and total N accumulated in the above-ground biomass*

In each trial year, grain samples were harvested and dried in order to determine the crude-protein concentration and the sedimentation value as a parameter for the baking quality. Moreover, the total N accumulated in above-ground organs (grains and straw) was also determined to caluculate the amount of N withdrawn by winter wheat during each year. The influence of N fertilization with different N forms, including the addition of UI on these parameters is shown in Figure 4-28A for Cunnersdorf and in Figure 4-28B for Gatersleben.

As expected, the crude-protein concentration at Cunnersdorf (Figure 4-28A) was significantly higher in grains of N-fertilized than of control plants. Among the N-fertilized treatments, crude-protein concentrations were in general not significantly different after application of NH_4NO_3 or urea + UI in all three experimental years. Thus, plants fertilized either with ammonium nitrate or urea + UI reached always a significantly higher level than those fertilized with urea alone. This was also reflected in total N accumulated in above-ground organs, which was up to 20% higher after fertilization with NH_4NO_3 or with urea + UI than after fertilization with urea alone. This indicated that the UI probably led to a longer and/or higher N availability and thus presumably supported protein synthesis during the grain filling phase. In the same way, sedimentation values were significantly increased by N fertilization, finding consistently higher values under fertilization with NH_4NO_3 or urea + UI, when compared to urea alone. Based on these observations, the UI was effective at this experimental site in all three experimental years.

Contrastingly, at Gatersleben only the influence of N fertilization but not of different N fertilizer forms was observed on crude-protein concentration, on total N accumulation and on sedimentation values (Figure 4-28B). None of the three parameters showed differences in dependence of UI addition to the urea fertilizer. Interestingly, sedimentation values were lower at Gatersleben, where sedimentation values reached only up to 36 cm^3 compared to 54 cm^3 at Cunnersdorf.

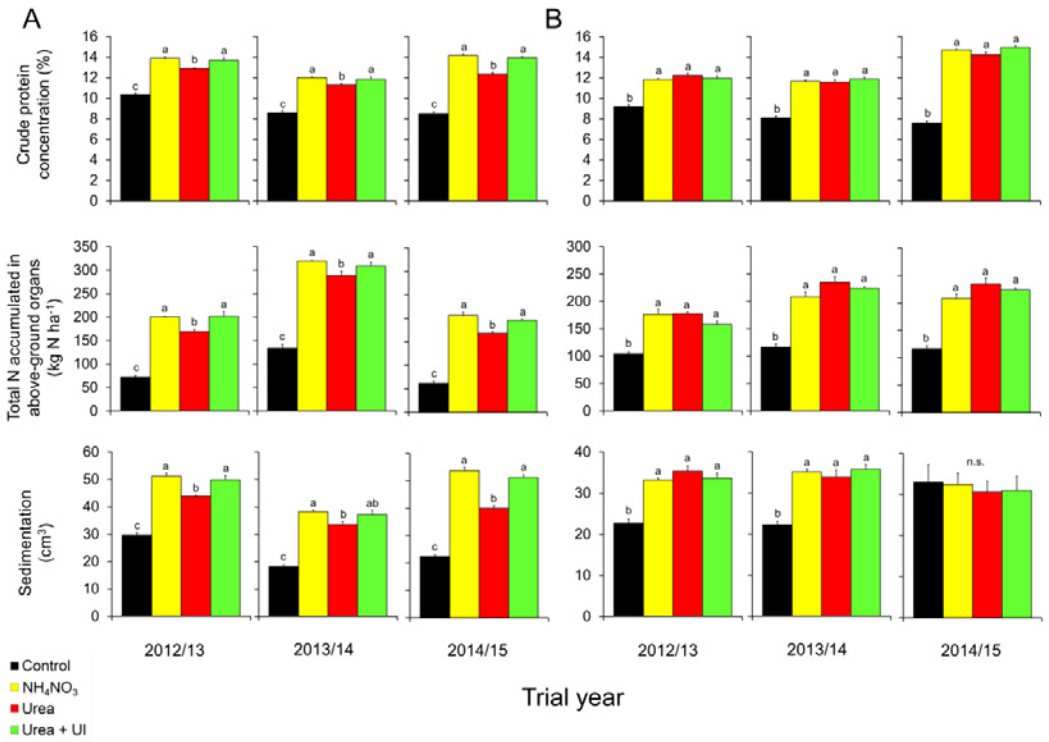


Figure 4-28. Influence of fertilization with different N forms (±UI) on crude-protein concentration, total N accumulated in above-ground organs and on sedimentation values in winter wheat. Results are shown for **(A)** Cunnersdorf and **(B)** Gatersleben over the three cropiing years 2012/13, 2013/14 and 2014/15. Bars indicate means ± SE, n=4. Different letters indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

5 DISCUSSION

Among the N forms being produced synthetically for fertilizers, urea is the cheapest. This economic advantage over NH_4^+- or NO_3^- -based fertilizers goes along with the disadvantage that urea is a short-lived N form when applied to soils. As the retardation of urea hydrolysis in soils is beneficial for plant production by reducing ammonia losses and thus making urea-N more available for root uptake, urea fertilizers are often amended with urease inhibitors. Urease inhibitors classified as amides and esters of phosphoric acid mainly consist of organophosphorus bound to amides and function as substrate analogues for urea by competing for the substrate-binding site in the enzyme, thus outcompeting urea from ureases persisting in soils (Shah & Soomro, 2012; Upadhyay, 2012). Most frequently used urease inhibitors belonging to this category are phenylphosphorodiamidates (PPD) and N-(n-butyl)thiophosphoric triamides (NBPT). Innovative urease inhibitors are required to be effective in reduction of the soil urease activity accompanied by other requirements, such as being harmless to plants or animals, having a good storage stability and obviously having low production costs. More recently, 2-NPT has been developed and introduced as urease inhibitor with the promise to act more efficiently in reducing the urea hydrolysis immediately after inhibition of soil urease activity and consequently lowering ammonia emissions, thus prolonging the uptake of urea-N by plant roots (Hucke et al., 2009). Indeed, previous studies on 2-NPT showed that its supplementation to urea fertilizers led to lower ammonia losses in field-grown winter wheat and perennial ryegrass, which was attributed to slower urea hydrolysis (Ni et al., 2014; Schraml et al., 2016). In incubation experiments, 2-NPT was found to reduce ammonia emissions by 89% during a period of 19 days (Ni et al., 2018). However, recent studies on NBPT (Zanin et al., 2015; 2016) suggested that other amides or esters of phosphoric acid like 2-NPT might also interfere with N uptake by roots or N metabolism in leaves, raising the question whether 2-NPT may exert so far unknown effects e.g. on primary metabolism or the phytohormone balance of crop plants and their yield composition. It was therefore a major aim of this first part of the study to investigate and quantify the influence of a urea fertilizer amended with the UI 2-NPT on N uptake, primary metabolism, plant development, yield formation and grain quality.

5.1 The effectiveness of 2-NPT in stabilizing urea depends on soil properties

As a first step in evaluating the effectiveness of 2-NPT addition to urea fertilizers, its impact on the conversion of reduced to oxidized N forms was investigated in soil samples. Shortly after the application of 90 kg N ha^{-1} in form of urea containing the urease inhibitor 2-NPT (Tables 3-4 and 3-7), soil urea-N contents increased strongly and significantly or at least in tendency at either location during all three experimental years, confirming the expected effect of this UI (Figures 4-1A, B). This held especially true when soil samples were collected at the second time point, namely when plants were around the developmental stage BBCH 32. Thus, stabilized urea in the soil remained available to plants for a longer time at both experimental sites. Only occasionally, the effectiveness of 2-NPT in preventing urea degradation also expressed in lower soil ammonium-N contents (Figures 4-1A, B). More drastic effects were reported by Herbst et al. (2006), who conducted pot experiments under controlled conditions in a growth chamber with oat plants, which were fertilized with urea amended with the urease inhibitor P204/98, a phosphoric acid triamide. When compared to urea without UI treatment, urea was still available 36 d after application, decreasing ammonium contents by up to 70%. Why the addition of 2-NPT was rarely accompanied by elevated ammonium levels here cannot be fully addressed, as downstream processes, such as ammonium adsorption to the soil matrix or rapid conversion to nitrate and subsequent leaching were not monitored at all or at sufficiently high time resolution. At least, soil nitrate levels were not affected by 2-NPT, suggesting that the subsequent nitrification of ammonium to nitrate was not dependent on the rate of urea hydrolysis.

The effectiveness of 2-NPT appeared to be higher in the Cunnersdorf than in the Gatersleben soil, which might be related, among several other reasons, to soil properties. In fact, the Gatersleben soil bears a higher ammonium fixation capacity, allowing ammonium to be immobilized by clay minerals (Scherer, 1993; Scherer et al., 2014). In general, soils with a high cation exchange capacity (CEC) and higher ammonium fixation capacity have a lower potential for ammonia losses (Jones et al., 2013). Hence, sandy soils such as in Cunnersdorf are prone to higher NH_3 losses than soils with higher clay contents such as that in Gatersleben, which had a more than 3-fold higher CEC (Table 3-1). Thus, the high CEC of the Gatersleben soil allows binding a large proportion of the ammonia-N released from urea hydrolysis and forming ammonium, whereas in the Cunnersdorf soil a large fraction of the ammonia can be volatilized, even though both soils have similar soil urease activities (Table 3-1).

It has been well documented that soil properties have an important impact on the potential for ammonia losses after urea fertilization (Fenn & Kissel, 1976; Ferguson et al., 1984). Concerning the differences between the two soils, an assay was conducted to determine the potential for NH_3 volatilization from urea under permanent loss conditions. Representative soil samples (0-30 cm) for this ammonia volatilization assay under standardized laboratory conditions were collected from Cunnersdorf and Gatersleben on the same day (Annex 34). Cumulated ammonia emissions from both soils were measured 7 and 14 d after urea application to soils. In the Cunnersdorf soil, up to 14-fold higher NH_3 losses than in the Gatersleben soil were measured 7 or 14 d after urea application. However, to which extent the potential for NH_3 losses of a soil determines the actual NH_3 volatilization after urea fertilization depends also on the interaction of soil properties with climatic factors, in particular temperature, wind, precipitation and moisture (Sommer et al., 2004; Schraml et al., 2005). After application of the urease inhibitor NBPT (N-(n-butyl)thiophosphoric triamide) to 16 different soils to reduce ammonia volatilization from surface-applied urea, Watson et al. (1994) concluded that the UI was most effective in those soils which have a high potential for NH_3 volatilization, which are soils with a high pH, low titratable acidity and a low organic matter content. This is in agreement with the present findings. In soils with high potentials for NH_3 volatilization, ammonia losses may raise above 20% of the applied urea fertilizer if soil conditions, especially pH, soil moisture and temperature, are favorable for microbial activity. Then, the use of urea may lead to a significant reduction of N uptake and fertilizer use efficiency, when compared to the use of other N forms (Sommer et al., 2004; Chadwick et al., 2005).

Under central European growth conditions, constellations which should trigger high ammonia emissions do not necessarily occur, even at sites with a comparatively high potential for NH_3 release. Döhler (2015) provided an overview of the NH_3 losses determined after urea fertilization under practical conditions in Germany. In part, the listed loss rates were very low. The use of urease inhibitors decreased NH_3 release from applied urea by more than 80% (Ni et al., 2014; Abalos et al., 2014). However, only when a significant N fertilizer loss really occurred, yield and N uptake were promoted. Considering this in Cunnersdorf, the annually varying effects of urease inhibition by 2-NPT appear plausible.

5.2 Uptake and leaf accumulation of the urease inhibitor 2-NPT in winter wheat plants

To address the question whether the UI 2-NPT initially combined with urea can be taken up by plants after its co-application with the fertilizer and whether this compound can accumulate in plant tissues, xylem sap and fully expanded leaves were collected a few days after each fertilization time point at either experimental location (Table 3-6). A newly established UPLC-MS/MS analysis showed that in xylem exudates this compound occurred only in those samples taken from plots fertilized with urea + UI, thus indicating root uptake of 2-NPT directly from soil (Figures 4-2A, B). In general, translocation rates of 2-NPT were higher in Cunnersdorf than in Gatersleben, although similar xylem exudation rates were measured at both locations. These rates strongly differed among years, especially between the first and the two following years at both locations. The reason for this difference was probably related to soil moisture and the irrigation conducted before xylem sap collection in the first year (Table 3-8). The soil type, especially when differing in structure, porosity, water holding capacity, and related soil properties, may also have a strong impact. Indeed, Sanz-Cobena et al. (2014) conducted an incubation experiment testing 2 UIs belonging to the thiophosphorotriamide and phosphorodiamidate families, respectively, namely NBPT (N-(n-butyl)thiophosphoric triamide) and PPD (phenylphosphorodiamidate), at different soil moisture conditions. These authors found that effectiveness of UIs in reducing N_2O (nitrous oxide) losses depended on soil moisture. While soil at 40% water-filled pore space (WFPS) did not show differences between control and UI treatments, soil at 60% WFPS exhibited approx. 87% less N_2O emissions after NBPT co-application than in case of urea alone. Although the impact of UIs on

N_2O emission is rather indirect and was not subject of the present study, the work by Sanz-Cobena et al. (2014) illustrates how UI efficacy depends on soil moisture conditions. In addition to weather conditions, soil properties might play an important role in particular how and to what extent UIs are being released from the surface of the fertilizer granules, mobilized or kept in the soil and from there reach plant roots. However, so far studies on the soil movement and root uptake behavior of UIs are rare. If translocation rates of the UI in the xylem sap of wheat plants are considered as an indicator of the released amount of the UI from the fertilizer, the availability of the UI in the sandy soil in Cunnersdorf was 4-5-fold larger than that in the Gatersleben soil. Even though these numbers were not directly derived from UI measurements in the soil, it is assumed that plants in the Cunnersdorf soil were subject to a higher availability of 2-NPT, leading to higher root uptake and xylem loading. This view is supported by the determined accumulation of 2-NPT in leaf tissues (Figures 4-3A, B). Similar as for the analyses conducted in xylem exudates, 2-NPT was detected in wheat leaves only in those plants supplied with the UI. Again, concentrations of 2-NPT in leaves were much higher in Cunnersdorf, exceeding those in Gatersleben by up to 35-fold. Taken together, these analyses showed that 2-NPT is taken up by roots and translocated to leaves in wheat, while absolute amounts of uptake and translocation appeared to be subject to soil and maybe weather conditions.

Up to date, there are no reports on the accumulation of the UI 2-NPT in plants or plant organs. So far, experiments with this UI were predominantly related to the investigation of ammonia losses, when co-applied with urea fertilizers (Ni et al., 2014; Schraml et al., 2016). By contrast, such studies have been conducted with other urease inhibitors, including those classified in the group of amides and esters of phosphoric acid, to which 2-NPT also belongs. For instance, a meta-analysis showed that addition of NBPT decreased volatilization rates of NH_3 in average by 52% and prolonged the period of ammonia losses from 4.8 to 8.3 d (Silva et al., 2017). This suggests that in general NBPT may be available for root uptake for more than one week. Studies on the physiological effects of NBPT were conducted in a wide range of plant species including wheat, sorghum, soybean, pea, spinach and maize (Krogmeier et al., 1989a; Krogmeier et al., 1989b, Cruchaga et al., 2011; Zanin et al., 2015; Zanin et al., 2016). For example, Zanin et al. (2015; 2016) conducted hydroponic experiments under controlled growth conditions with maize. After application of the UI NBPT, an inhibitory effect on urea uptake and its assimilation was observed to be triggered by the presence of the UI. Moreover, these authors concluded that activity of endogenous urease as well as of ammonium assimilatory enzymes was suppressed, leading to lower amino acid levels (Glu, Gln and Asn) in shoots after UI application. Since NBPT is a structural analogue of urea, it may be possible that urea uptake was inhibited by competition with NBPT. However, urea concentrations in the nutrient solution were at least 500-times higher than those of the applied UI, rendering a competitive effect unlikely. In the present study, a competitive effect cannot be excluded, since 2-NPT is also a structural analogue to urea (Hucke et al., 2010). In contrast to the observations made by Zanin et al. (2015; 2016) with NBPT, however, Glu, Gln and Asn concentrations in xylem exudates or in leaves of wheat were not consistently affected by 2-NPT (Figures 4-8, 4-9, 4-12 and 4-13). Moreover, endogenous urease activities in wheat leaves were also not altered by the uptake and accumulation of the UI at either studied location (Figures 4-14A, B). The latter is in agreement with Krogmeier et al. (1989b), who reported that both NBPT and PPD did not significantly decrease the urease activity in leaves of wheat and sorghum plants grown in soil for 21 days. On the other hand, Krogmeier et al. (1989a; 1989b) reported that the foliar application of either NBPT or PPD as UI in combination with a urea fertilizer caused leaf-tip necrosis in soybean, wheat and sorghum plants. Their investigations led to conclude that the use of these UIs as leaf sprays is phytotoxic due to an excess accumulation of urea in these tissues, because urea concentrations were much higher in necrotic areas as a consequence of reduced urease activities. The question whether elevated uptake and accumulation of urea in wheat plants after application of 2-NPT caused similar effects in the present study will be discussed in section 5.4. Among other studies listed above, only Cruchaga et al. (2011) determined the concentration of NBPT in leaf and root tissues and found that NBPT accumulated to substantial amounts in pea, while spinach plants contained approx. 35% less. Consequently, NBPT accumulation in pea leaves led to urea accumulation and leaf necrosis, whereas spinach plants remained less affected. The higher sensitivity of pea plants may be related to the fact that legumes synthesize and translocate ureides like allantoin and allantoate, which require urease activity for their catabolism (Winkler et al., 1988; Todd et al., 2006). However, some legumes such as soybean do not necessarily depend on the action of this enzyme for ureide degradation to urea but can employ alternative pathways (Stebbins & Polacco, 1995). Graminaceous plants do not employ ureides and are thus expected being less sensitive to UIs like NBPT. Considering the novelty of the UI 2-NPT, results provided in the present study show that under common agricultural practice, 2-NPT is indeed taken up by wheat roots and accumulating in leaves but not interfering with urease activity, urea accumulation or amino acid metabolism.

5.3 Uptake and leaf accumulation of the urease inhibitor 2-NPT did not alter the nutritional status of winter wheat plants

After confirming that winter wheat plants accumulated the UI 2-NPT in leaves (Figures 4-2 and 4-3), the question arose whether this may inhibit N metabolism. First, determination of chlorophyll concentrations in leaf samples demonstrated that the metabolic conversion of urea-N, at least of the amount that had been taken up in the form of intact urea molecules, was similarly efficient as that of N derived from ammonium nitrate fertilization (Figure 4-3). The presence of 2-NPT did not alter chlorophyll accumulation at any time point. When Kawakami et al. (2012; 2013) conducted field and growth chamber experiments with cotton plants fertilized with urea in combination with NBPT, they also observed that its addition did not decrease chlorophyll concentrations in leaves, when compared to plants fertilized just with urea alone. Chlorophyll concentrations in leaves of oat plants grown under controlled conditions were analyzed by Herbst et al. (2006) between the developmental stages BBCH 12 (2-leaf stage) and BBCH 31 (beginning of stem elongation), after fertilizing plants with urea combined with the UI P204/98. There, chlorophyll concentrations ranged between 1.7-2.2 mg g^{-1} FW, which was in concordance with the present results and indicated that plants were adequately supplied with N. Also these authors did not observe any significant differences in chlorophyll concentrations of oat leaves when plants were fertilized with urea alone or with the UI.

In the same way, determination of the effect of the UI on concentrations of macro- and micronutrients in leaves (Figures 4-4 and 4-5) confirmed that 2-NPT did not alter the mineral element accumulation in leaves. Initially, it was thought that the mineral elements in wheat leaves could be indirectly affected by the N fertilization, because N fertilizers affect soil pH significantly, thus altering nutrient availabilities (Vašák et al., 2015). Shortly after application of urea fertilizers to soils and in the presence of water, urea hydrolysis takes place releasing protons to the soil solution and thus forming ammonium-N and carbonate. This reaction is immediately followed by another one, in which protons are bound by bicarbonates, resulting in water and CO_2. As a consequence of these reactions, soil pH is increasing and promoting the conversion of ammonium to ammonia (Sommer et al., 2004), while decreasing the availabilities of phosphate and most metal micronutrients (Rayar & van Hai, 1977; Marschner, 2012). On the other hand, soil-pH changes also occur after nitrification when in oxygenated soils Nitrosomonas bacteria oxidize ammonia to nitrite and Nitrobacter use nitrite to form nitrate (Ward et al., 1982). In consequence of these two combined reactions, protons are released and reduce the soil pH (Fageria et al., 2010). In spite of the fact that soil-pH measurements were not conducted after fertilizations in the present study, the co-application of 2-NPT did not show any indication of altered macro- or micronutrient accumulation in leaf tissues as well. This observation held also true when plants were fertilized with urea alone, thus suggesting that uptake and accumulation of these essential minerals were not affected by applying this fertilizer, as it was the case with ammonium-nitrate treated plants.

5.4 Stabilizing urea nutrition by 2-NPT increases urea accumulation in leaves without affecting primary nitrogen and phytohormone metabolism

5.4.1 2-NPT increases the translocation of urea in xylem

An important step in evaluating the effectiveness of 2-NPT addition to urea fertilizers was to determine the translocation of urea in the xylem sap of winter wheat plants. A few days after each fertilization time point, xylem sap was collected at either experimental location and samples were promptly analyzed (Table 3-6). With regard to the xylem translocation of different N forms, there was an important and consistent influence of 2-NPT on translocation rates of urea at either location in all three experimental years (Figures 4-6A, B). Although translocation rates measured at Cunnersdorf were up to 7-fold higher than those determined in Gatersleben, translocation rates of urea were in general substantially increased after co-application of UI. Nonetheless, it is important to note that due to higher N reserves in Gatersleben soil than in Cunnersdorf soil (Table 3-2), plants in Gatersleben showed a weaker effect of stabilized urea nutrition on the metabolism of primary and secondary N-related compounds.

To date, little is known about root uptake and root-to-shoot translocation of urea in plants and their dependence on the addition of urease inhibitors. Recently, Zanin et al. (2015) conducted hydroponic experiments with maize plants treated with ^{15}N-labeled ammonium, nitrate or urea. In addition to these N sources, they included two more treatments combining the urease inhibitor NBPT either with nitrate or with urea. Then, they determined the accumulation of labeled N in shoots after incubating roots with the different N sources for 24 h. Interestingly, after

application of urea + UI maize plants accumulated up to 3-fold less ^{15}N than plants fed with urea alone, suggesting that the urease inhibitor affected the acquisition of urea. Moreover, when plants received ammonium or nitrate (± UI) as N source, they accumulated up to 4-fold more ^{15}N than plants treated with urea alone, most likely indicating preferred uptake of these two N forms over urea. However, the experiments conducted by Zanin et al. (2015) refer to maize plants grown in nutrient solution, and it is assumed here that the observed effects do not correspond to the action of urea under field conditions. First, plant-available UI concentrations in nutrient solution are most likely higher than those achieved in the soil solution. Although Zanin et al. (2014; 2015) followed common agricultural practice and used 0.5% NBPT relative to the mass of urea, the plant availability of the same proportion of UI co-applied with urea fertilizers to soil can be considered less, because of spatial effects restricting the access of roots to the UI, and immobilization effects as UIs will most likely interact with the soil matrix and may become immobilized. Second, UI concentrations in nutrient solution are held constant, while in soils UIs are subject to leaching and microbial degradation. Third, effects of UIs on plants also depend on the crop species (Cruchaga et al., 2011).

In the present study, winter wheat plants supplemented under field conditions with urea + 2-NPT exhibited higher translocation rates of urea not only when compared to urea but also to NH_4NO_3-treated plants, especially in Cunnersdorf after the second fertilization time point (Figure 4-6A). In a different study, Zhang et al. (2010) conducted a micro-plot field experiment with maize plants, in which they investigated effects of NBPT in combination with reduced application of ^{15}N-labeled urea as N source. Analyses of samples at maturity showed that translocation of urea-derived ^{15}N from stem to grains was promoted after application of NBPT, as the UI allowed maintaining a higher urea availability for root uptake as a consequence of inhibited urease activity. Testing the effects of other UIs on urea translocation, Xu et al. (2000) carried out a pot experiment containing a loamy-meadow brown soil with spring wheat. Analyses showed an increased uptake of urea-derived ^{15}N and promotion of N translocation from stems to grains after application of hydroquinone as urease inhibitor. From these studies it may be conjectured that depending on experimental conditions, sampling time or crop species, effects of the application of UIs on urea translocation will vary. However, it is very important to keep in mind that in these studies it remained unclear what was the form, in which ^{15}N had been taken up. In this regard, ^{15}N-labeling of applied urea is not suffient to determine the amount of urea taken up, because hydrolysis and nitrification convert an unknown amount of the labeled fertilizer form prior to root uptake. Anyways, in particular xylem translocation but also leaf accumulation data in the present investigation clearly showed that the tested urease inhibitor 2-NPT led to enhanced urea uptake, irrespective of contrasting soil properties and different weather conditions at the two locations (Figure 3-1, Table 3-1 and Figure 4-14).

Whether these high translocation rates of urea observed in the xylem sap of winter wheat after co-application of 2-NPT had an impact on other N forms was also investigated in the present study. Translocation rates of ammonium, nitrate or total amino acids in the xylem sap were not altered by UI application (Figure 4-6). Also Zanin et al. (2015) reported comparable concentrations of ammonium in shoots of hydroponically-grown maize 8 or 24 h after UI application. From their results it can be inferred that increased urea uptake after addition of NBPT observed once after 24 h did not repress the accumulation of ammonium in leaves. However, in presence of the UI these authors found slightly lower ammonium concentrations in roots after 24 h of NBPT exposure. The authors concluded that this was a consequence of lower urea acquisition due to NBPT. While root analyses were not conducted in the present study, leaf concentrations of ammonium, nitrate and total amino acids were similar in the xylem sap and in the leaf tissue irrespective of the presence of 2-NPT (Figure 4-14). Thus, regarding the impact of an UI on the accumulation of other N forms in leaves, 2-NPT remains without such secondary effects and may not substantially differ from NBPT.

5.4.2 Elevated urea concentrations in leaves upon 2-NPT supply do not alter primary nitrogen metabolism

The co-application of 2-NPT to urea fertilization was found to promote the translocation rate of urea in xylem sap of winter wheat at either location and at both time points, which led to increased accumulation of urea in leaves (Figures 4-6 and 4-14). Analyses of freeze-dried leaf samples showed increased urea concentrations only after application of the UI, which held especially true for experiments carried out in Cunnersdorf where differences were significant (Figure 4-6A). The low concentrations of urea observed in control and NH_4NO_3-supplied plants could have derived from the arginase reaction, which converts arginine to urea and ornithine and is the main route for urea synthesis in plants (Polacco & Holland, 1993; Cruchaga et al., 2011).

Among the different N pools determined in leaves, that of total amino acids made, with around 80% of total soluble N, the largest contribution to the total-N concentration measured in leaves (Figure 4-4). Relative to the contribution of total amino acids, other soluble N forms namely urea, NH_4^+ and NO_3^- represented only about 2.4, 0.2 and 1.0%, respectively. After suppression of Ni supply to cereal plants, inhibition of urease activity and subsequently elevated urea levels in leaves, Gerendás & Sattelmacher (1997b) reported concentrations of 65.4 and 96.9 µmol urea g^{-1} DW in wheat and rye leaves, respectively. This agrees with results in the present study, in which urea concentrations after UI application in Cunnersdorf mounted even up to 100 µmol g^{-1} DW, whereas in Gatersleben they were slightly lower (Figure 4-14). Previous studies reported leaf-tip necrosis in several crops triggered by high accumulation of urea in leaves. Bremner (1995) reviewed different publications referring to the problems associated to urea-fertilizer application. Among them, Krogmeier et al. (1989a) observed an accumulation of toxic amounts of urea in leaves, namely up to 0.1% after its application to soybean plants. This accumulation even increased up to 0.52% after addition of the urease inhibitor PPD. These toxic urea concentrations in leaves triggered leaf-tip necrosis and were associated with inhibited urease activity, because urease activity decreased by approx. three-fold, when urea was supplied together with PPD to the leaves. In another study, Krogmeier et al. (1989b) reported that addition of either NBPT or PPD to urea increased both leaf-tip necrosis and urea concentrations also in wheat and sorghum plants. These effects depended also strongly on soil type. Their pot experiments were carried out in a growth chamber by using two sandy soils. The first soil had pH 8.3, 0.7% organic carbon, 30% CCE (calcium carbonate equivalents) and a measured urease activity of 26 µg urea h^{-1} g^{-1} soil, whereas the second had a lower pH of 5.5, 0.8% org. carbon, 0% CCE and a slightly lower urease activity of 20 µg urea h^{-1} g^{-1} soil. When applied UI doses were increased from 0.10 to 1.0 µg g^{-1} soil, leaf-tip necrosis in wheat leaves substantially increased from 6.9 to 24.5 and from 6.1 to 9.8% in presence of NBPT, in the first and second soil, respectively. In comparison, after PPD application leaf-tip necrosis increased only from 0 to 2.1 and from 0.7 to 4.1% DW in the first and second soil, respectively. Very similar results were observed by these authors in sorghum plants. Although Krogmeier et al. (1989b) did not establish an association between reported effects and soil properties, the authors observed that NBPT worked more efficiently in the first soil, whereas PPD was more effective in the second soil in reducing urease activity in leaves of both crops, probably due to different pH and CCE levels. These experiments indicated that the application of UIs, either NBPT or PPD, trigger the accumulation of toxic amounts of urea through inhibition of leaf urease activity. Interestingly, when Krogmeier et al. (1989b) autoclaved the soil thus drestroying soil-urease activity, leaf-tip necrosis in wheat increased after soil fertilization with urea and application of PPD or NBPT. On the other hand, Cruchaga et al. (2011) reported contrary observations between pea and spinach plants in their hydroponic experiments. After application of 5 and 1.5 mM urea to pea and spinach plants, respectively, combined with 100 µM NBPT, only pea plants showed leaf-tip necrosis and scorch, whereas spinach plants remained unaffected. They also found larger amounts of the UI in pea plants than in spinach plants in both leaves and roots, after urea + UI application. Whereas urea concentrations increased up to around 220 µmol g^{-1} DW in pea leaves, urea concentrations in spinach leaves did not surpass 10 µmol g^{-1} DW after 7 d from application of urea + UI. They concluded that plant species may respond in a different way to UI application, and thus, to eventually elevated urea concentrations in leaves. Either necrotic leaf margins or leaf-tip necrosis have been reported also in zucchini, soybean and rape plants due to urea accumulation after inhibition of urease activity (Gerendás & Sattelmacher 1997a, b). Interestingly, Gerendás & Sattelmacher (1997b) did not observe necrotic leaf margins or leaf-tip necrosis in rye, wheat or sunflower leaves, in spite of urea concentrations of 97, 65 and 151 µmol g^{-1} DW, respectively. Although translocation and leaf concentrations of 2-NPT were higher in Cunnersdorf than in Gatersleben, wheat plants grown at both locations mostly showed no signs of leaf-tip necrosis (Figures 4-2 and 4-3), except for one time point in 2012/13 when wheat leaf tips turned yellow only in the urea + UI treatment but not when urea was applied alone (Annex 33). Actually, these symptoms coincided with higher translocation rates of the UI (Figure 4-2) and of urea (Figure 4-6), which may identify the observed leaf yellowing as urea toxicity. However, these symptoms were not present at later developmental stages of wheat and therefore plants grew further without toxicity symptoms. Thus, the doses of 2-NPT applied here were effective in conserving soil urea, while they did not provoke of toxicity in wheat plants.

In line with observations made by Cruchaga et al. (2011), it was also expected to observe a reduction in ammonium concentrations in leaf tissues as a consequence of UI application and repression of urea hydrolysis. At least, this was the case in the experiments conducted by Krogmeier et al. (1989a), who observed in UI-supplied soybean leaves approx. twofold lower ammonium concentrations. Nevertheless, in the present study the addition of 2-NPT did not significantly and consistently alter ammonium concentrations at either location (Figures 4-14A, B). In fact, ammonium concentrations in leaves were in general similar among N-fertilized plants, only in tendency higher in UI-supplied

plants. This agrees with ammonium concentrations reported in maize shoots (Zanin et al. 2015), which reached a similar level of approx. 8 µmol N-NH_4^+ g^{-1} FW in urea ± UI treatments, in spite of increased urea accumulation (1.6-fold higher) when 5-d-old plants were exposed to 0.5 mM urea and 0.9 µM NBPT for 24 h. Regarding the influence of enhanced urea accumulation on nitrate, nitrate concentrations remained also unaltered and only increased after ammonium-nitrate fertilization. This is in agreement with previous findings in rice and rape plants. Inactivation of urease activity increased urea concentrations in rice shoots from 3.2 to 177 µmol g^{-1} DW, while nitrate concentrations did not show significant alterations, increasing only from 0.1 to 0.5 µmol NO_3^- g^{-1} DW. Also in rape plants, nitrate concentrations remained unaffected, although urea concentrations increased from 10.8 to 580.4 µmol g^{-1} DW in leaves (Gerendás et al., 1998; Gerendás & Sattelmacher, 1999). When urease activity was repressed by omitting Ni supply to spring rape, Gerendás & Sattelmacher (1999) reported from their hydroponic experiments significantly increased urea concentrations in leaves. In leaves, urea concentrations were 54-fold higher than those of control plants as a consequence of repressed urease activity. Relative to 580.4 µmol urea g^{-1} DW determined in leaves, total amino acid concentrations in these tissues were found to be up to 3-fold lower, when compared to Ni-supplied plants with undisturbed urease activity. Decreasing concentrations of total amino acids in response to urea accumulation in leaves were also reported for zucchini, rye, wheat, soybean, sunflower and rice plants (Gerendás & Sattelmacher, 1997a, b; Gerendás et al., 1998). For instance, when urea concentrations increased from 2.8 to 201 µmol g^{-1} DW (72-fold higher) in leaves of zucchini plants as a consequence of inactivation of urease activity, total amino acid concentrations decreased from 108 to 24 µmol g^{-1} DW. On the other hand, repression of urease activity triggered an increase of urea concentrations in leaves of rye from 4.7 to 97 µmol g^{-1} DW (21-fold higher), of wheat from 2.3 to 65 µmol g^{-1} DW (28-fold higher), of soybean from 3.6 to 102 µmol g^{-1} DW (28-fold higher), of sunflower from 7.3 to 151 µmol g^{-1} DW (21-fold higher) and of rice plants from 3.2 to 177 µmol g^{-1} DW (55-fold higher), which consequently decreased concentrations of total amino acids in these tissues by 2-, 3-, 6-, 11- and 7-fold, respectively, when urease activity was repressed. Considering the high urea concentrations in leaves observed in the present study as a consequence of co-application of 2-NPT, it was expected to observe also lower concentrations of total amino acids in winter wheat. Nonetheless, concentrations of total amino acids were not depleted after application of the UI and thus after increasing urea concentrations in leaves at either location (Figure 4-14). The fact that the total accumulation of amino acids in leaves was not affected after fertilization with 2-NPT may be explained by an efficient hydrolysis of internal urea by urease in both treatments, urea with and without UI. This is supported by the observation of urease activity levels measured in leaves, which were not altered after 2-NPT co-application. It is thus concluded that winter wheat plants were able to hydrolyse the absorbed urea in spite of 2-NPT accumulation since the endogenous urease activity was not altered, whereas 2-NPT efficiently inhibited urea hydrolysis in the soil (Figures 4-14 and 4-1).

In source tissues, stored protein-N is mobilized via catabolism of arginine by arginase in the mitochondrial matrix, from where urea is released to the cytoplasm to be degraded by urease (Witte, 2011). Since endogenous urease activity in wheat plants was not altered after 2-NPT co-application to the urea fertilizer, it was expected that the urea cycle intermediates glutamate (Glu), ornithine (Orn), arginine (Arg) and glutamine (Gln) were also not modified. Indeed, this was the case (Figures 4-13 and 4-14). Gerendás et al. (1998) expected higher Orn and Arg levels after urea accumulation in rice leaves, as it has been suggested that urea accumulation increases concentrations of urea cycle intermediates via feedback inhibition. However, these authors observed the opposite in Ni-deprived rice plants. Nickel omission completely inhibited endogenous urease activity in leaves and increased urea concentrations from 3.2 to 177 µmol g^{-1} DW. In consequence, arginine levels in leaves fell from approx. 0.2 µmol g^{-1} DW to traces near zero, whereas Orn levels were below the detection limit. Additionally, they found 2- and 20-fold lower concentrations of Glu and Gln, respectively, as well as lower levels of all other amino acids. After NH_4NO_3 ± Ni supply, these authors did not observe such alterations in amino acid concentrations, which was explained by only slightly modified urea concentrations in leaves after Ni suppression. Lower concentrations of single amino acids from the ornithine cycle after high urea accumulation were also reported in rye, wheat, soybean rape, zucchini and sunflower plants (Gerendás & Sattelmacher, 1997a, b). Interestingly, Cruchaga et al. (2011) reported that addition of NBPT, which triggered high accumulation of urea in shoots of both pea and spinach plants, significantly decreased the accumulation only of Glu, Thr, Gly and Ser in pea plants, whereas in spinach only concentrations of Lys were significantly reduced. Therefore, it was suggested that there is a plant species-dependent influence. On the other hand, Zanin et al. (2016) observed a time-dependent influence of NBPT supply on amino acid concentrations in maize plants. An increase in urea accumulation in shoots, from 0.26 to 0.41 µmol urea g^{-1} FW after NBPT treatment, led to lower accumulation of Gln, Glu and Asn only after 8 h but not any more after 24 h. Thus, sampling time plays

an important role when investigating metabolic side effects of UIs. As in the present study samples were collected shortly after each fertilization time point, it is tought that all analyzed metabolites should still represent a short-term effect of the UI. The fact that in wheat neither ornithine cycle intermediates nor other investigated amino acids were consistently influenced by elevated urea concentrations after application of 2-NPT might indicate that plants could still utilize the provided urea-N. This is supported by unaffected levels of Gln and Asp as well as by Arg and Orn (Figures 4-12 and 4-13; Annexes 13, 14, 15, 16 and 17). Together with the unaffected levels of sugars and organic acids (Figures 4-10 and 4-11) the present analysis of the amino acid metabolism showed that the addition of 2-NPT does not alter primary nitrogen and carbon metabolism in winter wheat. In agricultural practice, this may constitute an advantage of 2-NPT over other investigated phosphorodiamidates.

5.4.3 Impact of urea-based fertilization in combination with the urease inhibitor 2-NPT on phytohormone metabolism

Another concern that needed to be addressed in this study was to investigate whether the co-application of 2-NPT to urea fertilizers could have altered the phytohormonal regulation of winter wheat plants, because there are different and partially contrasting reports about the effects of diverse N forms and fertilization doses on phytohormone levels. For instance, when Krouk et al. (2011) reviewed different studies, he reported that under low nitrate supply more auxin is translocated from shoots to roots, while higher nitrate concentrations repressed auxin levels in plants. In contrast, Pavlíková et al. (2012) reported an increase of IAA concentrations in Festulolium plants under NH_4^+ nutrition. According to Rahayu et al. (2005) and Garnica et al. (2010), levels of CKs in plants increase under nutrition with NO_3^-. Indeed, when N-depleted maize plants were resupplied with nitrate, Takei et al. (2001) observed higher levels of CKs in both xylem sap and leaves, suggesting that CKs can be considered as signaling compounds for an adequate N status of the roots (Kiba et al., 2011). On the other hand, also levels of ABA were found to increase after nitrate supply (Pavlíková et al., 2012). However, in barley this typical stress hormone was not consistently found to correlate with the plant-N status (Brewitz et al., 1995). A strong influence of urea on the hormonal balance in wheat and barley has been demonstrated by Bauer (2014). He observed a significant influence of urea nutrition on the CK balance, as urea strongly inhibited the root-to-shoot translocation of ribosylated CKs. This lead to a significantly lower tiller number at the end of the vegetative growth phase. Urea-based N nutrition during the generative growth phase decreased CK concentrations in senescing leaves much faster than under nitrate nutrition. In consequence, N translocation from leaves to grains was accelerated and supported an early end of the grain filling phase. This was considered beneficial in dry summer periods, when urea-fed plants showed higher crude-protein concentrations in the grains.

The present study found an influence of N fertilization only on some phytohormones. When wheat plants in Cunnersdorf were fertilized with N, translocation rates of major CK forms, namely tZ, tZR, tZROG, iPR, cZ and cZR, substantially increased irrespectively of the applied N form. In Gatersleben only concentrations of tZR were higher after N fertilization (Figures 4-19 and 4-20). This is in agreement with the study of Hirose et al. (2008), who reported that xylem sap in Arabidopsis plants contains mainly tZ-type CKs. Also Takei et al. (2001) observed that the dominant CK form in xylem sap of maize plants was tZR. In the present study, there was no consistent influence of N fertilization on leaf concentrations of CKs, except for the last trial year when tZR concentrations were higher under N supply in both locations (Figures 4-21 and 4-22). One reason for this inconsistency over years may lie in the sampling time points that may not have caught the same physiological state and response of the plants to the fertilizer supply.

It has been also demonstrated that phytohormones can modify the activity of plant urease. Recently, Shora & Ali (2016) observed increased urease activity in pumpkin cotyledons after addition of either auxins or cytokinins. Moreover, when urea-N was stabilized with NBPT, Zaman et al. (2013) proposed that phytohormones involved in N assimilation and photosynthesis could be altered, although they did not conduct phytohormone analyses to check whether this was the case. Regarding this, there is still a lack of investigations reporting effects of UIs on phytohormone levels. This holds especially true for 2-NPT and other phosphorodiamidates. Indeed, commercially available NBPT, introduced in the market already since 1995 (Watson, 2000; Silva et al., 2017) has not yet been characterized regarding this aspect. Phytohormone analyses in the present study, however, showed that addition of 2-NPT had no impact on the phytohormonal status (Figures 4-15 to 4-26). Consequently, endogenous urease activity in wheat leaves could also not be influenced by modified phytohormone levels (Figure 4-14). Notwithstanding, in Gatersleben there was a repeated influence of the addition of 2-NPT on translocation rates of tZ and tZR, which were

lower when urea was co-supplied with 2-NPT (Figure 4-20). Since tZ and tZR levels were then similar to those of nitrate-supplied plants, they most likely reflected the slower release of ammonium or an expanded availability of nitrate. Anyways, this observed site-dependent effect did not have any consequence for concentrations of tZR or other active CK forms in leaves (Figures 4-14 and 4-22). Finally, it can be concluded that 2-NPT does not interfere with phytohormone metabolism when applied to wheat under field conditions. Any observed changes in phytohormone translocation or metabolism is more likely due to the impact of 2-NPT on the balance of different N forms in the soils.

5.5 Efficacy of the urease inhibitor 2-NPT in promoting grain yield and grain protein levels depends on soil type and growth conditions

Several publications have reported increased grain yield after application of UIs to different crops. For instance, when maize was grown in a sandy clay loam at pH 8.1, there were 52% more grains per spike, 33% higher TKW and finally 10% higher grain yield after application of 115 kg ha^{-1} urea-N combined with NBPT than of urea alone (Khan et al., 2014). In wheat plants, Espindula et al. (2013) reported 14 or 15% higher yields after application of 90 or 120 kg ha^{-1} urea-N with NBPT, respectively, when compared to urea without UI. Moreover, the grain N content was 11 or 12% higher with NBPT application, respectively. This experiment was carried out on a clay soil with 14% organic matter, pH 5.4 and a CEC of 3.7 cmol L^{-1} and thus shows that NBPT is effective also on heavier soils. In rice plants, Phongpan et al. (1995) observed that application of NBPT together with PPD to the floodwater immediately before the addition of urea resulted in substantially higher grain yield in rice plants and was even more effective in retarding urea hydrolysis than the application of NBPT or PPD alone. This effect was explained by the rapid action of PPD and delayed action of NBPT, which must be converted to its oxygen analogue NBPTO before it becomes effective and thereby lasts for longer. Regarding this requirement for conversion, Freney et al. (1993) reported previously that algae in the floodwater needed to provide enough oxygen for NBPT conversion to be effective, which was not the case for PPD. Considering this, a higher efficacy was expected for 2-NPT, since this UI is usually added to the urea granules during industrial fabrication, thus without the necessity for chemical conversion as in case of NBPT (Schraml et al., 2016).

The present study clearly revealed a site-dependent effect of the efficacy of 2-NPT. Only in Cunnersdorf, 2-NPT significantly promoted grain yield above the level of urea alone and reached the same level as in treatments fertilized with ammonium nitrate (Figure 4-27A). Interestingly, the positive effect of 2-NPT was also translated into higher crude-protein concentrations, total N accumulation in above-ground organs as well as in more favorable sedimentation values (Figure 4-28). Interestingly, Barunawati et al. (2003) conducted field experiments with winter wheat on a silty to clayey loam at a location close to that of the present study in Gatersleben. Although soil properties were slightly different to those in the present study, (CEC 12.1 cmol kg^{-1}, pH of 7.3, organic carbon 2.4%; ref. to Table 3-1), these authors did not observe any differences in grain yield between control and N-fertilized plants. Moreover, also the TKW was not significantly influenced by N fertilization. This is in close agreement with the present findings, where N fertilization effects on yield composition and grain quality parameters were generally absent in Gatersleben (Figure 4-27B). There, the relatively weak increase in grain yield after N fertilization in 2012/13 and 2013/14 showed that N fertilization was just poorly effective probably due to a relatively high delivery of N from the organic matter. In addition, the pre-grown crop oilseed rape probably also transferred a considerable amount of N to wheat. These observations indicate that beneficial effects of 2-NPT become more apparent when the N fertilization effect on yield formation increases. This conclusion is supported by the grain yield data in the last year, when N fertilization in Gatersleben doubled grain yield and urea + 2-NPT tended to higher grain yield than urea alone.

In Cunnersdorf, grain yield was significantly increased by the addition of 2-NPT only in the first year, when urea + 2-NPT supply reached the same level as fertilization with NH_4NO_3 (Figure 4-27). In the following two years there was just a consistent trend. However, in all three years protein concentrations and total biomass-N profited significantly from 2-NPT addition to urea (Figure 4-28), leading to the same values as when fertilized with NH_4NO_3. These observations suggest that 2-NPT triggered not only a slower release of the fertilized urea-N to the soil solution, but in particular that 2-NPT-induced retardation of urea hydrolysis decreased N losses caused by ammonia emission. Actually, emission assays under standardized laboratory conditions conducted with the Cunnersdorf and Gatersleben soil confirmed higher ammonia losses from the Cunnersdorf soil (Annex 34). A site-dependent efficacy of the UI was also observed by Grant & Bailey (1999), who conducted field experiments over 3 years with barley in two different

soils, namely a clay loam of pH 7.8 and a fine sandy loam of pH 8.0. After application of urea amended with NBPT, grain yield was consistenly higher than in plants fertilized with urea alone only when trials were conducted in the fine sandy loam. Although the authors did not propose a mechanistic explanation for this effect, it is highly likely that their results were due to lower ammonia losses from the high-pH, sandy soil in presence of NBPT. In view of these and the present results, it is concluded that the effectiveness of 2-NPT is higher when 2-NPT is employed on lighter soils with larger porosity and higher pH that tend to elevated ammonia emission rates.

According to Abalos et al. (2014), soil texture and pH play an important role in determining the potential effect of UIs. Their meta-analysis indicated that UIs show better performance in alkaline soils (pH ≥ 8) than in neutral or acidic soils (pH < 6), since this soil parameter directly affects ammonia volatilization. On the other hand, they found that soil texture also defines the efficacy of a UI, since e.g. clayey soils are less susceptible to nitrate leaching than sandy soils, thus conferring to UIs greater chances to reduce N losses in sandy soils. Additionally, Kiss & Simihăian (2002) reported that urea applied to sandy rather than loamy soils is usually less efficient in improving grain yield, whereas the combination of urea and UI can become an efficient fertilizer strategy also in sandy soils. In another study, Abalos et al. (2012) observed neither significantly higher yield nor higher total N accumulation in above-ground organs after application of urea + NBPT, when growing barley in a clay loam soil of pH 7.6 and 8.2 g kg^{-1} total organic carbon. In this case, grain yield did not profit although NBPT significantly suppressed losses of gaseous N forms. When investigating the effects of a new UI consisting of 75% N-(n-butyl) thiophosphoric triamide (NBPT) and 25% N-(n-propyl) thiophosphoric triamide (NPPT) in winter wheat and maize, Li et al. (2015; 2017) obtained unexpected results on three different experimental locations in China. Although application of this new UI reduced ammonia losses substantially in average on 83% of the investigated sites, grain yields and total N accumulated in above-ground organs of winter wheat or maize plants were not significantly promoted (Li et al., 2015). Interestingly, all three silty or sandy soils in these experiments had a high CEC between 11.0 and 19.7 cmol kg^{-1}, a pH between 5.8 and 8.0 and a soil urease activity between 3.2 and 15.8 mg NH_4-N kg $soil^{-1}$ h^{-1}. Therefore, it cannot be predicted that yield or total N accumulated in above-ground organs will be always promoted as a consequence of reduced ammonia losses by the action of UIs. However, there are more factors interfering with the UI performance. Li et al. (2015) reported that initial mineral N levels in the soil also determine plant responses to UIs. If N levels in the soil are high, urea-derived N availability will be not a limiting factor for yield formation, and in consequence, the UI effect on reducing N losses will be not significant. Indeed, after reviewing more than 45 studies on NBPT, Silva et al. (2017) concluded that lack of UI-mediated yield promotion was mostly related to initial soil N, especially if soil N was not a limiting factor for yield formation. In agreement with this, initial soil mineral N levels were each year approx. 3-fold lower in Cunnersdorf than in Gatersleben (Table 3-2). Thus, elevated soil N levels in Gatersleben rendered lower ammonia emission rates by 2-NPT to become less effective. Using 2-NPT, denoted here as P101/04, Schuster et al. (2007) conducted field experiments with winter wheat at seven different locations in Germany, including one in Cunnersdorf as in the present study. Soil properties slightly differed from those of the present study. Similarly, these authors applied 170 kg N ha^{-1} in two doses at the beginning of vegetation phase (BBCH 11-19) and during the elongation phase (BBCH 32-39) (Table 3-4). In line with the present findings, their results showed that 2-NPT promoted total N accumulation in above-ground organs and crude-protein concentrations, when compared to urea alone (Figure 4-28). Their findings also stressed that among the soil properties, CEC is a dominant factor. Most likely, the higher CEC in the Gatersleben soil allowed more NH_4^+ to be fixed and thereby reduced the volatilization of NH_3. Thus, even when weather conditions for NH_3 volatilization were favorable in Gatersleben, i.e. dry soils and winds, the amount of volatilized NH_3 was probably too low to allow the UI becoming effective. From an agronomic perspective, farmers would be proposed to utilize a urea fertilizer combined with 2-NPT in soils with high potential for ammonia losses and to carry out broadcast urea fertilization immediately before precipitations to avoid or reduce ammonia emissions. Considering this together with initial mineral N levels in the soil, would certainly improve the efficacy of UIs in extending urea availability to plants. Whether the present findings can be also extrapolated to other crops, including mono- and dicots, requires further investigations in different settings.

CHAPTER 2

Characterizing the impact of the urease inhibitor 2-NPT on uptake and translocation in hydroponically-grown spring barley

CHAPTER 2: CHARACTERIZING THE IMPACT OF THE UREASE INHIBITOR 2-NPT ON UPTAKE AND TRANSLOCATION IN HYDROPONICALLY-GROWN SPRING BARLEY

6 CHAPTER 2: INTRODUCTION

To date, little is known about the effects of the addition of the urease inhibitor (UI) 2-NPT (N-(2-nitrophenyl)-phosphoric acid triamide) to urea fertilizers on plants, especially on their nitrogen (N) metabolism, whereas several reports have focused on the effects associated to ammonia (NH_3) volatilization (Ni et al., 2014; Schraml et al., 2016; Ni et al., 2018). Therefore, in the first part of the present study, field experiments were conducted at two different locations in Germany with different weather and soil properties during three consecutive years (ref. to section 3.1.2) to investigate the impact of urea-based N fertilization in combination with this UI on plant-N metabolism, phytohormone regulation, grain yield formation and grain quality of winter wheat. Results showed that the effectiveness of 2-NPT in stabilizing urea depended mainly on soil properties and that UI application had greater effects on plants, when soils had a high potential for NH_3 volatilization, assigning a crucial role to the cation exchange capacity (CEC) of the soil (ref. to section 5.1). In addition, it was shown through UPLC-MS/MS analyses that 2-NPT was able to enter plant roots and from there be translocated and accumulated in leaves after its release into the soil solution (ref. to section 5.2). Interestingly, it was also observed that the co-application of 2-NPT to urea fertilizer did not alter the nutritional status, primary N metabolism and also did not provoke changes in the phytohormone metabolism of winter wheat plants, despite of occurrence of high urea translocation rates as well as high accumulation of this nitrogenous compound in leaves after its addition (ref. to sections 5.3 and 5.4). Finally, analyses of the yield components showed that addition of 2-NPT promoted not only grain yield but also higher grain protein levels in this cereal crop (ref. to section 5.5).

As a structural analogue to urea, the addition of the already introduced and commercially available urease inhibitor (UI) NBPT (N-(n-butyl)thiophosphoric triamide) to maize plants has been recently shown to negatively affect the capacity of plant roots to acquire urea, by limiting the action of the high-affinity urea uptake system (Zanin et al., 2015). When added in conjunction with urea, these authors conjectured that this UI may compete with urea during the uptake process. The high-affinity uptake system for urea in plants involves the DUR3 transporter, which may allow the uptake of urea from the environment and mediate internal urea transport (Liu et al., 2003a; Kojima et al., 2007). This high-affinity transport system operates at low concentrations, usually below 0.5 mM (Hawkesford et al., 2012), finding its affinity constant (K_m value) in the lower µM range (Liu et al., 2003a). Therefore, to investigate whether the recently developed UI 2-NPT that may act as a structural analogue to urea may also affect the capacity of plants to acquire urea, ^{15}N-influx experiments were carried out by using two different nitrogen (N) sources (ref. to section 7.4). Accordingly, three independent hydroponic experiments differing in the length of incubation with the UI, either 2-NPT or NBPT, were established. Spring barley plants were precultured in axenic conditions for 12 days in a full nutrient solution under continuous supply of 4 mM N as either potassium nitrate (KNO_3) or urea + KNO_3. By conducting these experiments, possible conversions of the supplied N forms should be avoided during the course of the study (Mérigout et al., 2008). During the preculture period, UI was added at a concentration of 0.2 µM for either 2, 4 or 6 days. After UI addition, plants were subsequently incubated in a nutrient solution containing 200 µM ^{15}N-labeled source in presence of 0.01 µM UI for either 10 or 120 minutes, allowing calculations of uptake and translocation rates, respectively.

Hence, in this second chapter results of these hydroponical experiments will be presented to address whether the new urease inhibitor 2-NPT is affecting the acquisition, uptake and translocation of urea under nutrition with this N form. Thereby, focus was laid on distinguishing short-term effects of 2-NPT on the capacity to acquire urea and on the physiological implications of this UI for N metabolism.

7 MATERIALS AND METHODS

7.1 Plant material, culture and growth conditions

7.1.1 Plant material

Spring barley (*Hordeum vulgare* L.) cv. Golden Promise, was chosen, since it is known for its superior agronomic characteristics like its good malting performance in the brewing industry (Herb et al., 2017) or for its high salt tolerance (Kamboj et al., 2015). Although this cultivar has shown to be highly susceptible to pathogens, like powdery mildew (Douchkov et al., 2014) or cereal cyst nematode (Luo et al., 2017), its utilization as a model plant grown in hydroponic culture appeared to be effective in previous studies (Liu, 2018).

7.1.2 Plant culture and growth conditions

Sterilization of seeds

Barley seeds were first sterilized by using a mixture of two fungicides consisting in 530 g L^{-1} propamocarb ($C_9H_{20}N_2O_2$) and 310 g L^{-1} fosetyl ($C_6H_{18}AlO_9P_3$), the first being a systemic fungicide and the second an organophosphorus compound (Previcur® Energy, Bayer AG, Leverkusen, Germany). The sterilization procedure was carried out by shaking seeds with the fungicides at a concentration of 0.1% for 15 min and rinsing them afterwards two times with distilled water.

Plant culture and growth conditions

Plant culture was based on a method previously proposed by Podar (2013) with slight modifications. For this, sterilized seeds were transferred to a plastic box containing wet filter paper that was subsequently placed in a cold dark room at 4°C for 4 days until germination. Once germinated, seeds were relocated in a growth chamber set up under long day condition considering a day-night rhythm of 16 h/20°C and 8 h/16°C, a light intensity of 300 µmol m^{-2} s^{-1} and 60% humidity. Placed on the ground of the growth chamber, seeds were cultured for 8 days in a plastic box containing 5 cm soft-plastic needles moistened with a half-strenght nutrient solution (Table 7-1) without nitrogen (N) and iron (Fe), as described by Liu (2018). Since Ni is usually present as a contaminant of other chemicals, it was not directly supplied (Taiz & Zeiger, 2010). In order to avoid high-light intensity, seeds were covered with a semi-transparent plastic foil during the first 2 days inside the growth chamber. Three days after unconvering seeds, the germination box was placed in the middle of the growth chamber, but still on the ground. Later, 10-d-old plants of approximately 10-12 cm height were transferred to 5 L dark plastic pots containing a full-strenght nutrient solution, and pots were placed over metal tables (KANO Stahlbau und Transportgeräte GmbH, Riedstadt-Erfelden, Germany). This time, Fe and N were present in the nutrient solution (Table 7-1A, B), which was renewed every other day. Pots were provided with perforated plastic lids, in which a total of 12 plants pot^{-1} were carefully fixed by using 3 x 10 cm^2 polyurethane foam. The pH of the hydroponic solution was adjusted to 5.8 by using 5 M NaOH and buffered with MES monohydrate (2-(N-Morpholino)ethanesulfonic acid, Merck KgaA, Darmstadt, Germany). Finally, in order to well aerate the nutrient solution, an air pump (Typ 300/4, Husqvarna AB, Husqvarna, Sweden) connected to hosepipes allowed a vigorous air flow through pipettes directly and continuously pumping inside each pot and providing a sufficient supply of oxygen to roots.

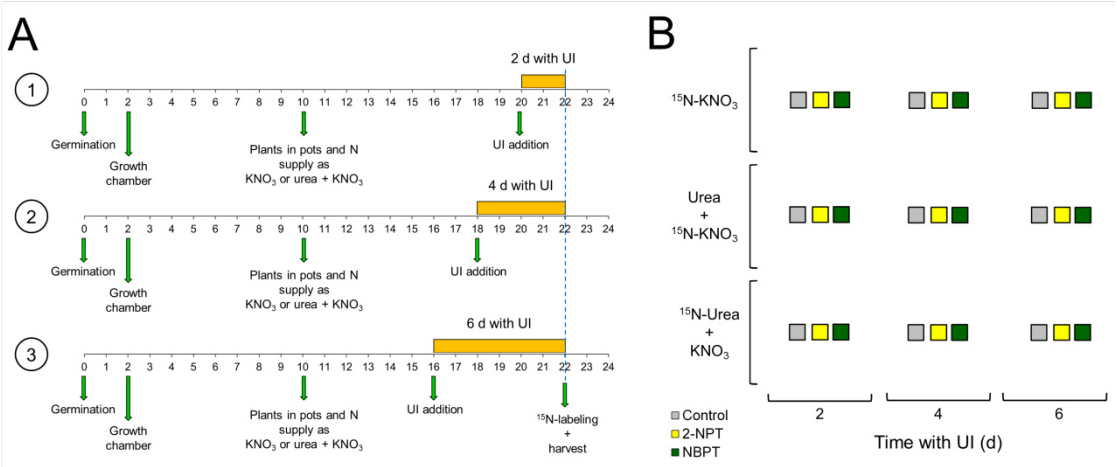
Table 7-1. Composition of the nutrient solution used for hydroponically-cultured spring barley. Macro- and micronutrient concentrations in the nutrient solution of hydroponically-grown barley plants. Nutrients were applied as salts, indicating for each one of them its i) molecular weight and ii) the final concentration used. Addition of the N source **(A)** and of iron **(B)** was carried out once 10-d-old plants were transferred to pots in the growth chamber. N was supplied either in form of potassium nitrate or KNO_3 + urea. When supplied together with urea, KNO_3 was prepared using a concentration of 2 mM, whereas urea at 1 mM due to its double amount of N, thus resulting in both cases in a final concentration of 4 mM N.

Nutrient	Salt	Molecular weight (g mol^{-1})	Final concentration
N form **(A)**			
Nitrate	KNO_3	101.10	4 mM
Nitrate + Urea	$KNO_3 + CO(NH_2)_2$	161.16	4 mM
Macronutrients			
Phosphorus	KH_2PO_4	136.09	0.1 mM
Potassium	KCl	74.55	0.1 mM
Calcium	$CaCl_2$	110.98	2 mM
Magnesium	$MgSO_4*7H_2O$	246.48	0.5 mM
Sulfur	K_2SO_4	174.27	0.5 mM
Micronutrients			
Iron **(B)**	Fe-EDTA	367.05	0.1 mM
Copper	$CuSO_4*5H_2O$	249.68	0.2 µM
Manganese	$MnSO_4*H_2O$	169.02	2.5 µM
Boron	H_3BO_3	61.83	1 µM
Molybdenum	$(NH_4)_6Mo_7O_{24}$	1235.86	0.01 µM
Zinc	$ZnSO_4*7H_2O$	287.54	0.5 µM

7.2 Hydroponic treatments and experimental setup

7.2.1 Hydroponical treatments

Three independent nutrient solution trials were conducted by establishing an aerated hydroponic system under controlled climatic conditions. Each experiment differed from the other by the time period, during which barley plants were exposed to the UI, either 2-NPT or NBPT, i.e. 2, 4 or 6 d of continuous supply with a N source in combination with one UI (Figure 7-1A). The UI was added at a final concentration of 0.005% (0.2 µM) relative to the total N concentration present in the nutrient solution. Control plants were supplied with a N source but in absence of UI. In order to investigate whether 2-NPT was affecting the capacity of barley plants to take up urea, labeled-N sources were used for N uptake and translocation analyses in 22-d-old plants (ref. to section 7.4 for ^{15}N influx analysis). Thus, the whole experiment consisted of 27 treatments as shown in Figure 7-1B.



► **Figure 7-1.** (For description of this Figure refer to next page).

◄ **Figure 7-1. Design of hydroponic experiments with treatments established for each nutrient solution trial.** After germination and relocalization in a growth chamber (ref. to section 7.1.2), spring barley plants were precultured for 12 d in full nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added during preculture at a concentration of 0.005% of total N in the nutrient solution (0.2 µM) for either 2, 4 or 6 days. Afterwards, for determination of uptake or translocation rates, labeled N sources were supplied to 22-d-old barley plants at 200 µM for low N conditions as ^{15}N-KNO_3, urea + ^{15}N-KNO_3 or ^{15}N-urea + KNO_3 in addition to 0.01 µM 2-NPT or NBPT **(A)**, resulting in 27 treatments as presented in **(B)**.

7.2.2 Experimental design of the nutrient solution trials

As shown in Figure 7-1B, hydroponic experiments were carried out with 9 treatments per time point, i.e. 27 treatments in total, each one with 6 replicates randomized in a complete block design (Figure 7-2) by using the software Research Randomizer 4.0 (Urbaniak & Plous, 2013). Nonetheless, analyses were performed by considering only 4 biological replicates (n=4) in order to have enough plant material. By renewing the nutrient solution every other day, pots were relocated among the different tables so that plants were not permanently in the same position during the course of the experiment. When plants were 22-days old, uptake and translocation studies were performed (ref. to section 7.4 for ^{15}N influx analysis).

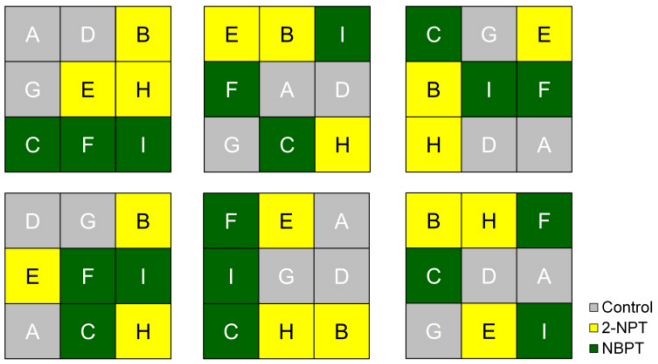


Figure 7-2. Spatial distribution of pots during hydroponic experiments. Pots were randomized in a complete block design, placing 6 pots per table, each one of them containing plants supplied either with **(A)** ^{15}N-KNO_3, **(B)** ^{15}N-KNO_3 + 2-NPT, **(C)** ^{15}N-KNO_3 + NBPT, **(D)** urea + ^{15}N-KNO_3, **(E)** urea + ^{15}N-KNO_3 + 2-NPT, **(F)** urea + ^{15}N-KNO_3 + NBPT, **(G)** ^{15}N-urea + KNO_3, **(H)** ^{15}N-urea + KNO_3 + 2-NPT or **(I)** ^{15}N-urea + KNO_3 + NBPT. Thus, **(A)**, **(D)** and **(G)** were the control treatments without addition of urease inhibitor. Exposure to ^{15}N-labeled N forms was conducted in 22-d-old spring barley plants, by transferring plants into different pots. From these hydroponically-grown barley plants, 4 biological replicates (n=4) were used for different analyses. Each square and sub-square represent one table and one pot, respectively.

7.3 Plant sampling and measurements

At harvest (ref. to section 7.2.1), 22-d-old barley plants were partitioned in shoots and roots and immediately frozen at -80°C before freeze-drying for ^{15}N measurements. Nonetheless, for determination of dry weight as well as concentrations of UI, urea, macro- and micronutrients in both shoots and roots, ^{15}N-labeling was not required and therefore plant samples were directly harvested, partitioned, homogenized by grinding them under liquid N and freeze-dried for these analyses, as described before in section 3.5. On the other hand, also fresh samples were required to determine the urease activity in shoots.

7.3.1 Determination of dry weight of shoots and roots

Dry weights of shoots and roots were gravimetrically determined using a balance.

7.3.2 Determination of macro- and micronutrient concentrations in shoots and roots

Macro- and micronutrient concentrations in shoots and roots were determined following the same procedure as described before in section 3.5.6. For these analyses, approximately 20-25 or 10-15 mg ground shoot or root material, respectively, were used.

7.3.3 Determination of 2-NPT in shoots and roots

Determination of the urease inhibitor N-(2-nitrophenyl)-phosphoric acid triamide (2-NPT) in shoots and roots was carried out as described before in section 3.5.9 for its determination in winter wheat leaves. For this analysis, approximately 30 or 15 mg of dried and ground shoot or root material, respectively, was used.

7.3.4 Determination of NBPT in shoots and roots

Determination of the urease inhibitor N-(n-butyl)thiophosphoric triamide (NBPT) in shoots and roots was based on the procedure carried out for determining concentration of 2-NPT in plant tissues (ref. to section 3.5.9). For this analysis, approximately 30 or 15 mg of ground shoot or root material, respectively, were used. As standard, the green liquid formulation of NBPT in different concentrations, was used (Koch Agronomic Services LLC, Wichita, USA).

7.3.5 Determination of urea concentrations in shoots and roots

Urea concentrations in shoots and roots were determined as described before for measurements in winter wheat leaves (ref. to section 3.5.2).

7.3.6 Determination of urease activity in shoots

Urease activity in shoots of spring barley was determined similarly as described before in section 3.5.5 for its determination in leaves of winter wheat. Main difference to here was the determination of the urease activity in whole shoot instead of fully expanded leaves. For this purpose, fresh shoots were cut into 1.0-1.5 cm pieces.

7.4 ^{15}N influx analyses

For influx analyses, spring barley plants from each experiment were transferred from pots containing non-labeled nutrient solution to different pots containing ^{15}N-labeled N forms. Before starting with the ^{15}N-influx measurements, 1 plant from each treatment was harvested and used as background to determine its natural ^{15}N abundance. For ^{15}N-influx measurements, barley roots were first rinsed in 1 mM $CaSO_4$ solution for 1 min. Subsequently, roots were incubated either for 10 or 120 min for determination of uptake or translocation rates, respectively, in a full nutrient solution (pH adjusted at 5.8) containing 200 µM of ^{15}N-labeled nitrate or urea + nitrate (95-98 atom% ^{15}N, Merck KGaA, Darmstadt, Germany) as the N source. Addition of either 0.01 µM 2-NPT or NBPT was carried out in UI treatments. After the incubation, roots were rinsed once again in 1 mM $CaSO_4$ solution for 1 min to exchange the ^{15}N tracer from the root apoplast, roots were carefully dried with paper tissue and plants were partitioned in shoots and roots, which were kept first in liquid nitrogen, then stored at -80°C, grinded under liquid N and freeze-dried. Aliquots of approx. 1.0-1.5 mg ground sample material were used for ^{15}N analyses by isotope ratio mass spectrometry (IRMS; NU Instruments Ltd., Wrexham, United Kingdom). Afterwards, these analyses allowed calculating ^{15}N influx in roots during 10 min, total ^{15}N uptake after 2h, the ^{15}N accumulation in roots and shoots and finally the root-to-shoot translocation rate (Annexes 35A, B, C, D, E, respectively).

7.5 Statistical analyses

Data were analyzed by analysis of variance (ANOVA), and treatment means were compared by using Tukey test at $P<0.05$ (n=4). Statistical analyses were performed using Statgraphics Centurion XV software version 15.2.05 (Statgraphics Technologies, Inc., The Plains, USA).

8 RESULTS

8.1 Influence of preculture with different N forms on growth, nutritional status and urease inhibitor accumulation in barley plants

8.1.1 Influence of supply with different N forms and addition of urease inhibitor on plant growth

To assess whether barley plants were able to be subjected to ^{15}N-influx analyses, biomass of shoots and roots were determined. Shoot and root DW are shown in Figures 8-1A and 8-1B, respectively.

Plants from all treatments reached a similar biomass (Figures 8-1A, B), corresponding to their visual appearance (Annexes 36 and 37). Taken together, results on shoot and root dry weight indicated that plants from all treatments had highly similar growth, without any significant difference due to the preculture.

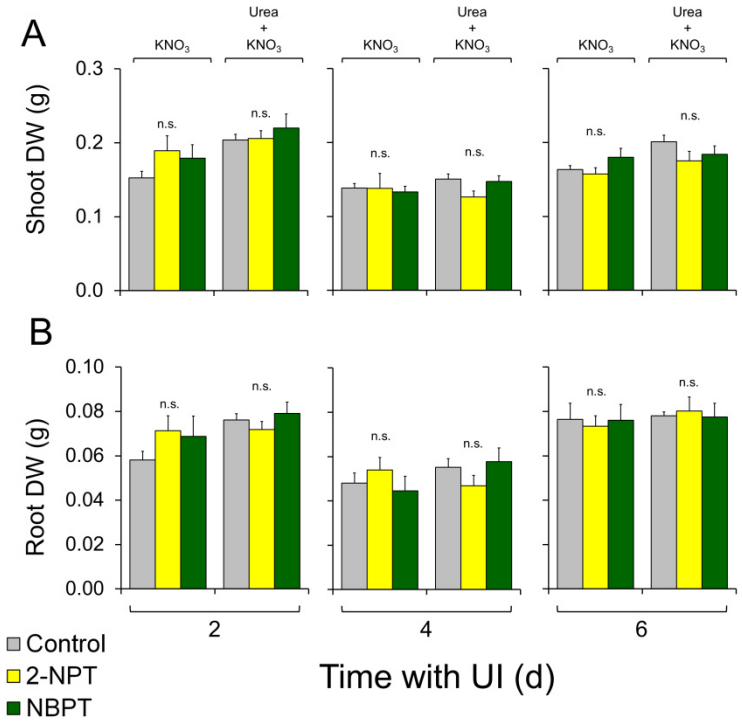


Figure 8-1. Shoot and root dry weights of barley plants after 2, 4 or 6 d of incubation with different urease inhibitors. (A) Shoot dry weight and **(B)** root dry weight of barley plants. Plants were precultured for 12 d in full-nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added during preculture at a concentration of 0.2 µM for either 2, 4 or 6 days. Results are shown for three independent experiments, which differed in their time period of incubation with urease inhibitors. Bars indicate means ± SE, n=4. Different letters indicate significant differences among means ($P<0.05$ by Tukey's test; n.s., not significant).

8.1.2 Influence of supply with different N forms and addition of urease inhibitor on macro- and micronutrient concentrations in shoots and roots

To assess whether the accumulation of the UI altered concentrations of nutrients in hydroponically-grown barley plants, 22-d-old spring barley plants were dissected in shoots and roots and analysed by ICP-MS and by an elemental analyzer. Concentrations of macronutrients in shoots and roots are presented in Figures 8-2A and 8-2B, respectively, whereas concentrations of micronutrients are in Figures 8-3A and 8-3B for shoots and roots, respectively.

In general, concentrations of all determined macronutrients were above threshold levels and indicated that plants were adequately supplied. Concentrations of calcium in roots were approx. half of those in shoots in all three hydroponic experiments (Figure 8-2). An influence of the supplied N form, i.e. either potassium nitrate or urea together with KNO_3, on concentrations of macronutrients was not found. This held true for any time of exposure to UI.

Regarding the UI treatments, concentrations of macronutrients in shoots and roots did not differ to those observed in control plants. Exceptionally, barley plants accumulated 8 and 22% more calcium than control plants when 2-NPT or NBPT was added, respectively, continuously for 4 d (Figure 8-2B). However, such an effect did not occur when plants were exposed to UIs for 2 or 6 days.

Contrastingly, concentrations of micronutrients strongly differed between shoots and roots (Figure 8-3). This held especially true for Fe, Cu, Mn, Ni and B. Concentrations of Fe, Cu, Ni and Mn in roots were up to 30-fold higher than those measured in shoots, whereas concentrations of B and Mo were approx. 3-fold higher in shoots than in roots. In general, concentrations of zinc were slightly higher in roots than in shoots. Elevated accumulation of metal microelements is due to their strong fixation in the root apoplast (Marschner, 2012). Again, neither an influence of the supplied N form nor of 2-NPT or NBPT was observed in any of the seven analyzed micronutrients. Although Bergmann (1988) did not refer to critical levels of barley plants at such an early developmental stage as assessed here (approximately BBCH 22, beginning of tillering phase), macro- and micronutrients reflected an adequate nutritional status of the plants, which was in agreement with their visual appearance (Annexes 36 and 37).

Taken together, neither the supplied N form nor the addition of 2-NPT or NBPT influenced concentrations of macro- and micronutrients in hydroponically-grown barley plants, suggesting that their presence in the nutrient solution had no adverse short-term impact on plant growth.

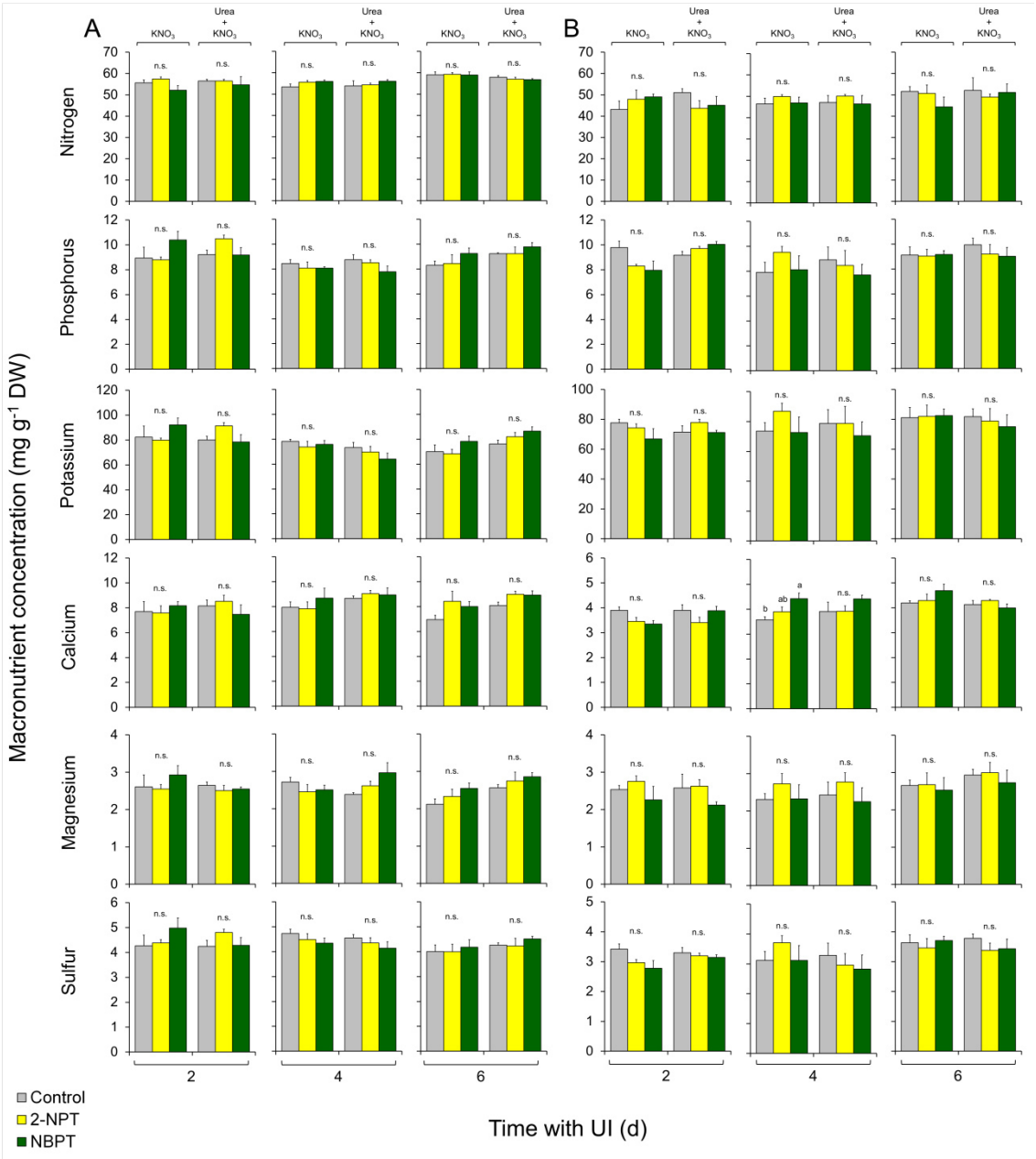


Figure 8-2. Influence of N form and urease inhibitor on macronutrient concentrations in shoots and roots. Results are shown for three independent experiments, which differed in their time period of incubation with urease inhibitor. Spring barley plants were precultured for 12 d in full-nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added during preculture at a concentration of 0.005% of total N in nutrient solution (0.2 µM) for either 2, 4 or 6 days. Concentrations of macronutrients are shown in **(A)** and **(B)** for shoots and roots, respectively. Bars indicate means ± SE, n=4. Different letters indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

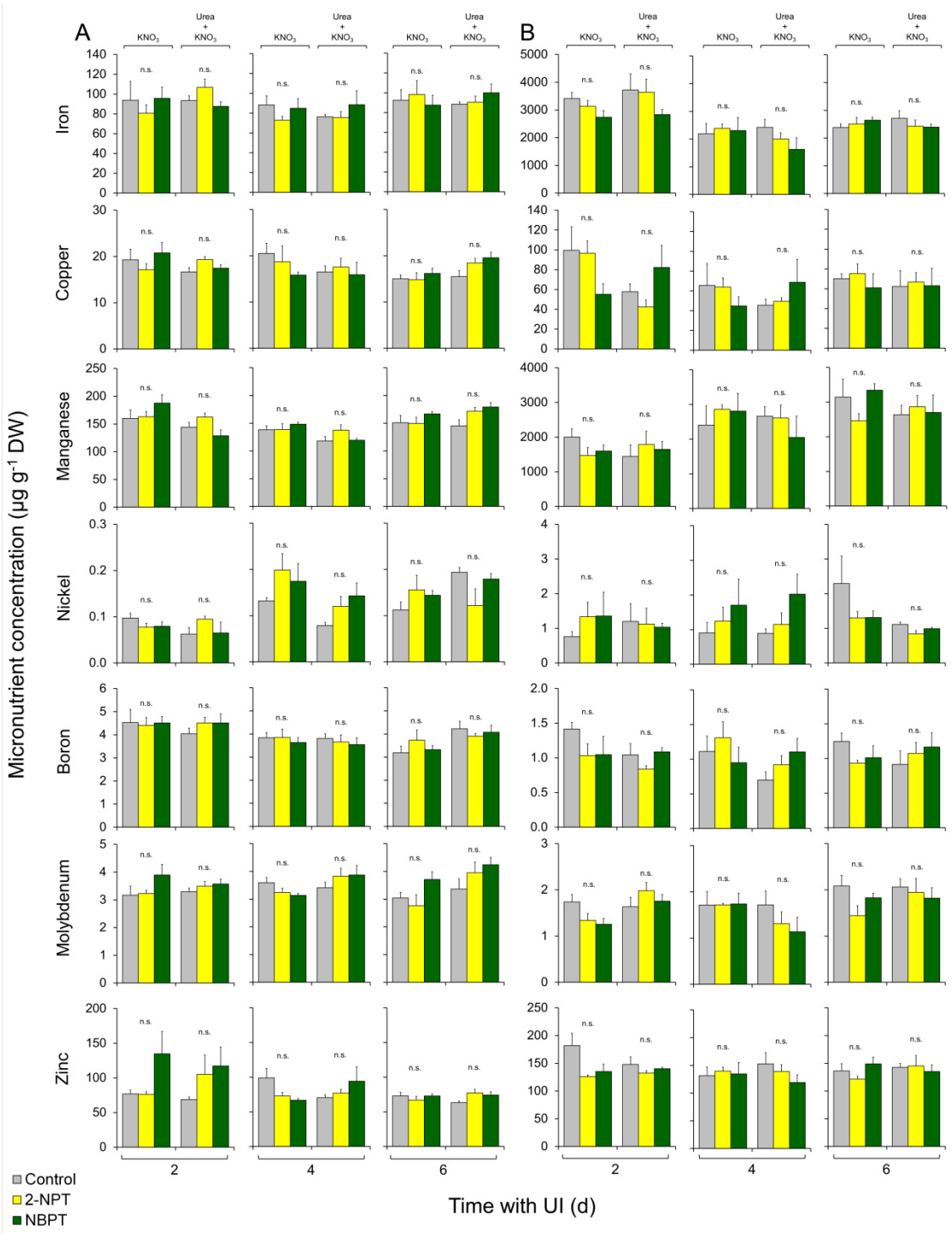


Figure 8-3. Influence of N form and urease inhibitor on micronutrient concentrations in shoots and roots. Results are shown for three independent experiments, which differed in their time period of incubation with urease inhibitor. Spring barley plants were precultured for 12 d in full-nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added during preculture at a concentration of 0.005% of total N in nutrient solution (0.2 µM) for either 2, 4 or 6 days. Concentrations of micronutrients are shown in **(A)** and **(B)** for shoots and roots, respectively. Bars indicate means ± SE, n=4. Different letters indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).

8.1.3 Detection of the urease inhibitors 2-NPT and NBPT in shoots and roots and their individual impact on N metabolism

Knowing that 2-NPT can accumulate in leaves after its uptake by wheat roots (ref. to section 4.2.2), it was important to check whether addition of this UI to hydroponically-grown barley plants also led to detectable accumulation in shoots and roots. Concentrations of both 2-NPT or NBPT are shown in Figures 8-4A and 8-4B or 8-4C and 8-4D, respectively. Since UIs were not detectable in control plants (without UI addition), these treatments are not represented.

Accumulation of UIs in plant tissues followed an effective addition of these compounds to the nutrient solution, without that adverse effects on plant growth occurred, as verified by visual inspection of shoots (Annex 36) and roots (Annex 37). Although 2-NPT accumulated to approx. 10-fold higher levels in shoots and roots than NBPT, there were no significant differences in growth. It was expected that a longer incubation time with the UI will lead to higher concentrations of the UI in the plant tissue. However, this expectation was not met, neither with 2-NPT nor with NBPT. Probably, the shortest incubation period (2, 4 or 6 d) was already sufficient to allow an almost maximum accumulation in roots and shoots. Comparing absolute levels of accumulation in roots versus shoots showed that NBPT concentrations ranged between 0.1-0.3 $\mu g\ g^{-1}$ in shoots and roots, whereas 2-NPT levels were between 3 and 8 $\mu g\ g^{-1}$, thus more than 10-fold higher. Moreover, 2-NPT had a slight tendency to accumulate to higher levels in shoots than in roots.

Although no significant differences were observed in the concentrations of either UI in shoots and roots, a tendency towards lower 2-NPT concentrations in both shoots and roots was found under urea nutrition. For instance, 2-NPT concentrations in shoots were approx. 30, 8 or 35% lower after 2, 4 or 6 d in presence of 2-NPT, when urea was supplied together with KNO_3 (Figure 8-4A). This could be explained by 2-NPT acting as an urea analogue, which may have competed with urea for uptake by plant roots.

Taken together, detection of either UI, 2-NPT and NBPT, in barley tissues showed that their accumulation did not coincide with visual symptoms. In this sense, 2-NPT and NBPT did not differ, except that 2-NPT concentrations in root and shoot tissues were approx. 20 times greater than those of NBPT.

The impact of UIs on N metabolism was further investigated in dependence of the supplied N form. For this purpose, aside of determining N concentrations in both shoots and roots (Figure 8-2), urea concentrations in these tissues were measured.

Despite the fact that N concentrations in both shoots and roots were neither affected by the supplied N form (KNO_3 or urea together with KNO_3) nor by UI treatment, urea concentrations appeared to be strongly affected in dependence of the N source applied (Figure 8-4E, F). As expected, the lowest urea concentrations were observed after supply of KNO_3 as the sole N source, irrespective of the period for which plants were exposed to either UI. In addition, no significant differences were observed among UI treatments, as shoot and root concentrations of urea in control plants were similar to those after addition of either 2-NPT or NBPT, when plants were grown for 2 d in presence of the UI (first experiment). Nonetheless, when plants were cultured for 4 or 6 days in nutrient solution supplied with urea + KNO_3 containing either 2-NPT or NBPT, concentrations of urea in shoots were significantly higher, when compared to those of control plants (Figure 8-4E). This may suggest that both UIs promoted urea uptake. When plants were cultured for 6 days in a full-nutrient solution containing NBPT, urea concentrations in roots under urea nutrition were significantly reduced. Maybe, the uptake capacity of roots was repressed after addition of NBPT (Figure 8-4F). However, such observations would require deeper investigation.

With regard to the enzymatic activity of urease in shoots, similar levels were found among plants supplied either with KNO_3 or with urea + KNO_3 when plants were exposed for 2 days to either UI (Figure 8-4G). Contrastingly, when barley plants were exposed for 4 or 6 days to the UIs, significant differences were found. Urease activity after supply of KNO_3 as the sole N source and addition of NBPT for 4 days was strongly increased, reaching 52% higher levels than control or 2-NPT-fed barley plants. The reason why NBPT increased the urease activity in shoots in KNO_3-fed plants remains unclear. Under supply of urea together with potassium nitrate, this effect was not observed. However, when barley plants were exposed to the UI for 6 days, significant differences among supplied N forms as well as between UI treatments were found. Interestingly, the presence of UI under KNO_3 as the sole N source appeared to

repress the enzymatic activity of urease, leading to 46 or 35% lower activity after addition of 2-NPT or NBPT, respectively, relative to control plants. When supplied with NBPT and urea + KNO_3, urease activities strongly decreased (up to 2-fold less). The latter suggested that the presence of NBPT provoked adverse physiological effects as reported in other studies (Cruchaga et al., 2011; Zanin et al., 2015).

Taken together, notwithstanding the absence of alterations on N concentrations in shoots and roots after supply of different N forms in addition to a UI (Figure 8-2), urea concentrations in shoots were especially promoted after 4 or 6 days of continuous UI supply to the nutrient solution, but only when urea was present. On the other hand, urease activity in shoots was strongly affected after addition of UI for 6 days, irrespective of the supplied N source. However, the latter held true only for NBPT and not for 2-NPT, when urea was supplied together with potassium nitrate.

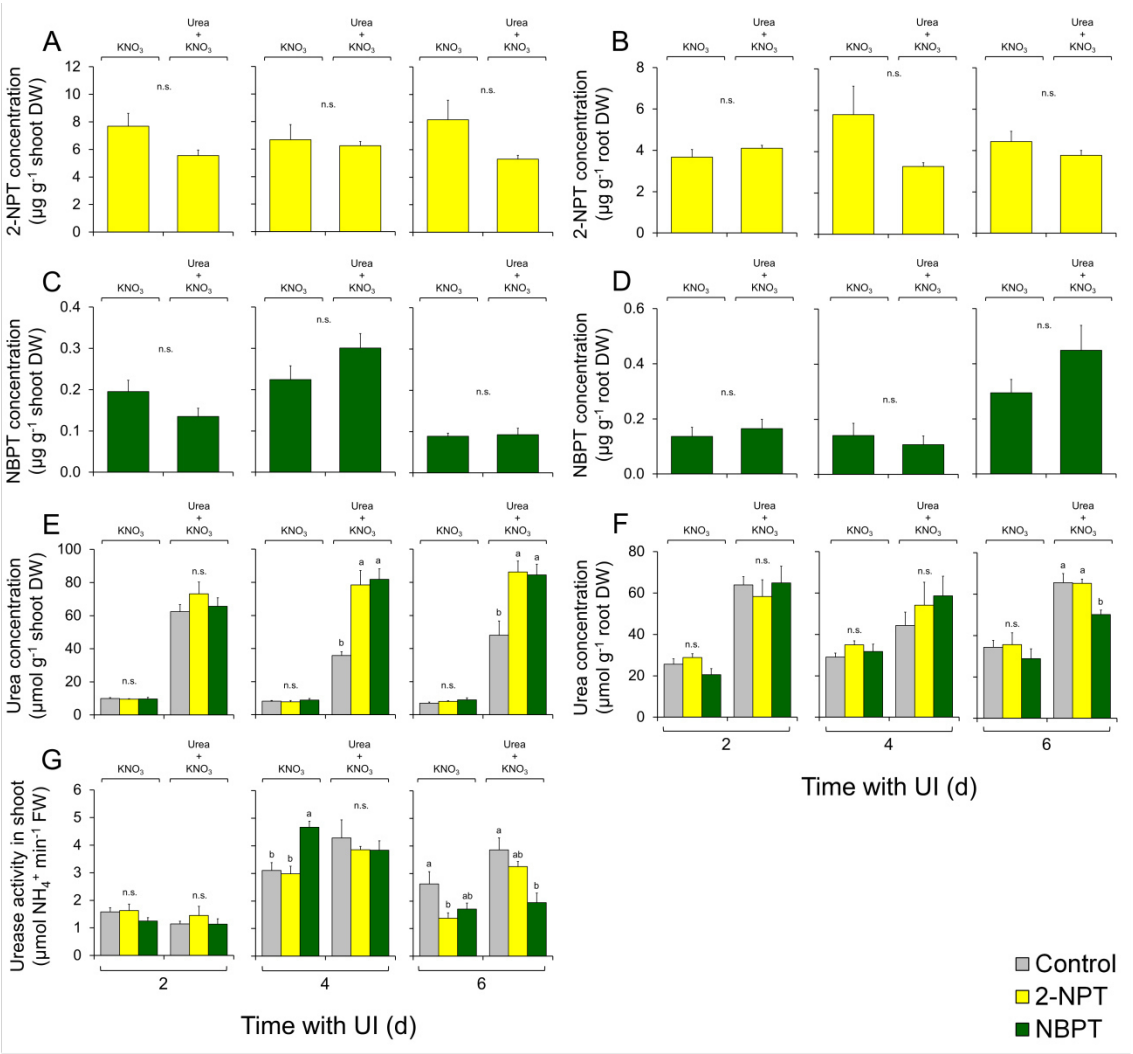


Figure 8-4. Concentrations of the urease inhibitor 2-NPT or NBPT in shoots and roots of spring barley plants grown in presence of KNO_3 or urea + KNO_3 and their influence on urea concentrations and on enzymatic activity of urease. Results are shown for three independent experiments, which differed in their time period of incubation with urease inhibitor. Spring barley plants were precultured for 12 d in full-nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added during preculture at a concentration of 0.005% of total N to the nutrient solution (0.2 µM) for either 2, 4 or 6 days. **(A, B)** Concentrations of 2-NPT and **(C, D)** of NBPT in shoots and roots, respectively. **(E, F)** Urea concentrations in shoots and roots, respectively. **(G)** Urease activity in shoots. Bars indicate means ± SE, n=4. Different letters indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant). Since both 2-NPT and NBPT were not detectable by UPLC-MS/MS in control plants, these concentrations are not shown.

8.2 Influence of preculture with different N forms on short- and long-term uptake of different ^{15}N-labeled N forms in barley plants

The previous results presented here raised some open questions, like whether the uptake capacity of roots is repressed after addition of NBPT when exposed for 6 days. To address this open question, uptake studies with ^{15}N-labeled KNO_3 or urea were performed. Barley plants were first precultured for 12 d in a full-nutrient solution containing 4 mM N as either potassium nitrate or as urea + KNO_3. To this nutrient solution, 0.2 µM of either 2-NPT or NBPT were added for 2, 4 or 6 days. Later, spring barley plants were transferred to different pots and incubated in nutrient solution containing 200 µM of ^{15}N-labeled sources, i.e. i) ^{15}N-KNO_3, ii) urea + ^{15}N-KNO_3 or iii) ^{15}N-urea + KNO_3. To these ^{15}N-labeled sources supplied to the nutrient solution, 0.01 µM of either 2-NPT or NBPT were also added, except for control plants, which remained without UI. Finally, barley plants were incubated for either 10 or 120 min with the corresponding ^{15}N-labeled sources for determination of ^{15}N influx in roots, of the total ^{15}N uptake, of the ^{15}N accumulation in roots or shoots and of the root-to-shoot translocation rate.

8.2.1 Influence of preculture with different N forms and addition of urease inhibitor on short-term uptake of ^{15}N-labeled sources in barley plants

When barley plants were exposed to ^{15}N-labeled KNO_3, root influx was at approx. 0.4 µmol ^{15}N g^{-1} root DW min^{-1} while no significant influence of the UI was observed (Figure 8-5). This observation held true irrespective of the time period for which roots were exposed to either UI. When root uptake of ^{15}N-labeled NO_3 was examined in the presence of urea, nitrate influx remained unaltered after exposure to UIs in all three hydroponic experiments. Nevertheless, when urea was the ^{15}N-labeled form supplied together with KNO_3, influx was generally 2-4 times lower than for nitrate and significant differences among treatments were observed after 2 or 6 d of exposure to either UI. After 2 d exposure, ^{15}N influx was lower in 2-NPT-treated plants, whereas NBPT reached the highest ^{15}N influx, namely about 45% more than after 2-NPT. These results suggested that urea uptake may have been negatively influenced by addition of 2-NPT, but only when referred to NBPT incubation for 2 d, while this effect was absent when added for 4 days. Nonetheless, when added for 6 days, both UIs showed substantially lower ^{15}N influx (approx. 30% less), when compared to control plants. However, alterations in short-term N influx by UIs occurred only in urea-fed plants.

Taken together, significant effects on N influx were observed after addition of 2-NPT or NBPT to plants for 6 days, if ^{15}N-urea was supplied together with KNO_3. However, either UI had no consistent impact on ^{15}N influx from nitrate but only from urea after longer incubation with the UI.

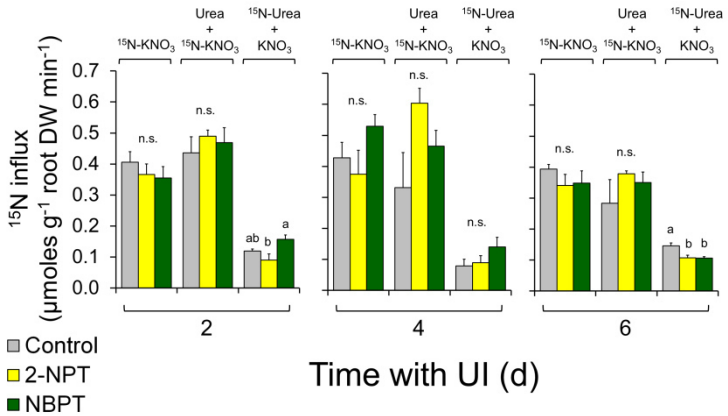


Figure 8-5. Influx of different ^{15}N-forms and addition of urease inhibitor in roots of barley plants. Results are shown for three independent experiments, which differed in their time period of incubation with urease inhibitor. Spring barley plants were precultured for 12 d in full-nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added during preculture at a concentration of 0.005% of total N to the nutrient solution (0.2 µM) for either 2, 4 or 6 days. Afterwards, ^{15}N influx was measured during 10 min of labeling with 200 µM N supplied as ^{15}N-KNO_3, urea + ^{15}N-KNO_3 or ^{15}N-urea + KNO_3 in presence of 0.01 µM 2-NPT or NBPT. Bars indicate means ± SE, n=4. Different letters indicate significant differences among means ($P<0.05$ by Tukey's test; n.s., not significant).

8.2.2 Influence of preculture with different N forms and addition of urease inhibitor on total ^{15}N uptake, on the ^{15}N accumulation in shoots or roots and on the root-to-shoot translocation rate in barley plants

After incubating spring barley plants for 120 min in a full-nutrient solution containing 200 µM ^{15}N-labeled sources and addition of 0.01 µM UI either for 2, 4 or 6 days, total ^{15}N uptake was determined in roots of 22-d-old plants (Figure 8-6A). In addition, obtained data allowed the calculation of the ^{15}N accumulation in roots and shoots (Figures 8-6B and 8-6C, respectively) and the root-to-shoot translocation rate (Figure 8-6D).

Total ^{15}N uptake from nitrate was not affected by the UI, irrespective of whether labeled nitrate was supplied together with urea or not. However, after 6 days of incubation with either UI, nitrate uptake dropped. Since this decline was also observed in control plants not exposed to UI, it was likely due to a lower demand or decreasing nitrate uptake activity.

Overall N uptake from ^{15}N-labeled urea was more than 10 times lower than for nitrate. Here, the presence of either UI for 2 or 6 days suppressed urea-N uptake, indicating that short- and long- term exposure of roots to the UI may interfere with the capacity of plant roots to take up urea. Maybe, as both UIs share similar chemical structures to urea, uptake transporters could have been blocked by the presence of either UI, thus impeding urea uptake by plant roots.

While the ^{15}N accumulation in roots contributed to approx. 80% and that in shoots to approx. 20% to overall accumulation of ^{15}N in the plants, they more or less reflected the same differences among treatments as the overall ^{15}N accumulation. Accumulation of N from urea was more than 10 times lower in roots as well as in shoots than from nitrate, and neither UI affected N accumulation from nitrate. By contrast, both NBPT and 2-NPT consistently inhibited N accumulation from urea in roots whereas in shoots their negative impact was only observed after 2 days. This indicated that roots by be exposed to a primary inhibitory effect of either UI on urea uptake.

Finally, although not always significant, when supplied alone or together with urea, there was a tendency to lower root and shoot accumulation of ^{15}N from ^{15}N-KNO_3 (both up to approx. 30% less) after addition of NBPT, when compared to the 2-NPT, especially when the UI was present for 2 or 6 days. This suggests that NBPT may interfere with N uptake and accumulation in the presence of urea whenever nitrate is supplied. This may point to an advantage of 2-NPT over NBPT.

Root-to-shoot translocation rates of ^{15}N were highest when ^{15}N-KNO_3 was supplied alone or together with urea (Figure 8-6D). The reason may be that overall uptake capacity of nitrate is higher than for urea or that urea is assimilated directly in roots, while nitrate is mainly translocated to the shoots (Mérigout et al., 2008). As shown for influx and root or shoot accumulation, also N translocation rates were lowest for urea-bound N. Interestingly, when UI was added for 6 days and N was supplied as urea + ^{15}N-KNO_3, translocation rates were approx. 20 or 40% less than those of control plants after addition of 2-NPT or NBPT, which was also reflected in the ^{15}N accumulation in shoots. Maybe, when the UI is present together with nitrate for a longer period, plants tend to take up and accumulate N preferentially in roots rather than translocating it to shoots (Figures 8-6C, D).

Correlations between ^{15}N accumulation in roots and ^{15}N accumulation in shoots were calculated for each hydroponic experiment, namely when barley plants were exposed either for 2, 4 or 6 days to an UI (Annex 38). Close correlations of up to $r^2 = 0.99$ were found between ^{15}N accumulation in roots and shoots when plants were grown for 2 or 6 d in the presence or absence of UI. When the UI was added for 4 d, correlations were not consistent, indicating that maybe in the 4 days-measurements there was a general technical inconsistency.

Very close and consistent correlations of $r^2 = 0.94$, 0.88 or 0.99 were also found between root ^{15}N accumulation and the root-to-shoot translocation rate when plants were supplied with urea + ^{15}N-KNO_3 with addition of UI for either 2, 4 or 6 days, respectively (Annex 39). When the UI was present or absent for 6 d in urea + ^{15}N-KNO_3-fed plants, a very close correlation of $r^2 = 0.99$ was found. Nevertheless, when N was supplied in form of either ^{15}N-KNO_3 or ^{15}N-urea + KNO_3 with addition of UI for 4 days, negative correlations were obtained ($r^2 = -0.94$ or -0.76, respectively). Somehow, these observations almost coincide with the previous correlations calculated between root ^{15}N accumulation and the root-to-shoot translocation rate ($r^2 = -0.33$ or -0.65, respectively) and also with the following calculated between the total ^{15}N uptake and the root-to-shoot translocation rate (Annex 40, $r^2 = 0.23$ or -0.58, respectively). The latter is not fully understood but might be ascribed to a weaker effect triggered after labeling plants with these specific ^{15}N forms,

which was not the case when N was supplied as urea + ^{15}N-KNO_3. In contrast, strong correlations between the total ^{15}N uptake and the root-to-shoot translocation rate of r^2 = 0.98, 0.97 or 0.99 were found in urea + ^{15}N-KNO_3-fed barley plants after addition of UI for either 2, 4 or 6 d, respectively. Finally, when UI was added either for 2 or 6 days, close correlations with r^2 between 0.76 and 0.99 were found after supply with the different ^{15}N sources, especially in presence of urea.

Taken together, addition of 2-NPT or NBPT triggered a lower accumulation of ^{15}N-urea mainly in plant roots, which was consistently observed irrespective of the UI incubation period. In shoots, this effect occurred mostly when the UI was present for 2 days and tended to become weaker with the extension of the exposure to UI. In contrast, when the N source was supplied as ^{15}N-KNO_3, alone or together with urea, no consistent influence on the ^{15}N accumulation was observed in shoots and roots. Therefore, it could not be clarified whether the uptake or translocation under urea nutrition is repressed by presence of either UI.

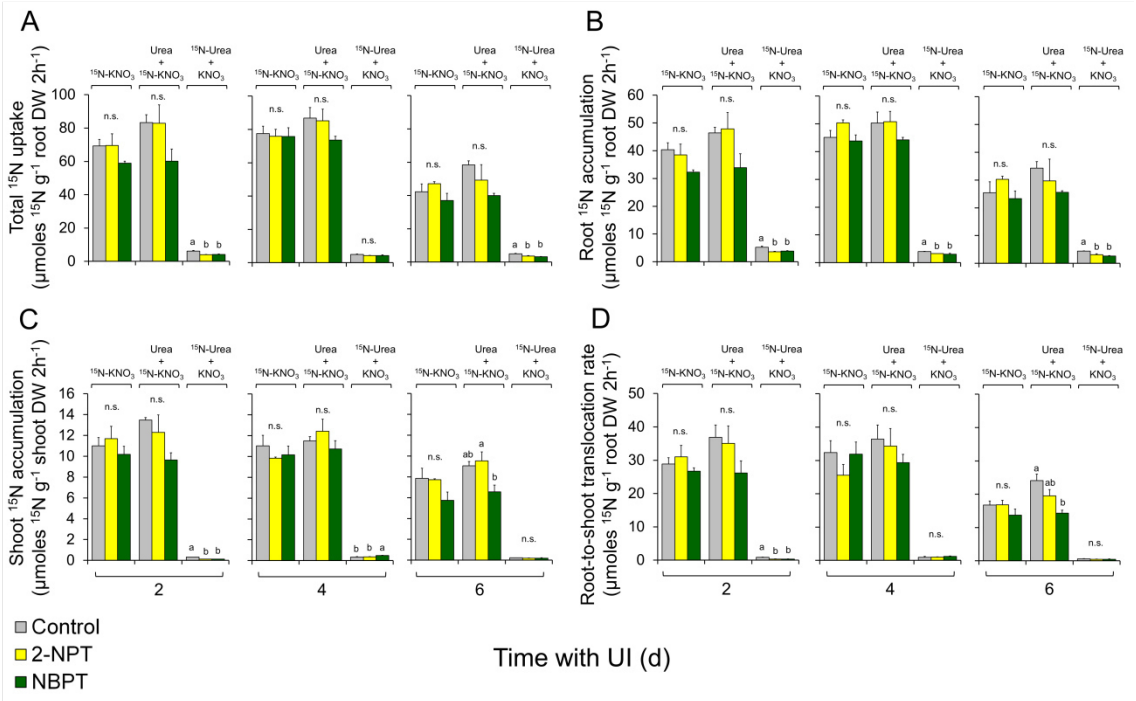


Figure 8-6. Influence of different ^{15}N-forms supplied in addition to urease inhibitor on total ^{15}N uptake, ^{15}N accumulation in roots and shoots and on the root-to-shoot translocation rate of barley plants. Results are shown for three independent experiments, which differed in their time period of incubation with urease inhibitor. Spring barley plants were precultured for 12 d in full-nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added during preculture at a concentration of 0.005% of total N to the nutrient solution (0.2 µM) for either 2, 4 or 6 days. Afterwards, **(A)** total ^{15}N uptake, **(B, C)** ^{15}N accumulation in roots or shoots, respectively, and **(D)** root-to-shoot translocation rate were measured after 120 min of labeling with 200 µM N supplied as ^{15}N-KNO_3, urea + ^{15}N-KNO_3 or ^{15}N-urea + KNO_3 in presence of 0.01 µM 2-NPT or NBPT. Bars indicate means ± SE, n=4. Different letters indicate significant differences among means ($P<0.05$ by Tukey's test; n.s., not significant).

9 DISCUSSION

As learnt from the first chapter of the present study, application of urea fertilizer in combination with the urease inhibitor 2-NPT to field-grown winter wheat plants triggered several effects on plant N metabolism, mainly as a consequence of its uptake by plant roots and accumulation in leaves (Figures 4-2 and 4-3). Among these effects, most relevant were the increased urea translocation rates in xylem sap (ref. to section 5.4.1) and the subsequent accumulation of this compound in leaf tissues (ref. to section 5.4.2). However, it was also learnt that such effects may depend on soil properties (Watson et al., 1994; Abalos et al., 2014; ref. to section 5.1). Whether other grain cereals like spring barley can also take up this UI, accumulate it in shoots and thus, trigger effects on growth and N metabolism as well as on the uptake and translocation of N forms was still an open question. However, such a verification needed to be conducted by excluding weather and soil factors that may alter the UI performance itself. Therefore, spring barley plants were cultivated in an aerated hydroponic system under controlled climatic conditions. As an urease inhibitor, 2-NPT belongs to the family of the amides and esters of phosphoric acid, thus representing a structural anologue to urea, which likely competes with urea for uptake by plant roots. Previous reports with NBPT, another structural analogue to urea, reported several adverse effects to plants after its addition. For instance, Zanin et al. (2015) treated hydroponically-grown maize plants under urea nutrition with NBPT and observed that the UI significantly reduced the uptake capacity of these plants, as shown by a decrease in the uptake rate as well as in the accumulation of ^{15}N derived from urea. On the other hand, Cruchaga et al. (2011) reported lower ^{15}N-urea accumulation in roots of pea and spinach plants after addition of NBPT than in control plants. Hence, it was a major aim of this second part of the study to investigate in cereals the short-term effects of 2-NPT on the capacity to acquire urea and to investigate the physiological implications of this UI for N metabolism.

9.1 Addition of 2-NPT to urea-fed barley plants increases urea concentrations in shoots without affecting urease activity

The present study revealed that the addition of the urease inhibitor 2-NPT at least for 4 days to a nutrient solution containing urea in presence of KNO_3 led to higher accumulation of urea in shoots (Figure 8-4E). The latter was in agreement with previous results with field-grown winter wheat (Figure 4-14). Moreover, after determining urease activity in shoots it became clear that addition of this UI did not alter endogenous urease activity (Figure 8-4G). This result confirmed previous observations described for winter wheat in the first part of this study, in which internal enzymatic activities of urease were not consistently affected (Figure 4-14).

When NBPT was added and urea concentrations in shoots were already higher, urease activity in shoots was suppressed (Figures 8-4E, G). However, this observation held true only for the 4 days UI treatment. Regarding the observed effect on urease activity after addition of NBPT, recently Zanin et al. (2016) investigated the implications after the addition of this UI to a nutrient solution supplied with 0.5 mM urea on 5-d-old maize plants. After exposure to UI for 1 d, these authors reported approx. 50% lower urease activity in shoots than without NBPT. In the present study, this decrease was only observed after 6 d of exposure. This may suggest, that regardless of the plant species, addition of NBPT may interfere with endogenous urease on the long run, while such an inhibitory effect may not occur after addition of 2-NPT.

Unaffected activity of endogenous urease, by 2-NPT is expected to result in less impact on N metabolism in plants, especially when considering that urea is internally and continuously produced by plants through the arginase reaction (Figure 1-5). Although Zanin et al. (2016) reported significantly higher urea concentrations in shoots of 5-d-old maize seedlings treated with NBPT compared to untreated plants, concentrations of urea were markedly lower than those reached by 22-d-old barley plants, i.e. 0.41 µmol urea g^{-1} FW and 86 µmol urea g^{-1} DW for maize and spring barley plants, respectively. Maybe, UI-mediated changes in urea levels are more difficult to be seen, because overall urea levels are much higher.

Uptake of 2-NPT by plant roots induced an accumulation of this UI up to approx. 6 and 4 µg g^{-1} DW in shoots and roots of barley plants supplied with urea + KNO_3, respectively (Figures 8-4A, B). As observed previously in the first chapter of this investigation, depending on the soil type, 2-NPT was found to accumulate in leaves of winter wheat up to either approx. 6 or 0.3 µg g^{-1} DW (Figures 4-3A, B). Interestingly, these concentrations of 2-NPT obtained from

field-grown winter wheat were measured in leaves harvested 4 or 5 d after UI addition (Table 3-6). Obviously, in the hydroponic experiment, concentrations of the UI in the nutrient solution are more stable, which is not the case when the UI is applied directly to soils via surface coating of urea granules. In the soil, 2-NPT may have leached to deeper soil layers and dispersed across the soil, decreasing its concentration close to the root surface. Thus, in the nutrient solution plant roots may have been in direct contact and permanent exposure to 2-NPT. It is noteworthy that both barley and wheat showed similar UI concentrations when urea was present. However, the first part of this study involved analyses in leaves, whereas the second part involved analyses considering the whole shoot. This might also contribute to the differences found between soil- and hydroponically-grown plants.

Concentrations of NBPT in shoots and roots were also determined in barley plants (Figures 8-4C, D). Concentrations of NBPT were roughly 25- and 15-fold lower in shoots and roots, respectively, than those observed for 2-NPT (Figures 8-4A, B). Cruchaga et al. (2011) reported slightly higher concentrations in leaves of pea (0.43 µg g^{-1} DW) and spinach (0.28 µg g^{-1} DW) after 2 days of NBPT addition. Nevertheless, these authors reported concentrations of NBPT of approx. 0.01 µg g^{-1} DW in both pea and spinach roots, whereas in barley plants from the present study concentrations of this UI in roots were around 15-fold higher than these reported concentrations after 2 days from UI application. Thus, the higher accumulation of 2-NPT relative to NBPT may indicate that 2-NPT allows plants to increase internal urea concentrations without altering activity of endogenous urease. Whether this has further metabolic implications remains open.

9.2 Growth and nutritional status of barley plants are not affected by addition of 2-NPT

The significant increase of urea concentrations observed mostly in shoots under urea nutrition and addition of 2-NPT did not provoke negative consequences on plant growth of both barley shoots and roots, neither in plants from these treatments nor in KNO_3-fed plants (Figures 8-1A, B). These plant tissues were harvested 22 days after germination of barley seeds and did not show any visible alterations after addition of 2-NPT (Annexes 36 and 37). The latter held true also after addition of NBPT. The addition of 2-NPT to the nutrient solution followed several preliminary experiments (data not shown), in which the amount to be added was tested and re-calculated in order to simulate the amount of 2-NPT contained in urea granules applied under field conditions. Formulated as a fertilizer, urea granules are coated with about 0.075% 2-NPT. Defined concentration of 0.005% 2-NPT of total N present in the nutrient solution did not induce toxicity in barley plants and indeed, this was the case in all three conducted hydroponical experiements of the present study. Similarly, Zanin et al. (2015) did not observe visible toxicity symptoms, such as yellowing or necrosis in maize seedlings, after addition of NBPT, which also triggered a significant increase of urea concentrations in shoots and roots. Nonetheless, these authors reported reduced shoot biomass when plants were supplied with urea and NBPT. In the present study, such an effect was observed neither after addition of 2-NPT nor of NBPT. In another investigation conducted by the same research group, Zanin et al. (2016) supplied maize seedlings with urea and 0.897 µM NBPT. In agreement with the present results, they did not observe changes in the dry weight of both shoots and roots between control- (without N), urea- and urea + UI-plants. In the present study, 0.2 µM 2-NPT was added, which was the same concentration used as for NBPT. Thereby, using even a slightly higher UI concentration as that applied by Zanin et al. (2016) turned out being effective and not detrimental to plants.

In other investigations, dry weight of shoots and roots of different crop species were shown to be strongly altered when urea concentrations increased due to repressed urease activity triggered by Ni deficiency. For instance, hydroponically-grown 6-week-old-rice plants showed 85 or 56% less biomass of shoots or roots, respectively, than control plants, while urea concentrations increased from 3.2 or 2.7 to 177 or 5.1 µmol g^{-1} DW in shoots or roots, respectively, under urea nutrition (Gerendás et al., 1998). Also dry weights of 5-week-old wheat, soybean, rape and zucchini plants dramatically decreased by 1.5-, 1.4-, 1.2- or 2.8-fold, respectively, compared to plants with unaltered urea concentrations due to repressed urease activity. Among the studied plant species in that study, dry weight of rye and sunflower plants were however not influenced by increased urea concentrations (Gerendás & Sattelmacher, 1997b). Although in the present study, dry weight of spring barley was not influenced by the supplied N form, neither as KNO_3 alone nor as urea + KNO_3, other studies reported lower biomass of shoots and roots under urea nutrition than under ammonium nitrate. For instance, under urea nutrition shoot or root dry weight of rice plants decreased 1.8 or 1.5-fold in shoots or roots, respectively, relative to NH_4NO_3 nutrition (Gerendás et al., 1998). In a similar experiment, 6-week-old spring rape plants were grown in a nutrient solution containing either urea or ammonium nitrate. When grown in urea, shoot or root dry weight were also reduced by 70 or 20%, respectively, relative to

NH_4NO_3 nutrition (Gerendás & Sattelmacher, 1997a). In contrast, Arabidopsis plants precultured hydroponically for 5 weeks in 1 mM NH_4NO_3 and then transferred to 1 mM NH_4NO_3 or 0.5 mM NH_4NO_3 and 0.5 mM urea did not show significant differences in their dry weight of shoots or roots after 1 week (Mérigout et al., 2008). Also Cruchaga et al. (2011) reported no differences between NBPT-treated and non-treated pea and spinach after 9 days. Thus, this may indicate that an influence of the N form on the dry weight of shoots and roots might depend on the plant species. When comparing such studies with the present work, however, a major difference lies also in the fact that here urea was co-supplied with nitrate, whereas in most studies cited above, urea was supplemented as a sole N source. Sole supply of urea-N definitely suppresses plant growth and does not reflect a likely scenario as found in most soils, where also other N forms will be present even directly after urea application.

Regarding the influence of UI addition on total N concentrations, Zanin et al. (2016) reported substantially lower concentrations of total N in shoots, when comparing urea + UI- with urea-treated plants. Moreover, they reported lower total N concentrations (approx. 68-70% less) in urea-fed plants than in control plants, irrespective of addition of UI. In the present study, such effects did not occur (Figures 8-2A, B). Probably, this can be ascribed to plant age, since these authors applied the UI to 5-d-old maize plants, whereas the present study employed further developed plants. Therefore, it is assumed that plants may respond differently to UI application depending on plant age, since early developmental stages may be more susceptible. Moreover, while these authors applied the UI just for 1 d, in the present study plants were exposed to either UI at least for 2 d. When continuously supplied for up to 6 days, 2-NPT does not have such an impact on concentrations of total N in shoots and roots.

In the same way, nutrient concentrations in both shoot and root tissues from the present study, were neither influenced by the N form nor by addition of either UI (Figures 8-2A, B; 8-3A, B). Nutrient concentrations remained consistently unaffected in all three experiments, regardless of the time of exposure to the UI (for 2, 4 or 6 days). As well as for 2-NPT, addition of NBPT to KNO_3- or to urea + KNO_3-treated spring barley did not trigger alterations neither on macronutrient nor on micronutrient concentrations in shoots or roots. Interestingly, different publications reported an influence of the N form, either as urea as the sole N source or ammonium nitrate on concentrations of macro- and micronutrients. For example, in rice plants concentrations of macronutrients like K or Ca were approx. 6-8% higher in shoots when plants were supplied with urea, whereas micronutrients like Cu, Zn, Fe or Mn levels decreased by approx. 33-50% when supplied with ammonium nitrate (Gerendás et al., 1998). In roots of rape plants, Gerendás & Sattelmacher (1999) reported in general the highest macro- and micronutrient concentrations under urea nutrition than after NH_4NO_3 supply. The same was also reported in roots of zucchini plants, finding for most nutrients the highest concentrations in plants grown under urea nutrition (Gerendás & Sattelmacher, 1997a). These higher nutrient levels in urea-supllied plants may be mostly due to suppressed biomass formation, leading to a concentration effect of the minerals accumulated in roots and shoots. From the present study it can be conjectured, that addition of 2-NPT to spring barley plants grown under nutrition of nitrate supplied as KNO_3 or under nutrition with urea supplied together with KNO_3 does not lead to alterations in nutrient concentrations, which may represent an advantage when using this UI.

9.3 Impact of 2-NPT on urea uptake and accumulation

The incubation of spring barley plants in a nutrient solution containing 0.01 µM 2-NPT or NBPT resulted in a significant impact on influx, uptake, translocation and accumulation of N, when the ^{15}N-labeled source was ^{15}N-urea (Figures 8-5 and 8-6). For instance, a substantial decrease of ^{15}N-urea accumulation in roots was observed after addition of 2-NPT or NBPT, when applied for 2, 4 or 6 d, compared to control plants (Figure 8-6B). Also a short-term effect with impaired ^{15}N accumulation in shoots was observed, when the UI was added for 2 d (Figure 8-6C). Therefore, as conjectured by Zanin et al. (2015), it is assumed that 2-NPT may also compete with urea during the uptake process, since 2-NPT like NBPT is a structural analogue to urea and may interfere with the high-affinity transport system DUR3, mediating urea uptake in plants (Kojima et al., 2007). After identifying DUR3 as a H^+/urea-coupling co-transporter mediating urea uptake when expressed in yeast or oocytes (Liu et al., 2003), ^{15}N-influx experiments and detection of ^{15}N-urea in roots confirmed that urea is taken up as an intact molecule by roots of Arabidopsis and of maize plants (Kojima et al., 2007; Mérigout et al., 2008; Zanin et al., 2015), which indicates that plants are indeed able to use urea as a sole N source (Kojima et al., 2007). Probably, the low ^{15}N uptake rates observed after supply of ^{15}N-urea + KNO_3 is due to the low uptake capacity for urea compared to that of nitrate or ammonium (Figure 8-5A; Gazzarrini et al., 1999; Yuan et al., 2007; Arkoun, et al., 2012). Moreover, Mérigout et al.

(2008) reported that the uptake of urea is stimulated when urea is supplied as the sole N source, whereas its supply together with ammonium nitrate results in lower uptake as a result of alterations in the transcriptional regulation of the DUR3 transporter.

In agreement with the present results, Zanin et al. (2015) reported no significant differences in ^{15}N accumulation in shoots and roots of 5-d-old maize plants when nitrate was supplemented alone or with NBPT (Figures 8-6B, C). Moreover, under nutrition with urea these authors reported a much lower (approx. 5-fold less) accumulation of N. Comparing urea and urea + NBPT, ^{15}N accumulation in shoots and roots was significantly impaired (approx. 2-fold less), when the UI was added. The resulting lower accumulation of urea compared to nitrate may be ascribed to a preference of plants to assimilate urea directly in roots, whereas nitrate is usually rapidly translocated to shoots (Mérigout et al., 2008; Figure 8-6D). Also in line with the present study, Zanin et al. (2015) did not find any effect after addition of NBPT on ^{15}N accumulation and on the root-to-shoot translocation, when hydroponically-grown maize plants were supplied with nitrate. Moreover, these authors reported that ^{15}N accumulation in both shoots and roots were dramatically reduced under urea nutrition, by 67 and 52%, respectively, after addition of NBPT. On the other hand, Cruchaga et al. (2011) reported for hydroponically-grown pea and spinach plants strongly decreased total ^{15}N concentrations in roots after co-application of NBPT with urea. This inhibitory effect was weaker in shoots, especially in pea leaves. The latter suggested that 2-NPT may decreases the uptake and accumulation of urea only when the supplied N form is urea, whereas such an impact does not occur under nitrate nutrition. Thus, not only long-term urea accumulation but also short-term root uptake of urea may become compromised when 2-NPT is co-applied with urea (Figure 8-6A).

Taken together, the supplementation of 2-NPT as a new UI together with urea can lead to an inhibition of the urea uptake capacity in spring barley roots, at least within a short time frame as examined here. On the other hand, 2-NPT leads to elevated urea concentrations however without altering the enzymatic activity of endogenous urease or macro- and micronutrient concentrations in roots and shoots. These results should be considered, when 2-NPT is considered to be applied together with urea, especially in other cereal grain crops.

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ANNEXES

Annex 1. Correlations between UI translocation rates in xylem exudates and urea translocation rates in xylem exudates. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

UI translocation rate (pmol 2-NPT h^{-1} $plant^{-1}$)		Urea translocation rate (pmol h^{-1} $plant^{-1}$)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.99				
2013/14	T1			0.55			
	T2				0.99		
2014/15	T1					0.99	
	T2						0.99
Gatersleben							
2012/13	T1	0.95					
	T2		0.99				
2013/14	T1			0.91			
	T2				0.79		
2014/15	T1					0.98	
	T2						0.87

Annex 2. Correlations between urea translocation rates in xylem exudates and content of urea-N in soil. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

Urea translocation rate (pmol h^{-1} $plant^{-1}$)		Urea-N content in soil (kg N ha^{-1})					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.97				
2013/14	T1			0.98			
	T2				0.96		
2014/15	T1					0.98	
	T2						0.44
Gatersleben							
2012/13	T1	0.67					
	T2		-0.37				
2013/14	T1			0.01			
	T2				0.99		
2014/15	T1					0.03	
	T2						0.99

Annex 3. Correlations between UI translocation rates in xylem exudates and ammonium translocation rates in xylem exudates. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

UI translocation rate (pmol 2-NPT h^{-1} $plant^{-1}$)		Ammonium translocation rate (µmol h^{-1} $plant^{-1}$)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.00				
2013/14	T1			-0.01			
	T2				0.08		
2014/15	T1					0.05	
	T2						0.08
Gatersleben							
2012/13	T1	-0.06					
	T2		0.32				
2013/14	T1			-0.58			
	T2				0.02		
2014/15	T1					0.00	
	T2						0.37

Annex 4. Correlations between ammonium (NH_4^+) translocation rates in xylem exudates and content of NH_4-N in soil. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

NH_4^+ translocation rate (µmol h^{-1} $plant^{-1}$)		NH_4-N content in soil (kg N ha^{-1})					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		-0.21				
2013/14	T1			0.31			
	T2				0.93		
2014/15	T1					0.69	
	T2						0.60
Gatersleben							
2012/13	T1	0.12					
	T2		0.78				
2013/14	T1			0.34			
	T2				0.92		
2014/15	T1					0.39	
	T2						0.01

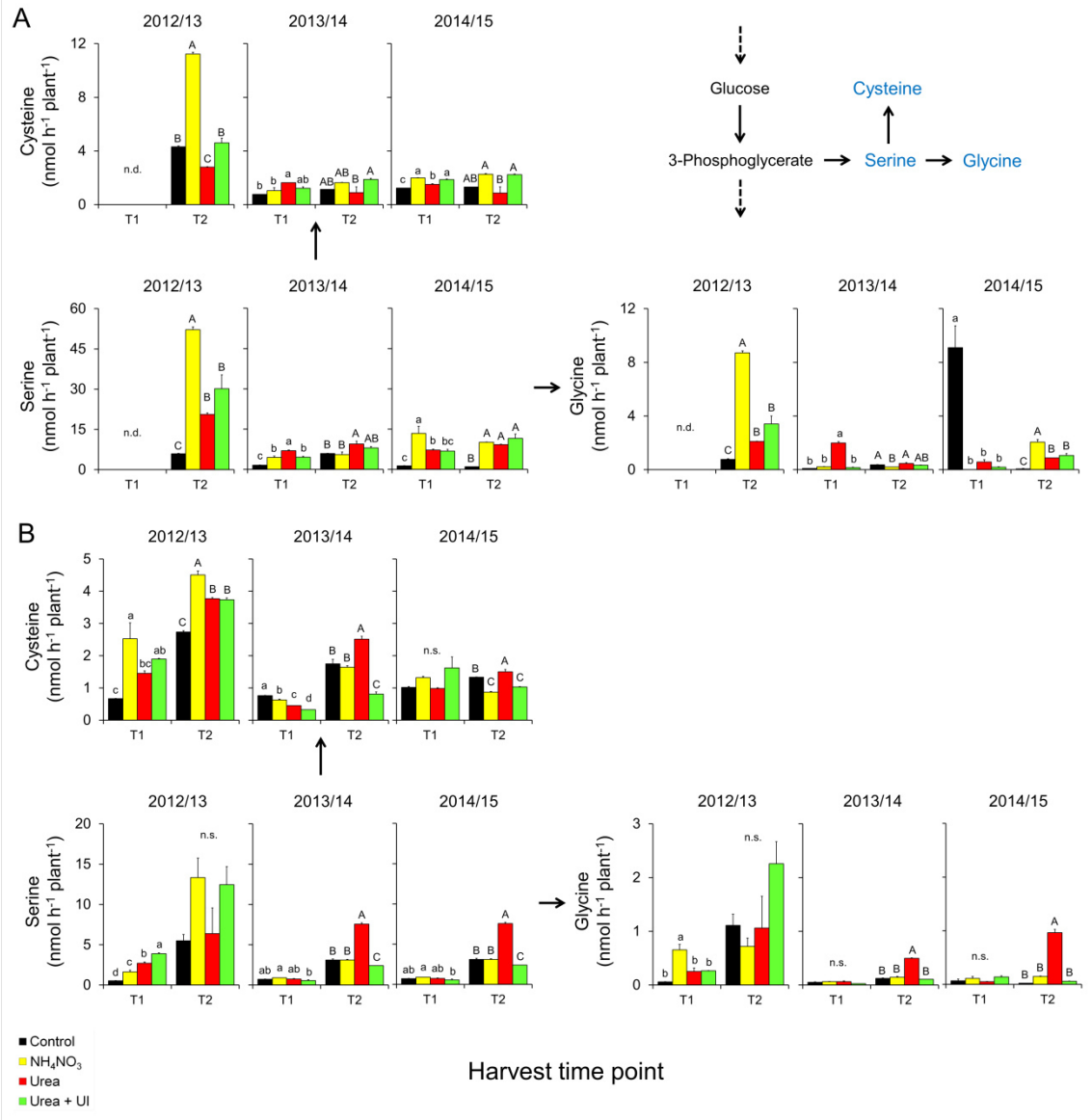
Annex 5. Correlations between UI translocation rates in xylem exudates and nitrate translocation rates in xylem exudates. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined

UI translocation rate (pmol 2-NPT h^{-1} $plant^{-1}$)		Nitrate translocation rate (µmol h^{-1} $plant^{-1}$)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.02				
2013/14	T1			0.00			
	T2				-0.25		
2014/15	T1					0.09	
	T2						0.00
Gatersleben							
2012/13	T1	0.14					
	T2		-0.01				
2013/14	T1			-0.26			
	T2				-0.14		
2014/15	T1					-0.01	
	T2						-0.19

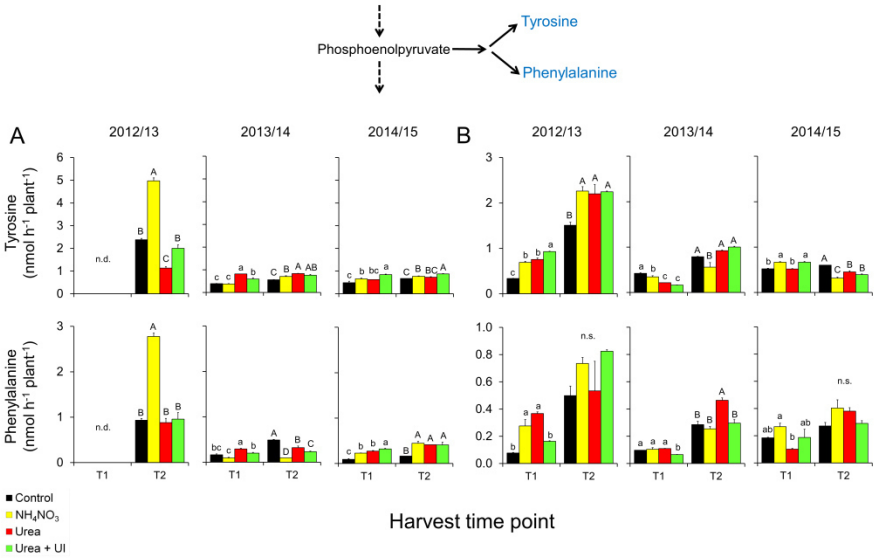
Annex 6. Correlations between nitrate (NO_3^-) translocation rates in xylem exudates and content of NO_3-N in soil. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

NO_3^- translocation rate (µmol h^{-1} $plant^{-1}$)		NO_3-N content in soil (kg N ha^{-1})					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.88				
2013/14	T1			0.47			
	T2				0.75		
2014/15	T1					0.44	
	T2						0.71
Gatersleben							
2012/13	T1	0.65					
	T2		0.65				
2013/14	T1			0.80			
	T2				0.78		
2014/15	T1					0.86	
	T2						0.10

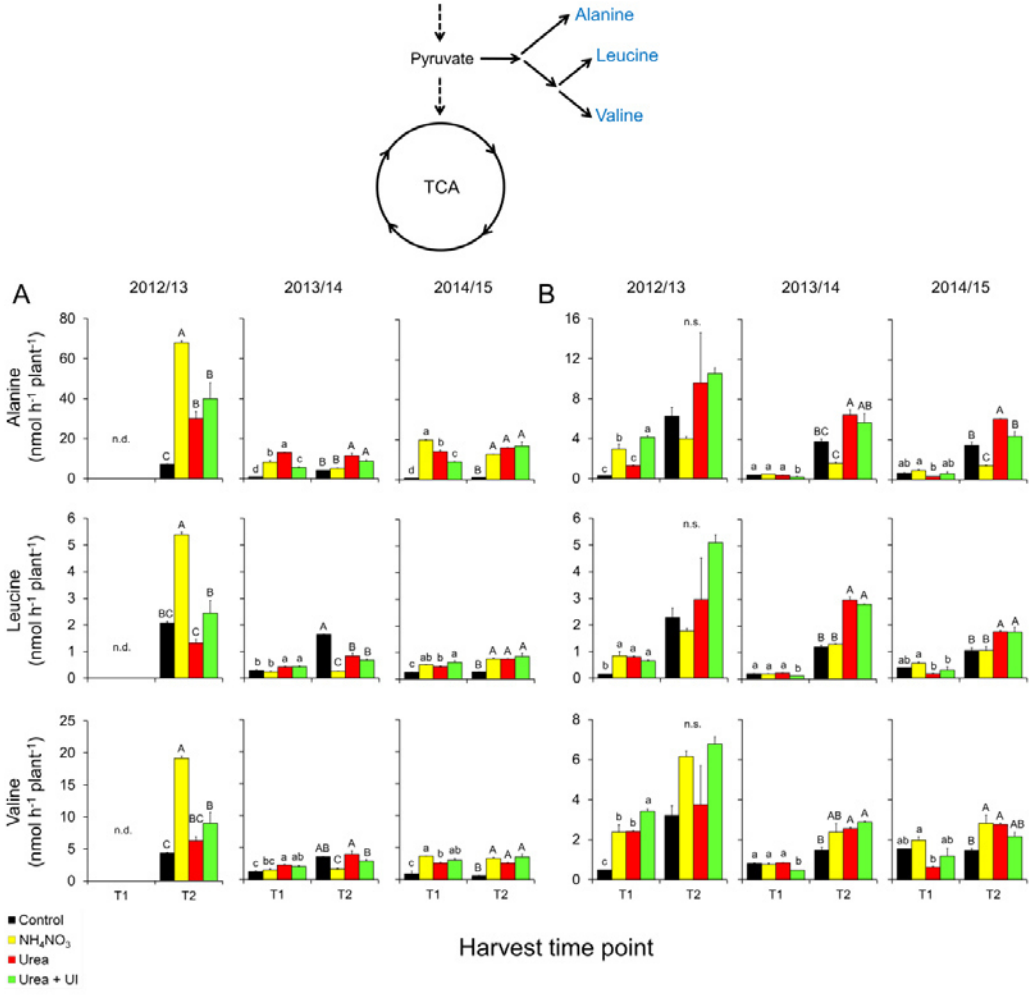
Annex 7. Influence of fertilization with different N forms (±UI) on translocation rates of cysteine, serine and glycine in the xylem sap. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined).



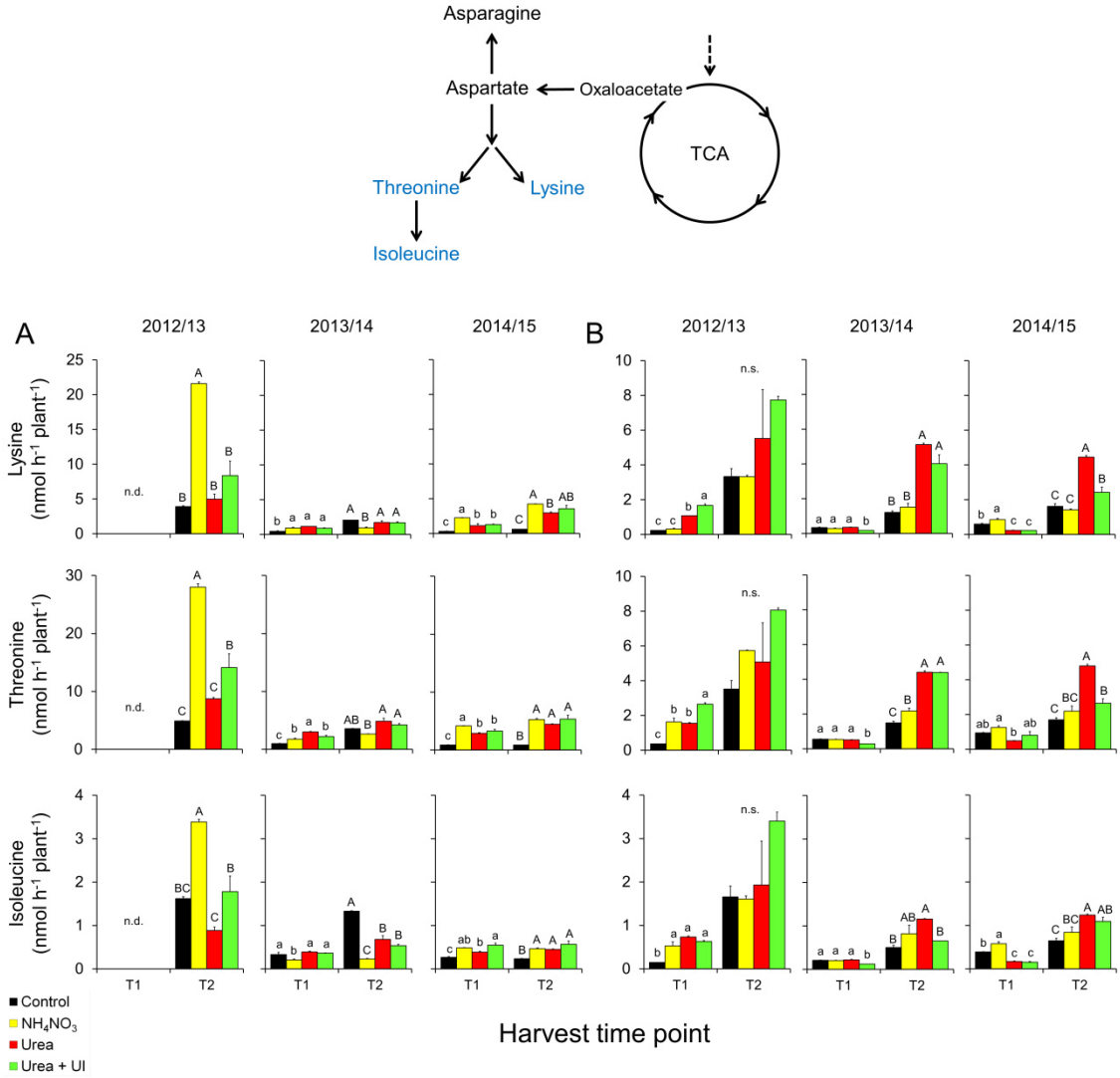
Annex 8. Influence of fertilization with different N forms (±UI) on translocation rates of tyrosine and phenylalanine in the xylem sap. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined).



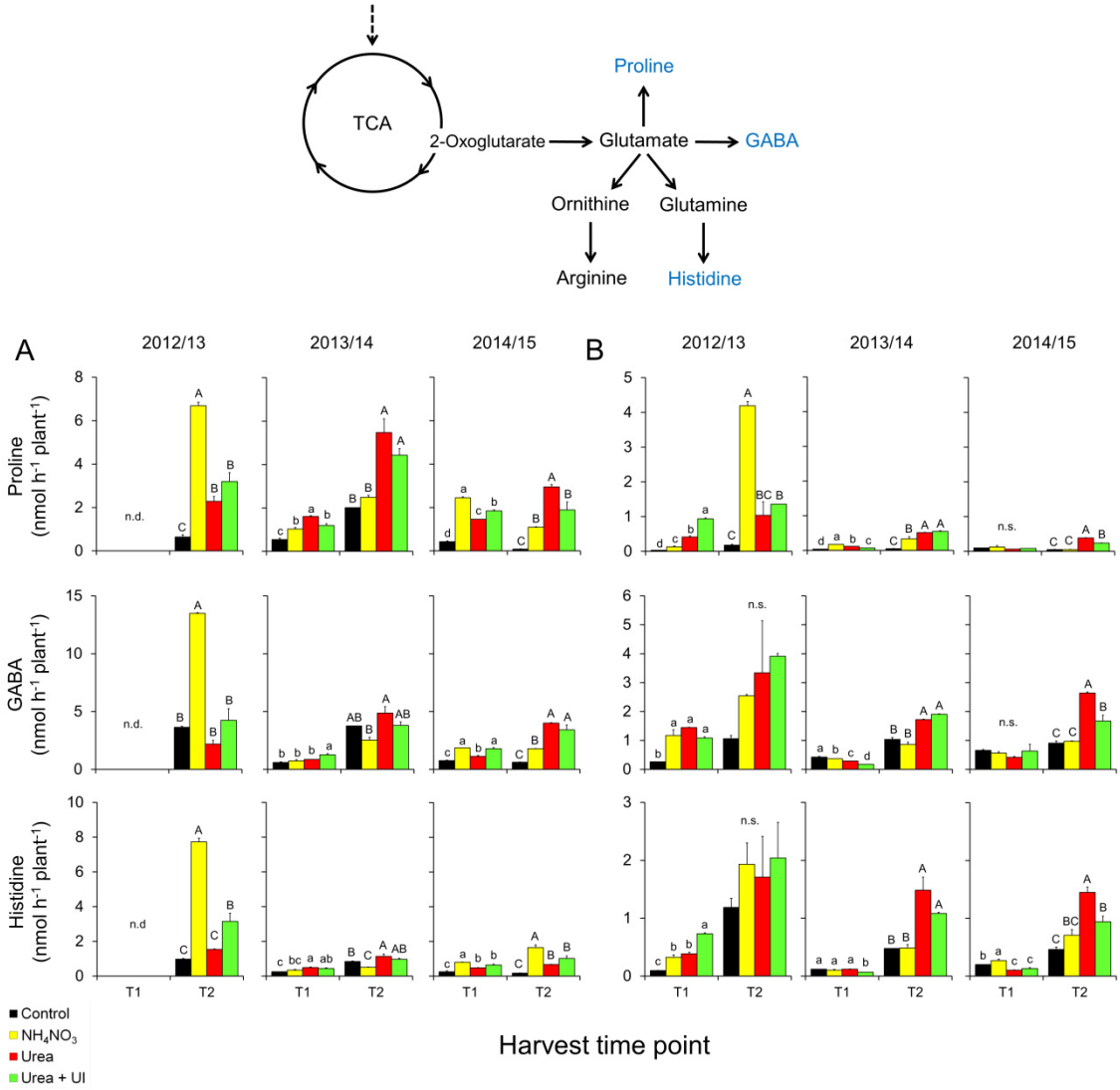
Annex 9. Influence of fertilization with different N forms (±UI) on translocation rates of alanine, leucine and valine in the xylem sap. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined). TCA indicates tricarboxylic acid cycle.



Annex 10. Influence of fertilization with different N forms (±UI) on translocation rates of lysine, threonine and isoleucine in the xylem sap. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined). TCA indicates tricarboxylic acid cycle. Ref. to Figure 4-8 for influence on aspartate and asparagine.



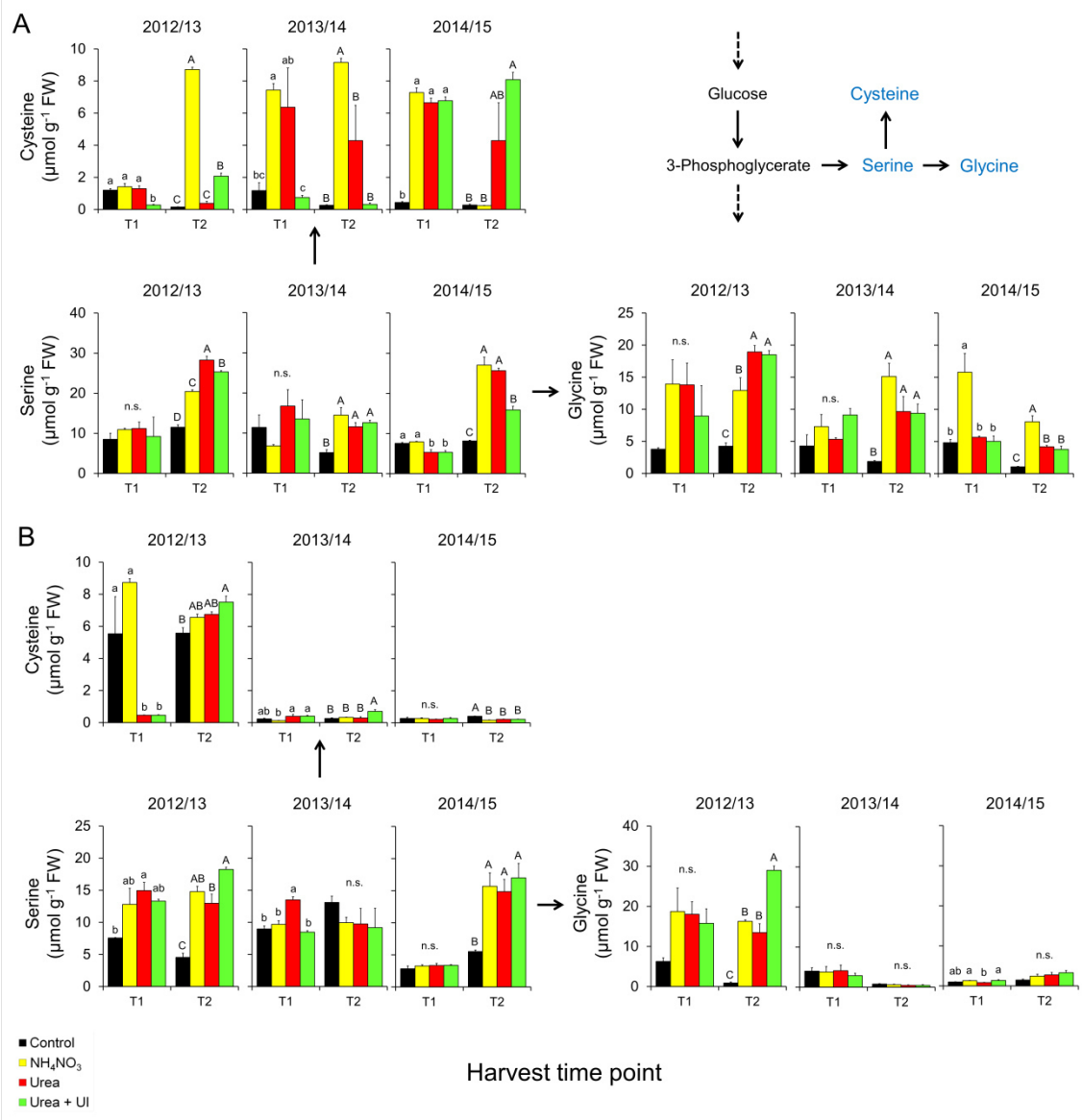
Annex 11. Influence of fertilization with different N forms (±UI) on translocation rates of proline, GABA and histidine in the xylem sap. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant; n.d., not determined). TCA indicates tricarboxylic acid cycle. Ref. to Figure 4-9 for influence on glutamate, ornithine, arginine and glutamine.



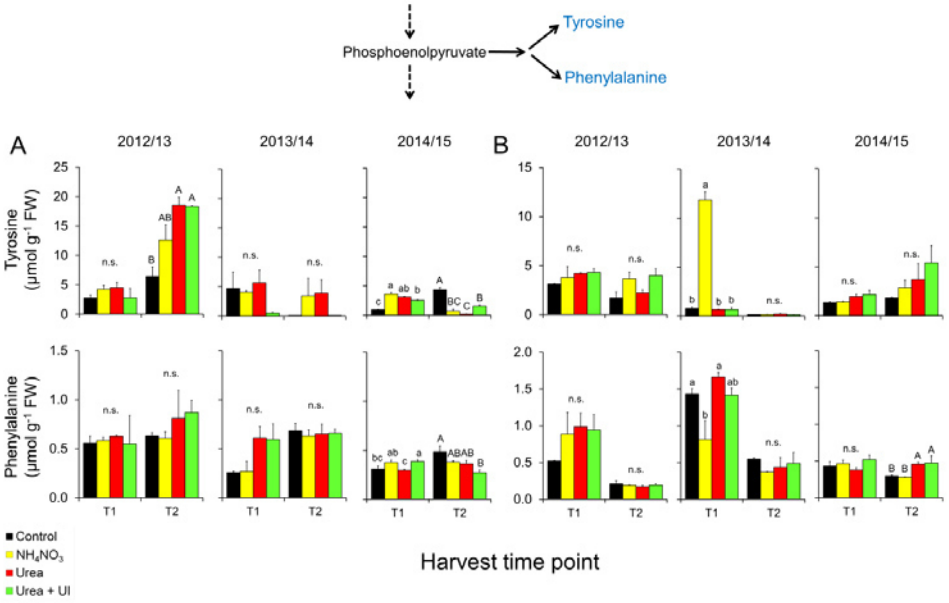
Annex 12. Correlations between translocation rates of AA in xylem exudates and concentrations of corresponding AA measured in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

	Cunnersdorf						Gatersleben					
	2012/13		2013/14		2014/15		2012/13		2013/14		2014/15	
	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Ala	n.d.	0.33	0.72	0.20	0.15	0.64	0.47	0.33	-0.08	-0.44	-0.36	-0.19
Arg	n.d.	-0.50	-0.10	-0.18	0.09	-0.14	0.75	0.00	-0.61	0.43	0.39	-0.08
Asn	n.d.	0.00	0.54	0.06	0.00	-0.15	0.22	0.00	-0.40	0.04	-0.64	-0.15
Asp	n.d.	0.00	0.58	0.08	0.49	0.36	0.06	0.90	-0.04	-0.75	0.86	0.54
Cys	n.d.	0.95	0.06	0.01	0.67	0.01	0.00	0.48	-0.60	-0.70	-0.19	0.17
GABA	n.d.	0.37	0.16	-0.44	0.00	0.80	-0.19	0.82	-0.42	0.80	-0.61	-0.04
Gln	n.d.	0.46	0.39	0.39	0.51	0.76	0.08	0.53	0.91	-0.11	0.05	0.14
Glu	n.d.	-0.11	0.43	0.19	0.15	0.12	-0.03	0.83	-0.37	-0.87	0.54	0.84
Gly	n.d.	0.04	-0.15	-0.26	-0.17	0.96	0.58	0.42	0.65	-0.20	0.94	0.01
His	n.d.	0.14	0.86	0.00	0.45	0.89	0.60	0.70	-0.52	-0.45	0.02	-0.19
Ile	n.d.	-0.34	0.61	0.71	0.04	-0.93	0.98	0.45	-0.25	-0.68	-0.13	0.23
Leu	n.d.	-0.22	0.87	-0.90	-0.27	-0.64	0.64	0.35	0.61	0.01	0.09	0.22
Lys	n.d.	0.46	-0.90	0.12	0.57	-0.75	0.45	0.45	-0.33	-0.60	-0.40	0.05
Orn	n.d.	0.00	0.14	0.37	0.01	0.62	0.44	0.04	-0.01	0.63	0.01	0.13
Phe	n.d.	-0.42	0.81	0.15	0.09	-0.60	0.63	-0.10	0.01	-0.05	0.44	0.13
Pro	n.d.	-0.12	0.32	0.49	0.94	0.17	0.47	0.20	0.81	0.01	-0.25	0.31
Ser	n.d.	0.17	0.26	0.00	0.01	0.66	0.68	0.60	0.25	-0.03	0.09	0.18
Thr	n.d.	0.04	0.34	-0.02	0.01	0.64	0.53	0.79	0.11	-0.73	0.24	0.20
Tyr	n.d.	-0.16	0.02	0.32	0.31	-0.25	0.80	0.53	0.11	0.01	0.00	-0.24
Val	n.d.	-0.19	0.62	-0.77	-0.07	-0.10	0.75	0.61	-0.09	-0.11	-0.08	0.90

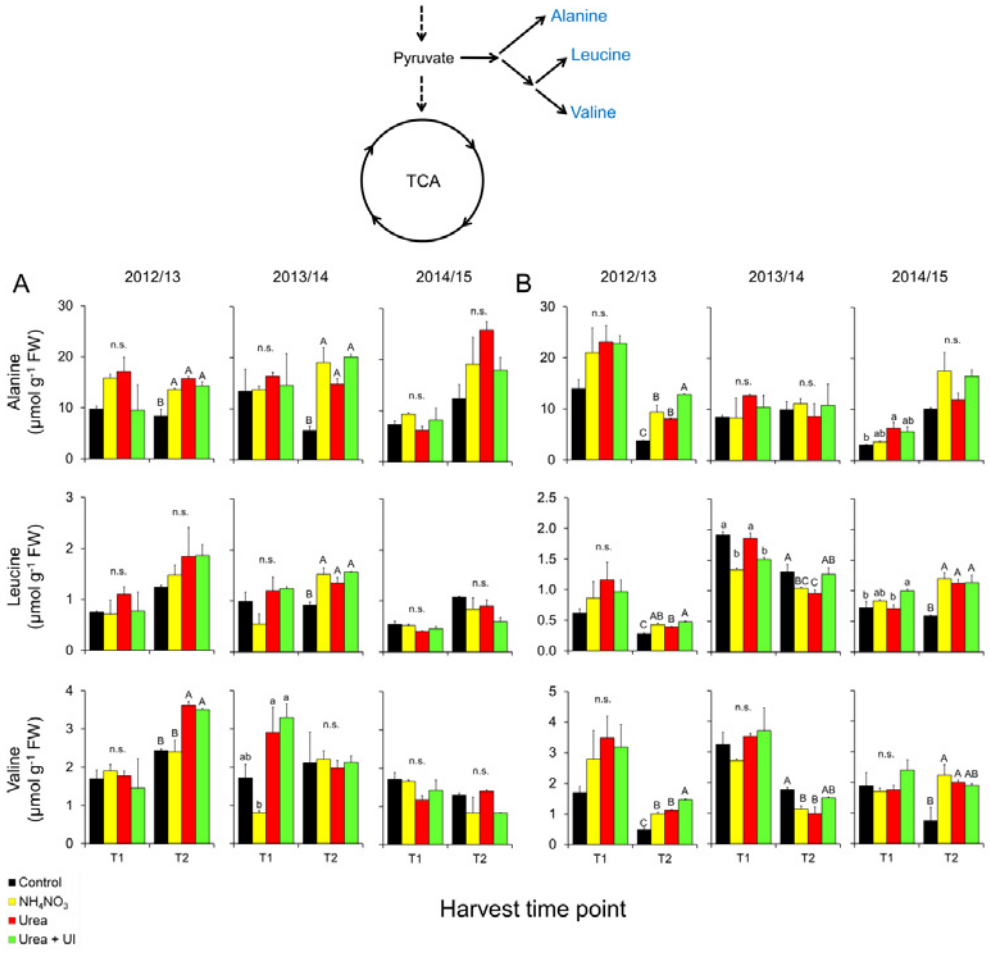
Annex 13. Influence of fertilization with different N forms (±UI) on concentrations of cysteine, serine and glycine in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).



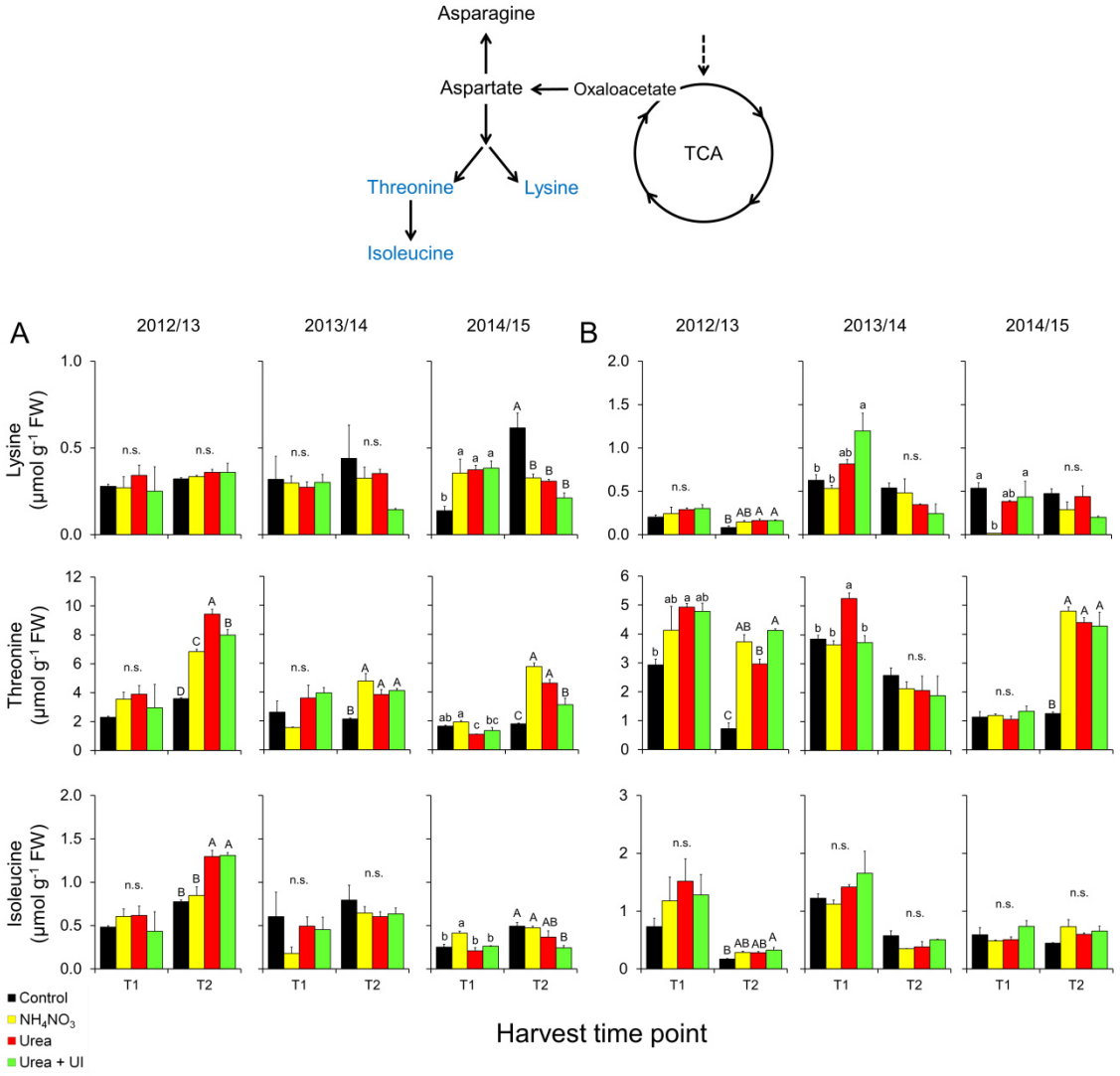
Annex 14. Influence of fertilization with different N forms (±UI) on concentrations of tyrosine and phenylalanine in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant).



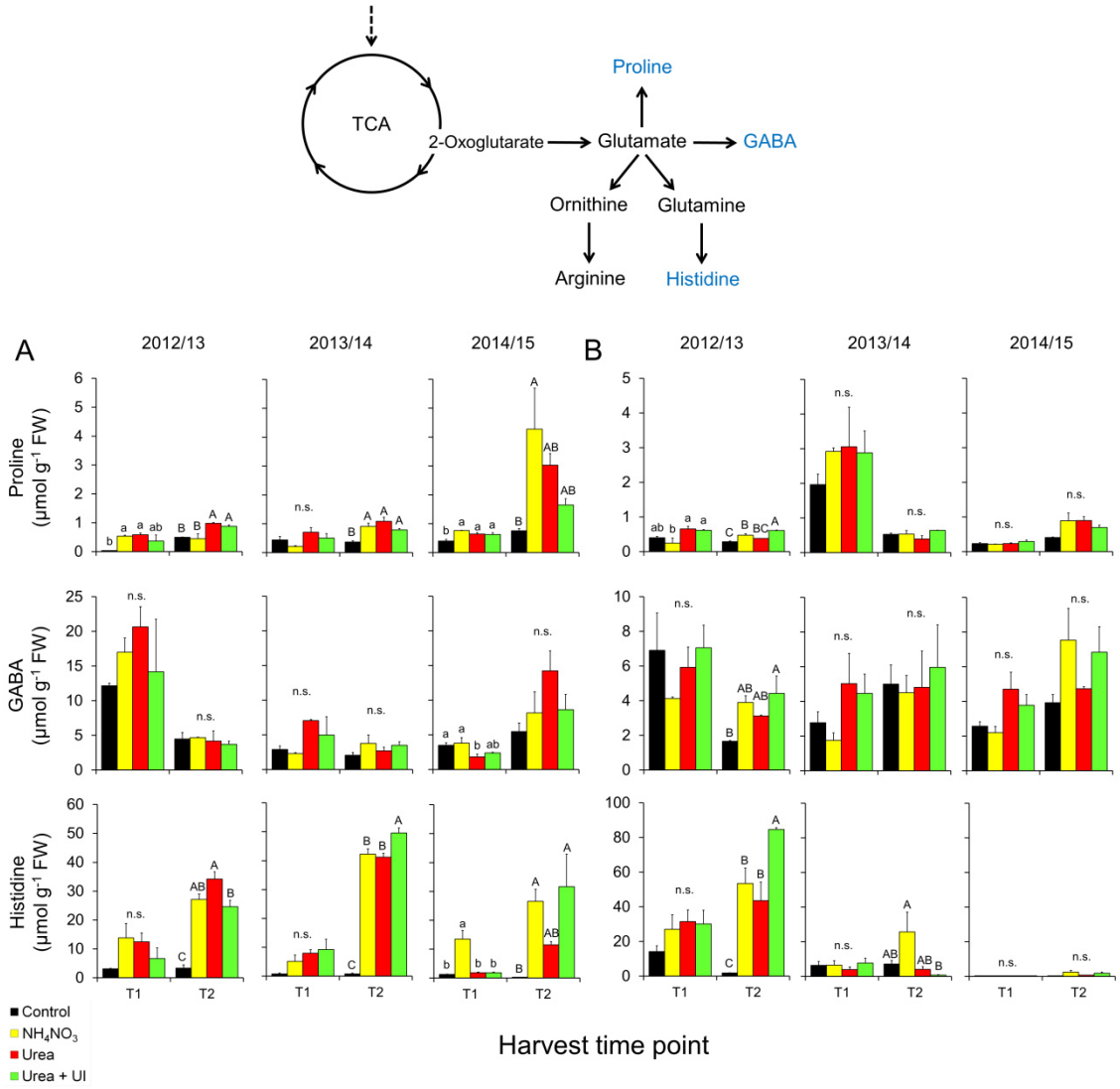
Annex 15. Influence of fertilization with different N forms (±UI) on concentrations of alanine, leucine and valine in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant). TCA indicates tricarboxylic acid cycle.



Annex 16. Influence of fertilization with different N forms (±UI) on concentrations of lysine, threonine and isoleucine in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant). TCA indicates tricarboxylic acid cycle. Ref. to Figure 4-11 for influence on aspartate and asparagine.



Annex 17. Influence of fertilization with different N forms (±UI) on concentrations of proline, GABA, and histidine in leaves. Results are shown for the first (T1) and second (T2) harvest time point over the three cropping years 2012/13, 2013/14 and 2014/15 in **(A)** Cunnersdorf and **(B)** Gatersleben. Bars indicate means ± SE, n=4. Different letters (lower- and uppercase for T1 and T2, respectively) indicate significant differences among means (P<0.05 by Tukey's test; n.s., not significant). TCA indicates tricarboxylic acid cycle. Ref. to Figure 4-12 for influence on glutamate, ornithine, arginine and glutamine.



Annex 18. Correlations between UI and urea concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites.

UI concentration (µg 2-NPT g^{-1} DW)		Urea concentration (µmol g^{-1} DW)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	0.95					
	T2		0.96				
2013/14	T1			0.99			
	T2				1.00		
2014/15	T1					0.93	
	T2						1.00
Gatersleben							
2012/13	T1	0.48					
	T2		0.59				
2013/14	T1			0.86			
	T2				0.91		
2014/15	T1					0.70	
	T2						0.97

Annex 19. Correlations between urea translocation rates in xylem exudates and urea concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

Urea translocation rate (pmol h^{-1} $plant^{-1}$)		Urea concentration (µmol g^{-1} DW)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.98				
2013/14	T1			0.54			
	T2				1.00		
2014/15	T1					0.98	
	T2						1.00
Gatersleben							
2012/13	T1	0.62					
	T2		0.64				
2013/14	T1			0.99			
	T2				0.97		
2014/15	T1					0.66	
	T2						0.96

Annex 20. Correlations between ammonium (NH_4^+) translocation rates in xylem exudates and NH_4^+ concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

NH_4^+ translocation rate ($\mu mol\ h^{-1}\ plant^{-1}$)		NH_4^+ concentration ($\mu mol\ g^{-1}$ DW)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.86				
2013/14	T1			0.53			
	T2				0.52		
2014/15	T1					-0.83	
	T2						0.38
Gatersleben							
2012/13	T1	0.02					
	T2		-0.94				
2013/14	T1			-0.86			
	T2				0.41		
2014/15	T1					-0.82	
	T2						-0.80

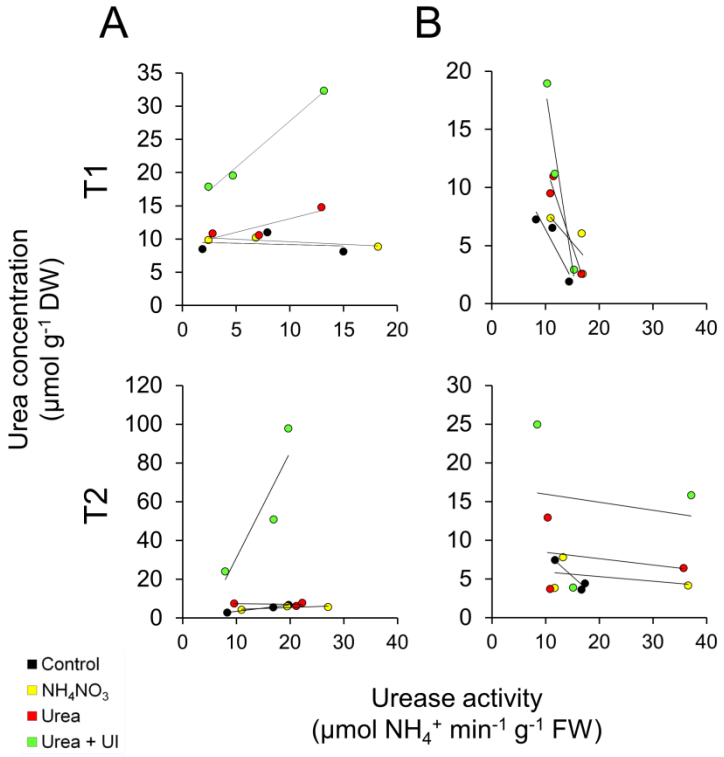
Annex 21. Correlations between nitrate (NO_3^-) translocation rates in xylem exudates and NO_3^- concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

NO_3^- translocation rate ($\mu mol\ h^{-1}\ plant^{-1}$)		NO_3^- concentration ($\mu mol\ g^{-1}$ DW)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.81				
2013/14	T1			0.64			
	T2				0.84		
2014/15	T1					0.32	
	T2						0.98
Gatersleben							
2012/13	T1	0.15					
	T2		0.38				
2013/14	T1			0.66			
	T2				0.92		
2014/15	T1					0.58	
	T2						-0.01

Annex 22. Correlations between total amino acids (AA) translocation rates in xylem exudates and total AA concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

Total AA translocation rate ($\mu mol\ h^{-1}\ plant^{-1}$)		Total AA concentration ($\mu mol\ g^{-1}$ FW)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.24				
2013/14	T1			0.53			
	T2				0.28		
2014/15	T1					0.57	
	T2						0.93
Gatersleben							
2012/13	T1	0.66					
	T2		0.59				
2013/14	T1			-0.05			
	T2				-0.52		
2014/15	T1					-0.07	
	T2						0.38

Annex 23. Correlations between urease activity and urea concentrations in leaves of winter wheat. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point at Cunnersdorf **(A)** and Gatersleben **(B)** experimental sites, where each point represents correlation during one trial year.



Annex 24. Correlations between translocation rates of auxins in xylem sap and content in soil as well as translocation rates of major plant-available N forms in xylem sap. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

		Cunnersdorf						Gatersleben					
		2012/13		2013/14		2014/15		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Urea-N content in soil	IAN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.96	n.d.	n.d.	n.d.	n.d.	n.d.
	IAM	n.d.	0.23	0.01	n.d.	0.19	n.d.	0.05	0.11	-0.44	-0.05	0.28	0.00
	IAA	n.d.	0.23	0.01	n.d.	n.d.	-0.26	0.33	0.15	0.01	0.02	0.47	-0.20
	OxIAA	n.d.	0.08	0.74	0.07	0.75	0.99	n.d.	0.00	-0.04	0.18	0.08	-0.20
Urea translocation rate in xylem sap	IAN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.84	n.d.	n.d.	n.d.	n.d.	n.d.
	IAM	n.d.	0.36	0.15	n.d.	0.16	n.d.	0.09	0.00	-0.63	-0.10	0.21	0.00
	IAA	n.d.	0.36	0.15	n.d.	n.d.	0.09	0.22	-0.45	-0.21	0.00	0.44	-0.27
	OxIAA	n.d.	0.12	0.63	0.05	0.72	0.37	0.00	n.d.	-0.23	0.22	0.80	-0.19
NH_4-N content in soil	IAN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.32	n.d.	n.d.	n.d.	n.d.	n.d.
	IAM	n.d.	-0.02	0.07	-0.05	0.58	0.01	0.68	0.83	0.23	-0.18	0.73	-0.10
	IAA	n.d.	-0.34	0.19	-0.14	0.51	-0.29	0.92	0.11	0.60	-0.04	0.35	-0.93
	OxIAA	n.d.	0.05	-0.03	0.60	0.18	0.28	n.d.	0.56	0.99	0.94	0.06	0.02
NH_4^+ translocation rate in xylem sap	IAN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-0.19	n.d.	n.d.	n.d.	n.d.	n.d.
	IAM	n.d.	0.69	0.89	-0.21	0.87	0.47	0.54	0.54	0.98	-0.43	0.35	0.13
	IAA	n.d.	0.61	0.95	-0.34	0.05	0.01	0.21	0.00	0.86	-0.20	0.24	0.02
	OxIAA	n.d.	0.54	0.26	0.83	0.61	0.43	n.d.	0.41	0.39	0.83	0.16	-0.89
NO_3-N content in soil	IAN	n.d.	n.d.	n.d.	-0.11	n.d.	n.d.	-0.15	n.d.	n.d.	n.d.	n.d.	n.d.
	IAM	n.d.	0.48	-0.11	n.d.	0.14	n.d.	0.65	0.78	0.33	-0.44	0.61	-0.27
	IAA	n.d.	0.48	-0.11	n.d.	n.d.	n.d.	0.27	0.05	0.67	-0.57	0.31	-0.90
	OxIAA	n.d.	0.64	-0.67	0.54	0.00	-0.25	n.d.	0.61	0.96	0.10	0.06	0.34
NO_3^- translocation rate in xylem sap	IAN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	IAM	n.d.	0.75	0.04	n.d.	0.83	n.d.	0.03	n.d.	0.77	-0.24	0.73	-0.57
	IAA	n.d.	0.75	0.04	n.d.	n.d.	-0.01	0.54	0.56	0.74	-0.20	0.59	0.00
	OxIAA	n.d.	0.90	-0.16	0.57	0.54	0.00	n.d.	0.95	0.70	0.46	0.17	0.91

Annex 25. Correlations between concentrations of auxins in leaves and major plant-available N forms concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

		Cunnersdorf						Gatersleben					
		2012/13		2013/14		2014/15		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Urea concentration	IAM	-0.09	-0.52	-0.27	-0.10	-0.56	-0.13	0.27	-0.35	0.00	0.68	0.31	-0.54
	IAA	0.07	-0.13	n.d.	0.00	-0.27	0.02	0.18	0.01	0.67	0.20	0.31	-0.87
	IAAla	-0.35	-0.63	0.01	0.00	-0.47	-0.23	0.69	-0.02	0.00	0.68	0.75	-0.15
	OxIAA	-0.49	-0.07	-0.92	0.21	-0.30	-0.02	0.00	-0.94	0.23	0.82	0.01	0.00
NH_4^+ concentration	IAM	0.73	-0.25	0.00	-0.84	0.89	-0.55	-0.77	0.02	0.00	0.08	-0.09	0.01
	IAA	0.70	-0.61	n.d.	-0.29	0.75	-0.22	-0.63	0.30	0.54	-0.04	-0.02	0.23
	IAAla	0.63	-0.29	-0.13	-0.76	0.65	-0.51	-0.67	0.32	-0.03	0.08	-0.08	0.01
	OxIAA	-0.02	-0.51	0.51	-0.17	0.95	-0.35	0.25	0.55	0.11	0.28	0.13	-0.13
NO_3^- concentration	IAM	-0.29	-0.11	-0.01	-0.54	0.00	-0.61	-0.89	0.09	0.20	-0.44	0.01	-0.18
	IAA	-0.28	-0.47	n.d.	-0.30	-0.03	-0.80	-0.99	-0.54	-0.20	-0.94	-0.01	-0.11
	IAAla	-0.38	-0.12	-0.31	-0.81	0.01	-0.42	-0.11	-0.31	-0.18	-0.45	0.03	-0.65
	OxIAA	0.25	-0.42	0.31	-0.58	-0.09	-0.66	0.87	-0.13	0.00	-0.11	-0.03	-0.49

Annex 26. Correlations between translocation rates of cytokinins in xylem sap and content in soil as well as translocation rates of major plant-available N forms in xylem sap. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

		Cunnersdorf						Gatersleben					
		2012/13		2013/14		2014/15		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Urea-N content in soil	tZ	n.d.	0.16	0.00	0.13	-0.67	0.61	-0.26	0.73	-0.26	0.00	0.17	0.27
	tZR	n.d.	0.27	0.03	0.11	-0.01	0.23	0.61	0.61	-0.10	0.03	0.75	0.02
	iPR	n.d.	0.77	0.16	0.16	0.47	0.66	0.54	0.25	0.00	-0.25	0.00	0.00
	cZ	n.d.	0.93	0.43	-0.50	0.48	0.98	-0.35	0.11	0.39	-0.83	0.00	-0.12
	cZR	n.d.	0.64	0.18	0.06	0.46	0.95	0.63	0.17	0.08	-0.40	0.01	-0.16
Urea translocation rate in xylem sap	tZ	n.d.	0.24	0.09	0.06	-0.55	0.66	-0.07	-0.09	-0.18	0.00	-0.13	0.37
	tZR	n.d.	0.35	0.16	0.04	-0.02	0.09	0.98	-0.14	-0.83	0.03	-0.01	0.07
	iPR	n.d.	0.73	0.39	0.04	0.35	0.27	0.90	-0.13	-0.99	-0.34	0.02	0.00
	cZ	n.d.	0.98	0.72	-0.62	0.36	0.60	-0.15	-0.17	-0.19	-0.86	-0.67	-0.07
	cZR	n.d.	0.71	0.45	0.00	0.34	0.39	0.74	-0.24	-0.87	-0.43	0.24	-0.09
NH_4-N content in soil	tZ	n.d.	0.01	0.83	0.94	0.04	0.09	0.08	0.70	0.87	0.19	-0.07	0.49
	tZR	n.d.	0.04	0.68	0.98	0.82	0.51	n.d.	n.d.	-0.29	0.82	0.52	0.20
	iPR	n.d.	0.34	0.53	0.85	-0.10	0.49	0.02	0.58	0.24	-0.09	-0.52	0.04
	cZ	n.d.	0.04	0.09	-0.16	-0.11	0.04	0.05	0.36	-0.21	-0.36	-0.06	0.00
	cZR	n.d.	0.08	0.14	0.86	0.00	0.26	0.00	0.35	0.14	0.07	-0.73	0.13
NH_4^+ translocation rate in xylem sap	tZ	n.d.	0.58	0.68	0.99	-0.15	0.67	1.00	0.26	0.44	0.03	-0.79	0.31
	tZR	n.d.	0.45	0.84	0.97	0.85	0.96	n.d.	n.d.	-0.72	0.66	0.39	0.02
	iPR	n.d.	0.01	0.93	0.62	0.07	0.95	-0.24	0.19	0.80	-0.30	-0.95	0.16
	cZ	n.d.	0.01	0.86	-0.35	0.06	0.34	0.98	0.08	0.01	-0.35	-0.34	0.01
	cZR	n.d.	0.17	0.95	0.64	0.26	0.62	-0.51	0.06	0.52	0.00	-0.62	-0.11

► **Annex 26.** (This Annex will be continued on next page).

◄ **Annex 26.** (This Annex begins on previous page).

NO_3-N content in soil	tZ	n.d.	0.58	0.10	0.22	0.23	0.00	0.97	0.45	0.98	0.05	-0.11	0.14
	tZR	n.d.	0.45	0.02	0.17	0.59	0.26	-0.12	0.45	0.59	0.01	0.99	0.00
	iPR	n.d.	0.01	-0.01	-0.01	-0.38	0.01	-0.24	0.44	0.23	-0.14	0.55	0.00
	cZ	n.d.	0.00	-0.29	-0.12	-0.37	-0.24	0.95	0.28	-0.29	0.05	-0.07	0.01
	cZR	n.d.	0.13	-0.23	0.00	-0.13	-0.09	-0.50	0.24	0.10	0.04	-0.08	0.19
NO_3^- translocation rate in xylem sap	tZ	n.d.	0.88	0.65	0.44	-0.11	-0.56	0.63	0.44	0.85	0.05	0.02	-0.49
	tZR	n.d.	0.78	0.49	0.44	0.77	0.79	0.07	0.64	0.85	0.13	0.94	-0.14
	iPR	n.d.	0.17	0.38	0.10	0.01	0.36	0.01	0.89	0.48	-0.05	-0.34	-0.38
	cZ	n.d.	0.09	0.05	0.01	0.02	0.00	0.50	0.85	-0.12	-0.01	-0.11	-0.13
	cZR	n.d.	0.43	0.04	0.21	0.16	0.06	-0.02	0.80	0.22	0.18	-0.44	0.01

Annex 27. Correlations between concentrations of cytokinins in leaves and major plant-available N forms concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

		Cunnersdorf						Gatersleben					
		2012/13		2013/14		2014/15		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Urea concentration	cZ	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.31	n.d.	n.d.	n.d.	n.d.	n.d.
	cZR	0.19	0.00	-0.04	-0.25	-0.35	0.00	0.15	-0.79	0.43	0.93	-0.07	0.01
	iPR	0.57	0.15	0.82	0.09	-0.10	0.06	0.08	0.54	0.04	0.68	-0.42	0.00
	tZOG	0.00	0.37	0.06	0.14	-0.22	0.82	0.68	0.41	1.00	0.61	0.01	0.12
	tZR	0.18	0.04	-0.17	0.03	-0.02	0.01	0.02	0.20	0.32	-0.05	-0.01	0.10
	tZROG	-0.87	0.72	-0.16	0.03	0.00	0.08	0.15	0.50	-0.79	0.04	0.16	-0.03
	tZ9G	-0.07	0.21	0.00	-0.03	-0.33	0.27	0.07	0.61	1.00	0.11	-0.62	0.01
	iP	0.17	-0.07	0.10	-0.08	-0.18	0.39	0.00	0.69	0.42	0.84	0.02	0.32
	iP9G	0.97	-0.40	-0.26	-0.10	-0.89	0.01	-0.02	0.17	0.22	0.85	-0.15	-0.04

► **Annex 27.** (This Annex will be continued on next page).

◄ **Annex 27.** (This Annex begins on previous page).

NH_4^+ concentration	**cZ**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-0.73	n.d.	n.d.	n.d.	n.d.	n.d.
	cZR	-0.28	-0.99	0.56	-0.37	0.41	0.01	-0.54	0.46	0.89	0.74	-0.05	0.08
	iPR	-0.40	0.28	-0.30	0.87	-0.19	0.76	-0.46	-0.99	-0.06	0.19	0.21	0.14
	tZOG	-0.17	0.53	0.25	0.39	-0.05	0.97	-0.43	-0.92	0.70	0.18	0.55	0.00
	tZR	-0.76	0.56	0.00	0.78	-0.40	0.55	-0.01	-0.65	0.13	-0.05	-0.16	-0.01
	tZROG	-0.13	0.18	-0.08	0.34	-0.52	0.78	-0.41	-0.85	-0.77	0.01	0.94	0.10
	tZ9G	-0.56	0.71	0.26	0.40	-0.05	0.93	0.03	-0.98	0.70	-0.11	-0.36	0.05
	iP	0.55	0.23	0.10	0.47	0.02	0.56	-0.11	-0.49	0.71	0.76	0.03	-0.18
	iP9G	-0.01	-0.24	0.24	0.50	0.54	-0.11	0.08	-0.01	0.46	0.33	-0.82	-0.04
NO_3^- concentration	**cZ**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	-0.59	n.d.	n.d.	n.d.	n.d.	n.d.
	cZR	0.62	-0.91	-0.51	-0.07	0.00	0.00	-0.55	-0.08	-0.46	0.00	0.00	0.50
	iPR	0.20	0.20	0.01	0.59	0.66	0.86	-0.61	0.84	0.02	-0.40	-0.23	0.42
	tZOG	-0.01	0.32	-0.38	0.14	0.84	0.61	0.00	0.92	-0.76	-0.40	-0.35	0.60
	tZR	0.74	0.49	0.99	0.65	0.77	0.98	0.10	0.86	-0.01	0.10	0.04	0.73
	tZROG	0.13	0.05	0.79	0.24	0.56	0.86	-0.76	0.56	0.97	0.05	-0.98	0.61
	tZ9G	0.15	0.49	0.01	0.63	0.99	0.60	0.61	0.72	-0.72	-0.83	0.53	0.57
	iP	-0.18	0.30	0.00	0.85	0.08	0.09	-0.68	0.29	-0.13	-0.01	-0.13	0.67
	iP9G	0.01	-0.10	0.60	0.95	0.23	-0.48	0.32	-0.08	-0.02	-0.26	0.95	0.95

Annex 28. Correlations between translocation rates of abscisic acid (ABA), phaseic acid (PA) and salicylic acid (SA) in xylem sap and content in soil as well as translocation rates in xylem sap of major plant-available N forms. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

		Cunnersdorf						Gatersleben					
		2012/13		2013/14		2014/15		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Urea-N content in soil	ABA	n.d.	0.18	n.d.	0.01	-0.19	-0.32	0.21	0.72	-0.05	0.03	0.00	-0.25
	PA	n.d.	0.19	0.05	-0.23	0.02	0.05	0.91	0.09	-0.02	-0.12	-0.10	-0.08
	SA	n.d.	0.03	0.28	-0.74	0.72	0.03	-0.05	0.15	0.40	-0.32	0.78	-0.19
Urea translocation rate in xylem sap	ABA	n.d.	0.30	n.d.	-0.08	-0.14	-0.05	-0.02	-0.78	0.91	0.01	0.84	-0.26
	PA	n.d.	0.30	0.22	-0.38	0.01	0.72	0.39	-0.03	-0.95	-0.17	0.78	-0.07
	SA	n.d.	0.10	0.52	-0.88	0.73	0.50	-0.06	-0.06	-0.51	-0.40	0.11	-0.15
NH_4-N content in soil	ABA	n.d.	-0.05	n.d.	0.72	0.88	-0.70	0.22	0.07	-0.15	0.24	-0.31	-0.65
	PA	n.d.	-0.01	0.00	0.36	0.63	-0.19	0.55	0.46	0.40	0.03	-0.12	-0.30
	SA	n.d.	-0.13	0.00	0.01	0.12	-0.03	0.40	0.50	0.01	-0.03	0.01	-0.20
NH_4^+ translocation rate in xylem sap	ABA	n.d.	0.68	n.d.	0.56	0.34	-0.99	-0.10	-0.04	-0.58	0.05	-0.08	-0.06
	PA	n.d.	0.65	0.68	0.28	0.94	0.05	-0.15	0.21	0.90	-0.01	0.04	0.21
	SA	n.d.	0.89	0.64	0.00	0.51	0.20	0.82	0.21	0.14	-0.18	-0.36	0.07
NO_3-N content in soil	ABA	n.d.	0.53	n.d.	0.10	0.99	-0.14	-0.05	0.00	-0.11	-0.08	-0.34	-0.80
	PA	n.d.	0.56	-0.47	0.25	0.34	0.05	0.08	0.43	0.41	-0.04	-0.12	-0.58
	SA	n.d.	0.74	-0.64	0.17	0.01	0.25	0.90	0.44	0.00	-0.06	0.00	-0.35
NO_3^- translocation rate in xylem sap	ABA	n.d.	0.77	n.d.	0.53	0.42	-0.66	-0.13	0.25	-0.26	0.03	-0.13	-0.10
	PA	n.d.	0.83	-0.04	0.73	0.82	0.05	0.00	0.95	0.67	0.02	-0.07	-0.35
	SA	n.d.	0.85	-0.03	0.47	0.58	0.30	0.54	0.95	0.00	-0.01	0.15	-0.20

Annex 29. Correlations between phaseic acid (PA) and chlorophyll concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

Phaseic acid (pmol g^{-1} DW)		Chlorophyll (mg g^{-1} FW)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	0.06					
	T2		-0.96				
2013/14	T1			0.32			
	T2				-0.91		
2014/15	T1					-0.96	
	T2						-0.75
Gatersleben							
2012/13	T1	-0.09					
	T2		0.00				
2013/14	T1			0.41			
	T2				0.65		
2014/15	T1					0.00	
	T2						-0.94

Annex 30. Correlations between concentrations of abscisic acid (ABA), phaseic acid (PA) and salicylic acid (SA) in leaves and major plant-available N forms concentrations in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

		Cunnersdorf						Gatersleben					
		2012/13		2013/14		2014/15		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2	T1	T2	T1	T2	T1	T2
Urea concentration	ABA	-0.37	0.00	0.10	-0.29	-0.58	0.02	0.00	0.36	0.09	0.00	0.51	-0.03
	PA	0.81	-0.17	0.29	-0.12	-0.12	-0.07	-0.03	-0.22	0.51	-0.07	0.00	-0.02
	SA	0.39	-0.28	0.40	0.00	0.09	0.07	0.01	0.00	0.57	0.89	0.74	-0.05
NH_4^+ concentration	ABA	-0.62	0.64	0.03	-0.04	0.35	0.37	-0.27	-0.93	0.50	-0.28	0.70	0.00
	PA	0.00	-0.38	-0.84	-0.46	0.85	-0.45	-0.05	0.84	0.94	-0.50	-0.62	0.00
	SA	0.00	-0.64	-0.24	0.39	-0.38	0.51	-0.34	-0.37	0.81	0.27	-0.08	0.87
NO_3^- concentration	ABA	0.60	0.62	-0.99	0.03	0.08	0.30	-0.91	0.95	-0.29	-0.32	-0.73	0.86
	PA	0.04	-0.28	-0.02	-0.21	-0.31	-0.62	-0.62	-0.96	-0.41	-0.74	0.41	-0.73
	SA	0.12	-0.43	0.17	0.48	0.13	0.37	-0.94	0.81	-0.82	-0.26	0.03	-0.08

Annex 31. Correlations between translocation rates of salicylic acid (SA) and translocation rates of phenylalanine (Phe) in xylem sap. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

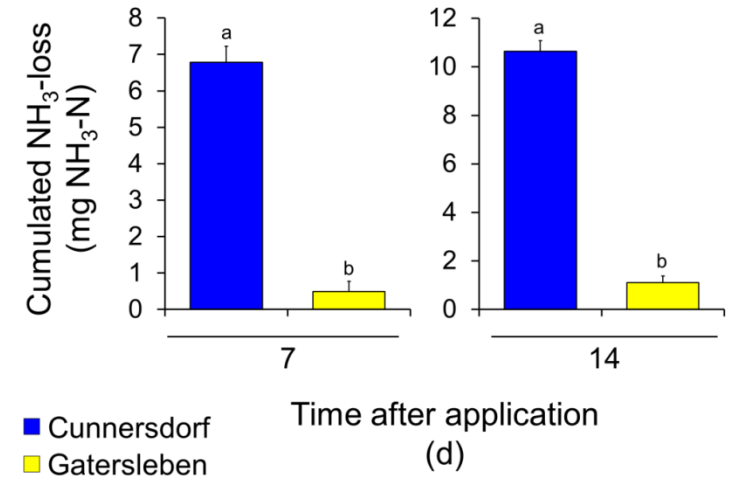
Phe translocation rate (nmol h^{-1} $plant^{-1}$)		SA translocation rate (fmol h^{-1} $plant^{-1}$)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	n.d.					
	T2		0.68				
2013/14	T1			0.99			
	T2				0.00		
2014/15	T1					0.73	
	T2						0.38
Gatersleben							
2012/13	T1	0.48					
	T2		0.23				
2013/14	T1			0.94			
	T2				0.30		
2014/15	T1					-0.05	
	T2						0.00

Annex 32. Correlations between concentrations of salicylic acid (SA) and concentrations of phenylalanine (Phe) in leaves. Correlations were performed by Pearson product moment and are shown at first (T1) and second (T2) harvest time point over the three crop years 2012/13, 2013/14 and 2014/15 at Cunnersdorf and Gatersleben experimental sites. n.d., not determined.

Phe concentration (µmol g^{-1} FW)		SA concentration (nmol g^{-1} DW)					
		2012/13		2013/14		2014/15	
		T1	T2	T1	T2	T1	T2
Cunnersdorf							
2012/13	T1	0.00					
	T2		-0.07				
2013/14	T1			0.04			
	T2				-0.79		
2014/15	T1					0.95	
	T2						-0.03
Gatersleben							
2012/13	T1	0.08					
	T2		-0.82				
2013/14	T1			0.82			
	T2				0.22		
2014/15	T1					0.48	
	T2						-0.09

Annex 33. The effect of high urea accumulation in winter wheat leaves in Cunnersdorf after fertilization with urea + UI. Picture taken 16 d (24/Mar./2013) after the second fertilization time point (T2) with urea + UI during the first trial year 2012/13. Leaf-tip necrotic aereas are present in most leaves.

Annex 34. Ammonia emissions from Cunnersdorf and Gatersleben soils after 7 and 14 d of urea fertilizer application. Soil samples (0-30 cm depth) from both locations were collected on the same day after the same preceding crop (winter wheat) in December 2015, homogenized and kept on ice until measurements. This test was conducted at SKW Stickstoff Piesteritz GmbH, where potential NH_3 release from urea was determined under permanent loss conditions. For this test, 50 mg urea were applied to 30 g dried soil, where available water capacity was adjusted to 50%. Released ammonia from urea was collected in sulfuric acid and quantitatively measured by flow analysis (colorimetry). Results are shown as cumulated losses of ammonia (NH_3) from soils. Bars indicate means ± SD, n=3. Different letters indicate significant differences among means ($P<0.05$ by Tukey's test). Source: SKW Stickstoffwerke Piesteritz GmbH (2017, unpublished results).



Annex 35. Calculations for ^{15}N influx in roots, total ^{15}N uptake, ^{15}N accumulation in roots or shoots and for the root-to-shoot translocation rate. Spring barley plants were precultured for 12 d in full-nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added during preculture at a concentration of 0.005% of total N to the nutrient solution (0.2 µM) for either 2, 4 or 6 days. Afterwards, ^{15}N-influx was measured after 10 min of labeling, whereas another set of plants was measured after 120 min of labeling with 200 µM N supplied as ^{15}N-KNO_3, urea + ^{15}N-KNO_3 or ^{15}N-urea + KNO_3 in addition of 0.01 µM 2-NPT or NBPT. N(%)correc. describes the correction for the natural abundance of ^{15}N.

A

$$^{15}\text{N influx } (\mu\text{moles } ^{15}\text{N g}^{-1}\text{ root DW min}^{-1}) = \left[\frac{(\text{Atom(\%)N root} - \text{Background}) \times \text{N(\%)correc.} \times 0.01 \times 1000000}{15 \times 0.98}\right] / 10$$

B

$$\text{Total } ^{15}\text{N uptake } (\mu\text{moles } ^{15}\text{N g}^{-1}\text{ root DW 2h}^{-1}) = \frac{^{15}\text{N in roots } (\mu\text{mol/2h}) + ^{15}\text{N in shoots } (\mu\text{mol/2h})}{\text{root DW (g)}}$$

C

$$\text{Root } ^{15}\text{N accumulation } (\mu\text{moles } ^{15}\text{N g}^{-1}\text{ root DW 2h}^{-1}) = \left[\frac{(\text{Atom(\%)N root} - \text{Background}) \times \text{N(\%)correc.} \times 0.01 \times 1000000}{15 \times 0.98}\right]$$

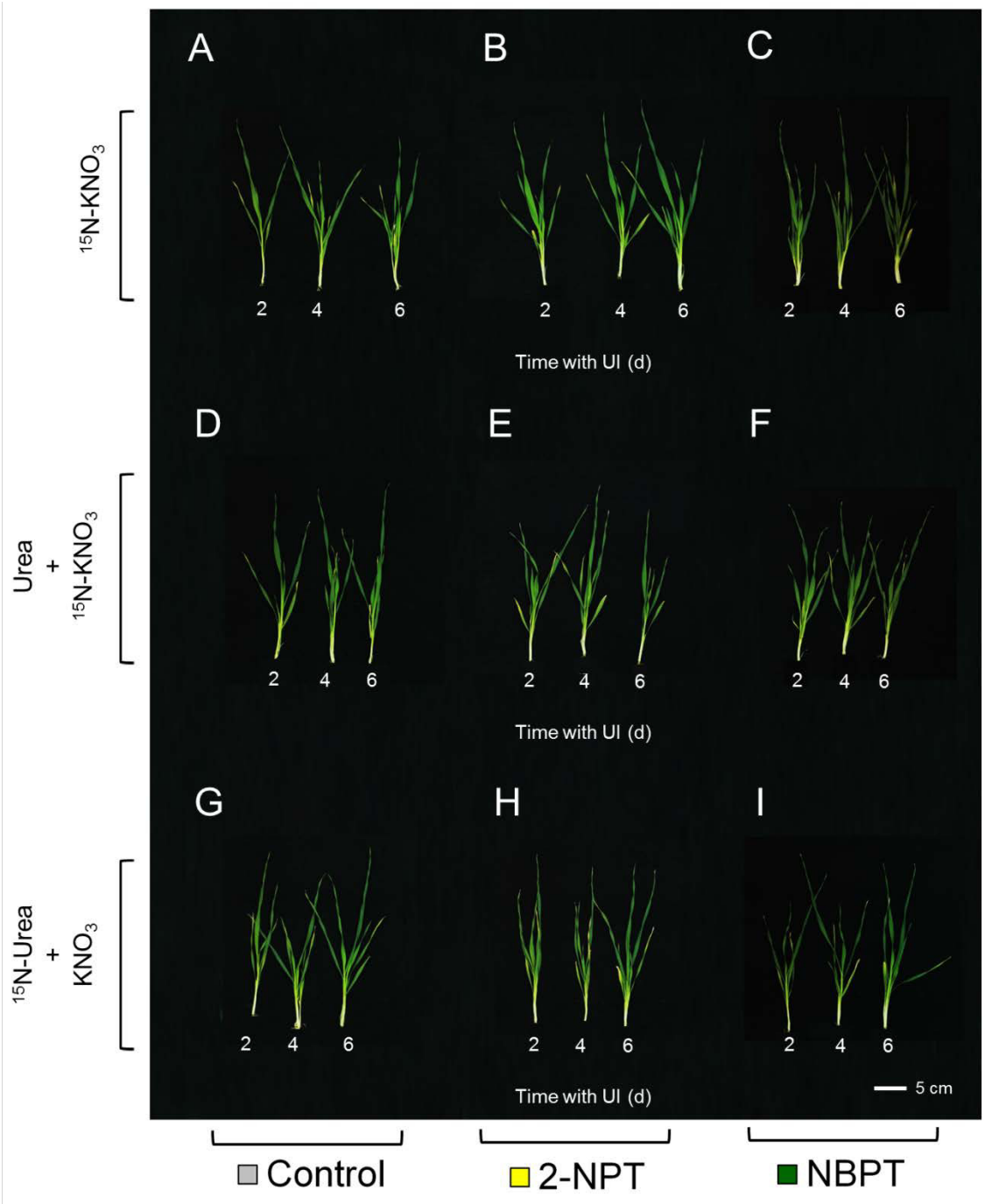
D

$$\text{Shoot } ^{15}\text{N accumulation } (\mu\text{moles } ^{15}\text{N g}^{-1}\text{ shoot DW 2h}^{-1}) = \left[\frac{(\text{Atom(\%)N shoot} - \text{Background}) \times \text{N(\%)correc.} \times 0.01 \times 1000000}{15 \times 0.98}\right]$$

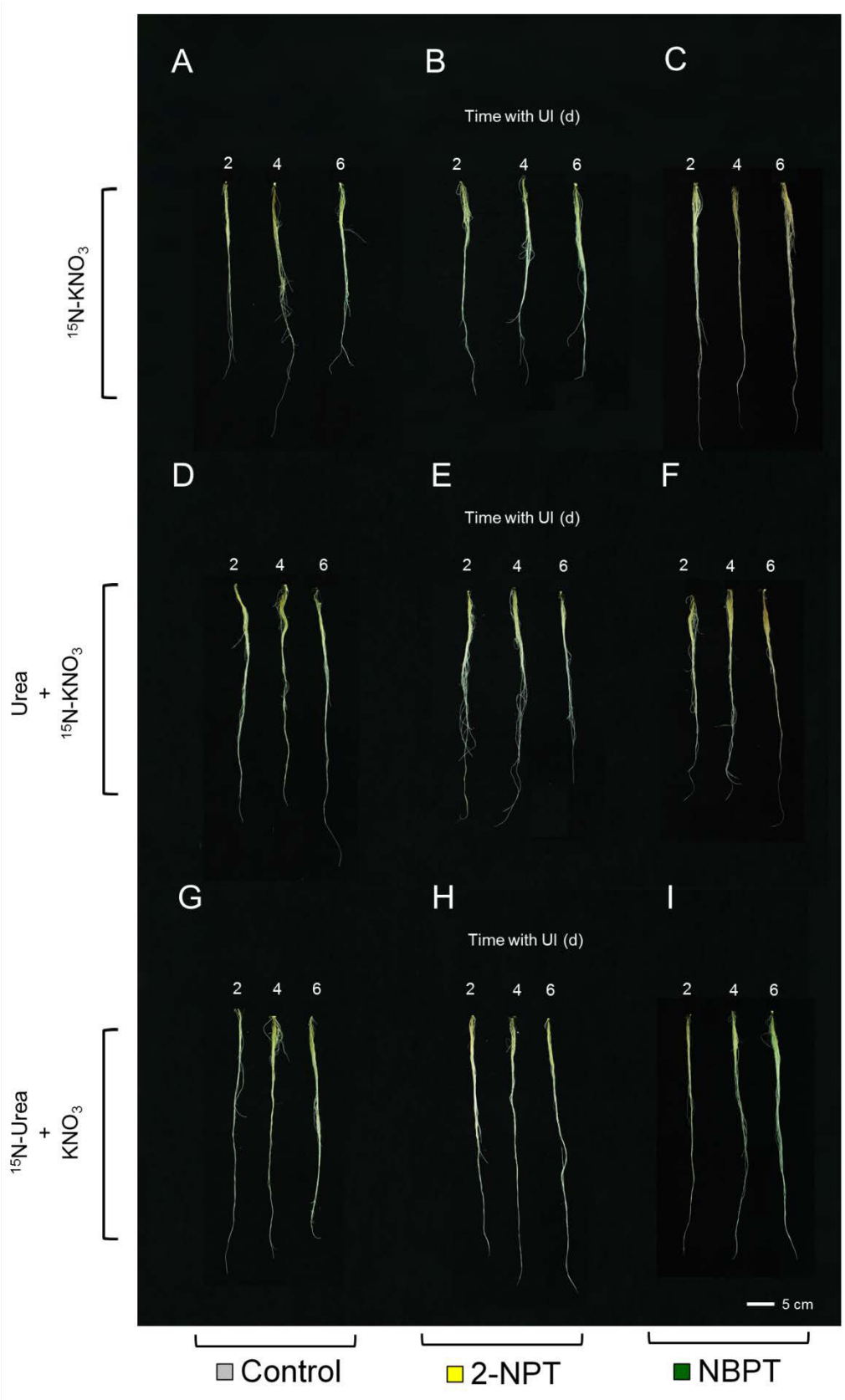
E

$$\text{Root-to-shoot translocation rate } (\mu\text{moles } ^{15}\text{N g}^{-1}\text{ root DW 2h}^{-1}) = \left[\frac{^{15}\text{N in shoots } (\mu\text{mol/2h})}{\text{root DW (g)}}\right]$$

Annex 36. Morphological effect on shoots of barley plants after N supply and addition of either 2-NPT or NBPT. In this picture are shown representative shoots of 22-d-old barley plants at harvest time point. Spring barley plants were precultured for 12 d in full nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added at a concentration of 0.005% of total N in nutrient solution (0.2 µM) for either 2, 4 or 6 days. Afterwards, plants were incubated for 10 or 120 min (for determination of uptake or translocation rates, respectively) in full nutrient solution containing 200 µM of ^{15}N-labeled urea or nitrate, namely ^{15}N-KNO_3 (**A**, **B** and **C**), urea + ^{15}N-KNO_3 (**D**, **E** and **F**) or ^{15}N-urea + KNO_3 (**G**, **H** and **I**), as well as 0.01 µM of either 2-NPT or NBPT.



Annex 37. Morphological effect on roots of barley plants after N supply and addition of either 2-NPT or NBPT. In this picture are shown representative roots of 22-d-old barley plants at harvest time point. Spring barley plants were precultured for 12 d in full nutrient solution under continuous supply of 4 mM N as either KNO_3 or urea + KNO_3. Urease inhibitor was added at a concentration of 0.005% of total N in nutrient solution (0.2 µM) for either 2, 4 or 6 days. Afterwards, plants were incubated for 10 or 120 min (for determination of uptake or translocation rates, respectively) in full nutrient solution containing 200 µM of ^{15}N-labeled urea or nitrate, namely ^{15}N-KNO_3 (**A**, **B** and **C**), urea + ^{15}N-KNO_3 (**D**, **E** and **F**) or ^{15}N-urea + KNO_3 (**G**, **H** and **I**), as well as 0.01 µM of either 2-NPT or NBPT.



Annex 38. Correlations between ^{15}N accumulated in roots and ^{15}N accumulated in shoots of spring barley plants. Correlations were performed by Pearson product moment and are shown for three independent hydroponical experiments, which differed in their time period of incubation with urease inhibitor (UI), either for 2, 4 or 6 days.

Time with UI (d)	^{15}N-supplied form		
	^{15}N-KNO_3	urea + ^{15}N-KNO_3	^{15}N-urea + KNO_3
2	0.58	0.85	0.98
4	-0.33	0.77	-0.64
6	0.49	0.59	0.99

Annex 39. Correlations between root ^{15}N accumulation and the root-to-shoot translocation rate in spring barley plants. Correlations were performed by Pearson product moment and are shown for three independent hydroponical experiments, which differed in their time period of incubation with urease inhibitor (UI), either for 2, 4 or 6 days.

Time with UI (d)	^{15}N-supplied form		
	^{15}N-KNO_3	urea + ^{15}N-KNO_3	^{15}N-urea + KNO_3
2	0.53	0.94	0.97
4	-0.94	0.88	-0.76
6	0.55	0.99	0.74

Annex 40. Correlations between total ^{15}N uptake and root-to-shoot translocation rate of spring barley plants. Correlations were performed by Pearson product moment and are shown for three independent hydroponical experiments, which differed in their time period of incubation with urease inhibitor (UI), either for 2, 4 or 6 days.

Time with UI (d)	^{15}N-supplied form		
	^{15}N-KNO_3	urea + ^{15}N-KNO_3	^{15}N-urea + KNO_3
2	0.77	0.98	0.98
4	0.23	0.97	-0.58
6	0.77	0.99	0.76

ABBREVIATIONS

$-NH_2$	Amide group
2-NPT	N-(2-nitrophenyl)-phosphoric acid triamide
2-OG	2-oxoglutarate
3PGA	3-phosphoglycerate
AA	Amino acids
ABA	Abscisic acid
ABAGlu	Abscisic acid-glucosyl ester
ACQ	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
AG	*Aktiengessellschaft* (Joint-stock company)
Ala	Alanine
AMT	Ammonium transporter
ANOVA	Analysis of variance
Approx.	Approximately
AQP	Aquaporin
Arg	Arginine
AS	Asparagine synthetase
Asn	Asparagine
Asp	Aspartate
AspAT	Aspartate aminotransferase
AUX	Auxin
BBCH	*Biologische Bundesanstalt, Bundessortenamt und Chemische Insdustrie*
BBOT	2,5-Bis(5-tert-butyl-benzoxazol-2-yl)thiophene
CAN	Calcium ammonium nitrate
CCC	2-chloroethyl trimethylammonium chloride
CCE	Calcium carbonate equivalents
CEC	Cation exchange capacity
CH	Column heater
Cit	Citrate
CK	Cytokinin
CO_2	Carbon dioxide
$CO(NH_2)_2$	Urea
Cys	Cysteine
cZ	*cis*-zeatin
cZOG	*cis*-zeatin-O-glucoside
cZR	*cis*-zeatin riboside
cZROG	*cis*-zeatin riboside-O-glucoside
dhZ	Dehydrozeatin
dhZR	Dehydrozeatin riboside
DMF	N,N-dimethylformamide
DW	Dry weight
e.g.	*exempli gratia* (for example)
FAO	Food and Agriculture Organization of the United Nations
Fe-EDTA	Sodium ferric ethylenediaminetetraacetate
Fru	Fructose
Fum	Fumarate
FW	Fresh weight
GABA	*gamma*-Aminobutyric acid
GDH	Glutamate dehydrogenase
Glc	Glucose
Gln	Glutamine
Glu	Glutamate

Gly	Glycine
GmbH	*Gesellschaft mit beschränkter Haftung* (private limited company)
GOGAT	Glutamate synthase or glutamine:2-oxoglutarate aminotransferase
GS	Glutamine synthetase
HATS	High-affinity transport system
His	Histidine
HLB	Hydrophilic-lipophilic-balanced
HPLC	High-performance liquid chromatography
i.a.	*inter alia* (among other things)
IAA	Indole-3-acetic acid
IAAla	Indole-3-acetyl-L-alanine
IAAMe	Indole-3-acetic acid methyl ester
IAM	Indole-3-acetamide
IAN	Indole-3-acetonitrile
ICP	Inductively coupled plasma
IFA	International Fertilizer Industry Association
i.e.	*id est* (that is)
Ile	Isoleucine
iP	Isopentenyl adenine
iP9G	Isopentenyl adenine-9-glucoside
iPR	Isopentenyl adenosine
IRMS	Isotope ratio mass spectrometry
Isocit	Isocitrate
KG	*Kommanditgesellschaft* (limited partnership business entity)
LATS	Low-affinity transport system
LC	Liquid chromatography
Leu	Leucine
LLC	Limited Liability Company
Lys	Lysine
Mal	Malate
MCX	Mixed-mode, reversed-phase/strong cation-exchange
Mel	Melatonin
MES	2-(N-Morpholino)ethanesulfonic acid
MIPs	Major intrinsic proteins, also called aquaporins
MS	Mass spectrometry
N	Nitrogen
NBPT	N-(n-butyl)thiophosphoric triamide (also known as NBTPT)
NBPTO	N-(n-butyl) phosphoric triamide (NBPT oxygen analogue)
NH_3	Ammonia
NH_4^+	Ammonium
NH_4NO_3	Ammonium nitrate
$(NH_4)_2SO_4$	Ammonium sulfate
NIPs	Nodulin 26-like intrinsic membrane proteins
NiR	Nitrite reductase
N_{min}	Mineralized nitrogen
NO_2^-	Nitrite
NO_3^-	Nitrate
NPPT	N-(n-propyl) thiophosphoric triamide
NR	Nitrate reductase
NRT	Nitrate transporter
NUE	Nitrogen use efficiency
OH^-	Hydroxide
Orn	Ornithine
OxIAA	2-oxindole-3-acetic acid

PA	Phaseic acid
PDA	Photodiode array detector
Phe	Phenylalanine
PIPs	Plasma membrane intrinsic proteins
PMSF	Phenylmethylesulfonyl fluoride
PPD	Phenylphosphorodiamidate (also known as PPDA)
PPM	Parts per million
Pro	Proline
PTFE	Poly tetra fluoro ethylene
QSM	Quaternary solvent manager
Ref.	Refer (to)
rpm	Revolutions per minute
S.A.	Anonymous company
SA	Salicylic acid
Sal	Salicin
Ser	Serine
SIPs	Small basic intrinsic proteins
SM-FTN	Sample manager – flow through needle
SPE	Solid-phase extraction
SSS	Sodium solute symporter
T1	First time point
T2	Second time point
TCA	Tricarboxylic acid
Thr	Threonine
TIPs	Tonoplast intrinsic proteins
TKW	Thousand kernel weight
Trp	Tryptophan
Tryp	Tryptamine
Tyr	Tyrosine
tZ	*trans*-zeatin
tZ9G	*trans*-zeatin-9-glucoside
tZOG	*trans*-zeatin-O-glucoside
tZR	*trans*-zeatin riboside
tZROG	*trans*-zeatin riboside-O-glucoside
UAN	Urea ammonium nitrate
UI	Urease inhibitor
UPLC	Ultra-performance liquid chromatography
USA	United States of America
Val	Valine
WFPS	Water-filled pore space

CURRICULUM VITAE

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ACKNOWLEDGEMENTS

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