THE ESSENTIAL AND BENEFICIAL ROLES OF NICKEL IN GROWTH OF SOYBEAN AND WHEAT PLANTS

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Submitted to the Graduate School of Engineering and Natural Sciences
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biological Sciences and Bioengineering

Sabanci University
August 2013

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ABSTRACT

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Biological Sciences and Bioengineering, PhD Thesis, 2013

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Keywords: glyphosate, nickel, nitrogen use efficiency, soybean, urea, urease, wheat

Nickel (Ni), which is known to be the cofactor of urease, was the last element to be included in the list of essential micronutrients for higher plants. Although the Ni requirement of plants is very low, Ni deficiency was documented to occur under field conditions. However, most of the studies on plant Ni nutrition were conducted in hydroponics and focused on urea metabolism. In order to investigate the essential and beneficial roles of Ni in plant growth, several nutrient solution and soil culture studies were conducted on two major crops, namely soybean and wheat, under growth chamber and greenhouse conditions. Nickel deficiency reduced the seed yield in nitrate-fed soybean and caused impaired growth and toxicity symptoms upon foliar urea applications. Moreover, Ni deficiency resulted in physiological nitrogen (N) deficiency and reduced the N uptake and N use efficiency (NUE) of urea-fed plants. Using high-Ni seeds was a highly effective alternative to external Ni supply for alleviating the problems caused by urea. In wheat, soil and/or foliar applications of Ni improved the grain yield and NUE under ample N supply, indicating that Ni may be beneficial at levels much higher than required to fulfill its essential roles, depending on the conditions. Furthermore, foliar Ni applications were shown to provide protection against sublethal glyphosate, which can cause developmental abnormalities and dramatic yield losses in wheat. The use of Ni as a micronutrient may have great impacts on agricultural productivity, NUE and crop tolerance to glyphosate drift. These effects should be further investigated under field conditions.

ÖZET

SOYA VE BUĞDAYIN BÜYÜMESİNDE
NİKELİN ESANSİYEL VE YARARLI ROLLERİ

Bahar Yıldız Kutman

Biyoloji Bilimleri ve Biyomühendislik, Doktora Tezi, 2013

Tez Danışmanı: Prof. Dr. İsmail Çakmak

Anahtar sözcükler: Azot kullanım etkinliği, buğday, glifosat, nikel, soya, üre, üreaz

Üreazın kofaktörü olduğu bilinen nikel (Ni), bitkiler için esansiyel (mutlak gerekli) mikrobesinler listesine son eklenen elementtir. Bitkilerin Ni ihtiyacı çok düşük olsa da, Ni eksikliğinin tarla koşullarında görülebildiği gösterilmiştir. Bununla beraber bitki Ni beslenmesi ile ilgili çoğu çalışma su kültürü ortamında yapılmış ve üre metabolizmasına odaklanmıştır. Nikelin bitkisel üretimdeki esansiyel ve yararlı rollerini araştırmak için, iki önemli tarım ürünü olan buğday ve soya üzerinde, iklim odası ve sera koşullarında, çok sayıda su kültürü ve toprak çalışması yapılmıştır. Nikel eksikliği nitrat ile beslenen soyada verimi düşürmüş, yapraktan üre uygulaması yapıldığında ise büyümeyi azaltmış ve toksisiteye sebep olmuştur. Ayrıca, üre ile beslenen bitkilerde fizyolojik azot (N) eksikliğine, N alımında ve N kullanım etkinliğinde (NKE) azalmaya yol açmıştır. Üre kaynaklı sorunların azaltılmasında Ni'ce zengin tohum kullanımı dışarıdan Ni sağlamak yerine etkin bir alternatiftir. Buğdayda, topraktan ve/veya yapraktan Ni uygulamaları dane verimini ve NKE'yi bol N ile beslenen bitkilerde arttırmıştır ve bu da Ni'in, koşullara bağlı olarak, esansiyel rollerini yerine getirebilmek için gerektiğinden çok daha yüksek düzeylerde bile yararlı olabileceğine işaret etmektedir. Öte yandan, Ni'in yapraktan uygulanmasının, buğdayda gelişim bozukluklarına ve şiddetli verim kayıplarına neden olabilen glifosat zararına karşı koruma sağladığı gösterilmiştir. Nikelin bir mikrobesin olarak kullanımının bitkisel verimlilik, NKE ve glifosata karşı bitki toleransı üzerinde büyük etkileri olabilir. Bu etkilerin tarla koşullarında daha detaylı olarak araştırılması gerekir.

This work is dedicated to

my *Imzadi*, **Ümit Barış KUTMAN**,
without whose love and support I would not have accomplished it.

ACKNOWLEDGEMENTS

I would like to start by expressing my gratitude to my thesis supervisor Prof. Dr. İsmail Çakmak, for his invaluable contributions to my PhD study, his guidance, inspiring ideas and constructive critics.

I would like to thank all members of my thesis committee, Assoc. Prof. Dr. Levent Öztürk, Prof Dr. Osman Uğur Sezerman, Prof. Dr. Ali Rana Atılgan and Prof. Dr. İsmail Türkan for their precious contributions to my thesis. I wish to express my special thanks to Assoc. Prof. Dr. Levent Öztürk for sharing his creative ideas with me during my studies and Prof. Dr. Osman Uğur Sezerman for encouraging me to choose the BIO program. I also wish to thank all professors of the BIO program, who greatly contributed to my academic development.

I wish to thank all the technical staff of the Plant Physiology group, particularly Veli Bayır, who passed away last year, Atilla Yazıcı, Özge Berber, Uğur Atalay and Yusuf Tutuş, for their friendship and support. I would also like to thank all my friends in the Biological Sciences and Bioengineering Program for sharing my five-year graduate experience.

Many thanks go to my husband Ümit Barış Kutman, who convinced me to be a plant biologist, for being in every part of this project and my life, his love, patience and care.

I would like to give my special thanks to all members of my family, especially my mother and father for their invaluable supports and to my little sister Lale who even shared the joy of watering plants with me on weekends.

Finally, I would like to acknowledge the Department of Science Fellowships and Grant Programmes of the Scientific and Technological Research Council of Turkey (www.tubitak.gov.tr/bideb) for supporting me by a scholarship throughout my PhD study.

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α-KG ..... α-ketoglutarate
ACC ..... 1-aminocyclopropane-1-carboxylic acid
ADP ..... adenosine diphosphate
Al ..... aluminum
ANOVA ..... analysis of variance
App ..... application
Arg ..... arginine
B ..... boron
Ca ..... calcium
$CaCl_2$ ..... calcium chloride
$CaCO_3$ ..... calcium carbonate
$Ca(NO_3)_2.4H_2O$ ..... calcium nitrate tetrahydrate
$CaSO_4.2H_2O$ ..... calcium sulfate dihydrate
Cd ..... cadmium
Cl^- ..... chloride
Co ..... cobalt
$CO(CH_2)_2$ ..... urea
CO_2 ..... carbon dioxide
Conc. ..... concentration
Cr. ..... chromium
Cu ..... copper
$CuSO_4.5H_2O$ ..... copper sulfate pentahydrate
cv. ..... cultivar
DAA ..... days after application
DAS ..... days after sowing
dH_2O ..... distilled water
DTPA ..... diethylenetriamine pentaacetic acid
DTT ..... dithiothreitol
DUR3 ..... high-affinity urea uptake transporter
DW ..... dry weight
e.g. ..... exempli gratia (for example)

EFE ..... ethylene-forming enzyme

EN ..... external nickel

EPSPS ..... 5-enolpyruvylshikimate 3-phosphate synthase

F Pr. ..... f value probability

F.Ni. ..... foliar Ni

Fe ..... iron

$FeCl_3.6H_2O$ ..... iron ferric chloride hexa hydrate

FeEDTA ..... iron ethylenediamine tetraacetic acid

FU ..... foliar urea

FW ..... fresh weight

Glu ..... glutamic acid

GLUD ..... glutamate dehydrogenase

Gly ..... glyphosate

GR ..... glyphosate-resistant

Gr. ..... grain

H_2 ..... molecular hydrogen

H_3BO_3 ..... boric acid

H_2O_2 ..... hydrogen peroxide

HI ..... harvest index

HNO_3 ..... nitric acid

HSD ..... Tukey's honestly significant difference

i.e. ..... id est (that is)

ICP-OES ..... inductively coupled plasma optical emission spectrometry

IFA ..... International Fertilizer Industry Association

IRT1 ..... iron-regulated transporter 1

K ..... potassium

KCl ..... potassium chloride

KH_2PO_4 ..... potassium dihydrogen phosphate

K_2HPO_4 ..... dipotassium hydrogen phosphate

KNO_3 ..... potassium nitrate

KOH ..... potassium hydroxide

K_2SO_4 ..... potassium sulfate

K-P buffer ..... potassium phosphate buffer

LSD ..... Fisher's least significant difference

Mg ..... magnesium

$MgSO_4.7H_2O$ ..... magnesium sulfate heptahydrate

Mn ..... manganese

$MnSO_4.H_2O$ ..... manganese sulfate monohydrate

Mo ..... molybdenum

MS ..... main stem

N ..... nitrogen

$(NH_4)_6Mo_7O_{24}.4H_2O$ ..... ammonium heptamolybdate (paramolybdate) tetrahydrate

n.d. ..... not determined

n.s. ..... not significant

N_2 ..... molecular nitrogen

Na_3EDTA ..... ethylenediaminetetraacetic acid trisodium salt

NADPH ..... nicotinamide adenine dinucleotide phosphate

NaOH ..... sodium hydroxide

NH_3 ..... ammonia

NH_4^+ ..... ammonium

$(NH_4)_2SO_4$ ..... ammonium sulfate

Ni ..... nickel

$NiCl_2.6H_2O$ ..... nickel chloride hexahydrate

NO_2^- ..... nitrite

NO_3^- ..... nitrate

NUE ..... nitrogen use efficiency

NUpE ..... nitrogen uptake efficiency

NUtE ..... nitrogen utilization efficiency

Orn ..... ornithine

P ..... phosphorus

Pb ..... lead

rec ..... recommended

S ..... sulfur

SAM ..... S-adenosyl-L-methionine

Sn. ..... tin

SN ..... seed Ni

$SnCl_2.2H_2O$ ..... stannous chloride dihydrate

Transloc ..... translocation

V.....vanadium

v/v ..... volume per volume

w/v ..... weight per volume

Zn.....zinc

$ZnSO_4 \cdot 7H_2O$..... zinc sulfate heptahydrate

ZS..... zadoks stage

(A) GENERAL INTRODUCTION

A.1. Nickel as an Essential Plant Micronutrient

In plant nutrition, a mineral is considered as an essential nutrient if it meets the following three criteria (Arnon and Stout 1939; Marschner 2012):

1. The mineral is directly involved in plant metabolism either as a structural or functional component.
2. The mineral can not be replaced by another element.
3. Plants can not complete their lifecycle unless the mineral is present.

The history of nickel (Ni) as a plant nutrient started when Dixon *et al.* (1975) showed Ni to be the cofactor of the enzyme urease isolated from jack beans (*Canavalia ensiformis*). This enzyme hydrolyzes urea ($CO(NH_2)_2$) to produce ammonia (NH_3). Accordingly, soybean (*Glycine max*) plants grown in hydroponics with urea as the sole nitrogen (N) source accumulated toxic concentrations of urea, which was prevented by Ni addition to the nutrient solution (Shimada and Ando 1980). The first subtle evidence for the essentiality of Ni for higher plants was reported in 1983 by Eskew *et al.*, who showed that Ni was required in nutrient solution to prevent the accumulation of toxic concentrations of urea in not only urea-fed but also mineral N-fed (nitrate, ammonium) or N-fixing soybean. The observation of toxicity symptoms in the absence of urea application indicated that the requirement of soybean for Ni was not conditional. Detrimental effects associated with the accumulation of internally produced urea was also observed in Ni-deprived cowpea (*Vigna unguiculata*), especially during reproductive growth and senescence of older leaves (Eskew *et al.* 1984; Walker *et al.* 1985). Nickel deficiency symptoms in a non-legume were first observed by Checkai *et al.* (1986), who reported chlorosis of youngest leaves and necrosis of meristem in Ni-

deficient tomato (*Lycopersicon esculentum*). Several studies were conducted to check the replaceability of Ni by other elements including Al, Co, Cr, Cd, Pb, Sn and V, and it was concluded that none of these elements was a substitute for Ni *in planta* (Klucas *et al.* 1983; Eskew *et al.* 1984; Gerendas *et al.* 1998a). The final criterion for the essentiality of Ni as a plant nutrient was met when barley (*Hordeum vulgare*) was shown to be unable to complete its life-cycle in the absence of Ni (Brown *et al.* 1987a). Seeds produced by Ni-deficient barley plants were inviable and failed to germinate even if imbibed in Ni-containing solution. Thus, among all essential mineral nutrients for plants, Ni was the last to be accepted as essential (Marschner 2012). The critical deficiency concentration for Ni was estimated at about 100 µg per kg dry matter in several crops including barley, rice (*Oryza sativa*) and zucchini (*Cucurbita pepo*) (Brown *et al.* 1987a, b; Gerendas *et al.* 1999). Due to the extremely low level of requirement, Ni was classified as an ultra-micronutrient, along with Mo (Asher 1991).

A. 2. Availability of Nickel in Soils: Deficiency and Toxicity

The typical range of total Ni concentration in agricultural soils is 5-500 mg kg^{-1}, but the plant-available Ni concentration, which is most commonly estimated by DTPA extraction, is much lower and highly dependent on soil and environmental conditions (Brown 2006). Sandy soils with low cation exchange capacities are most likely to be poor in Ni (Wood *et al.* 2004; Marschner 2012). The bioavailability of Ni is low in alkaline soils due to the formation of sparingly soluble Ni hydroxides (Brown 2006). Other factors reducing the Ni availability include high levels of $CaCO_3$ and high concentrations of competing divalent cations such as Zn, Cu and Mg (Dalton *et al.* 1985; Wood *et al.* 2004). Therefore, excessive applications of these elements and liming practices can induce Ni deficiency. The first clear evidence for Ni deficiency under field conditions was provided by Wood *et al.* (2004), who showed that Ni deficiency was the cause of the mouse-ear disorder commonly observed in pecan orchards. Container-grown river birch trees suffering from the same disorder were also reported to be Ni-deficient (Ruter 2004). Significant yield responses to soil Ni applications were also documented for other plants including potato, wheat, common bean (Roach and Barclay 1946), parsley (Atta-Aly 1999) and tomato (Gad *et al.* 2007).

In crop production, there has been more concern about the toxicity of Ni than its deficiency (Marschner 2012). Nickel toxicity can restrict plant yields in not only serpentine soils, which are naturally rich in Ni and some other heavy metals, but also Ni-contaminated soils (Brown 2006). Industrial pollution, atmospheric deposition, impurities in fertilizers and application of sewage sludge to field are the main factors contributing to Ni accumulation in agricultural soils. Moreover, agricultural practices causing soil acidification as well as acid rains can increase the availability of Ni and thus the risk of Ni toxicity to plants.

A.3. Urea as a Nitrogen Fertilizer and Plant Metabolite

Urea is the most commonly used N fertilizer, often preferred over mineral N fertilizers (*i.e.* NO_3^- and NH_4^+) due to its relatively low cost, high N content (46%) and ease of handling (Gilbert *et al.* 2006). The share of urea in the total N fertilizer consumption has increased from 40% to over 55% in the last 20 years (International Fertilizer Industry Association(IFA)), and there is still a global trend for increased urea consumption (Gilbert *et al.* 2006). Soil-applied urea can be directly taken up passively through channels or actively by urea uptake transporters (Witte, 2011). Alternatively, urea can be converted to mineral N by soil microbial activity and then absorbed by plants. However, urea hydrolysis in soils can cause significant portions of the applied N to be lost as a result of NH_3 volatilization. Nitrification of NH_4^+ can then lead to nitrite (NO_2^-) accumulation and nitrate (NO_3^-) leaching. Urease inhibitors are commonly added to urea fertilizers in order to minimize these problems and improve the nitrogen use efficiency (NUE) (Watson and Miller 1996; Dawar *et al.* 2011). The use of urease inhibitors can also lower the risk of NH_4^+ toxicity, which can be observed under field conditions when urea fertilizers are rapidly converted into NH_4^+ by soil urease activity (Bremner 1995). On the other hand, using urease inhibitors increases the amount of urea available for root uptake.

Urea is also used as a foliar N fertilizer for correcting N deficiency, improving the yield and enhancing seed protein levels (Gooding and Davies 1992; Dong *et al.* 2005; Yildirim *et al.* 2007). Compared to soil N applications, foliar urea treatment may be advantageous as it may reduce N losses and provide extra N to plants when root activity

is impaired late in the season or due to stress conditions such as drought (Gooding and Davies 1992). Urea is the preferred N form for foliar fertilization because of its low cost, high leaf penetration rate and lower salt index than NO_3^- and NH_4^+ reducing the risk of leaf burn (Gooding and Davies 1992).

Urea is not only taken up from the environment as an N source but also produced endogenously as an N metabolite (Witte 2011). The only confirmed source of metabolic urea in all higher plants is arginine (Arg) catabolism. Arginine break-down by arginase to urea and ornithine (Orn) is central to the mobilization of N from source to sink tissues, particularly during senescence or seed germination (Walker *et al.* 1985; Micallef and Shelp 1989; Witte 2011). This amino acid is also the most important metabolite for N storage in plant seeds, accounting for 17.3% of total seed N in a survey of 379 plant species (Van Etten *et al.* 1967). In developing soybean cotyledons, Arg constitutes 18% of total protein N and 60% of free amino acid N (Micallef and Shelp 1989). Apart from the Arg catabolism, another possible source of metabolic urea is the ureide degradation in ureide-transporting species such as tropical legumes and hydrophilic trees (Gerendas *et al.* 1999, Bai *et al.* 2006). There are two different pathways for ureide catabolism, and only one of them produces urea as an intermediate. The relative importance of these two pathways in plants is still debated and apparently species dependent.

Urease activity and thus Ni are required for both the assimilation of urea absorbed from the environment and recycling of N in endogenous urea (Polacco *et al.* 2013).

A.4. Urease: a Nickel Metalloenzyme

Urease, which is still the only known Ni metalloenzyme in higher plants, catalyzes the breakdown of urea to NH_3 and CO_2 (Dixon *et al.* 1975; Witte 2011; Polacco *et al.* 2013). The enzymatic hydrolysis of urea, which is reported to be at least 10^{14} times faster than its non-enzymatic degradation, occurs in two steps (Gerendas *et al.* 1999; Witte 2011):

(1) $CO(NH_2)_2 + H_2O \rightarrow NH_3 + NH_2COOH$ (catalyzed by urease)

(2) $NH_2COOH \rightarrow NH_3 + CO_2$ (spontaneous)

The best-characterized plant urease is isolated from jack bean, has a molecular weight of 590 kDa and contains six subunits with two Ni atoms in each (Dixon *et al.* 1980, Marschner 2012). Although Ni is essential for the structure and catalytic function of urease, the biosynthesis of the urease protein is apparently not dependent on Ni availability (Winkler *et al.* 1983, Klucas *et al.* 1983, Marschner 2012). Urease is known as a cytosolic enzyme and accordingly, most of the urease activity is detected in soluble fractions of cell extracts (Mobley *et al.* 1995; Sirko and Brodzik 2000, Witte 2011). Arguably, the critical deficiency levels reported for Ni in plant tissues are levels required for achieving full urease activity (Gerendas *et al.* 1999). According to several reports, plant urease production is constitutive and does not respond to externally supplied urea (Gerendas and Sattelmacher 1999; Witte *et al.* 2002), although induction of urease upon urea treatment was also reported by some contradictory studies (Chen and Ching 1988; Hine and Sprent 1988).

A.5. Nitrogen Use Efficiency and Nickel

The nitrogen use efficiency (NUE) is a measure of plant growth response to available N or applied N fertilizer (Moll *et al.* 1982; Good *et al.* 2004). According to the crop species and the exact definition chosen, either the seed yield or the total biomass is divided by the N supply to calculate the NUE. Increasing the NUE in crop production is a hot topic, because more than half of the N applied globally to agricultural soils is lost from the plant-soil system due to inefficiencies in N use (Good *et al.* 2004). Ammonia volatilization, nitrate leaching and denitrification are major processes behind these N losses (Fageria and Baligar 2005). Inefficient N fertilization is associated with huge economic losses as well as global environmental issues. Since the use of N fertilizers is one of the main costs in the production of high-yielding crops, increasing the NUE would have a big impact on the economy of agriculture (Good *et al.* 2004). Moreover, the non-utilized N fertilizers saturate ecosystems with N and cause soil, water and atmospheric pollution (Matson *et al.* 2002; Chardon *et al.* 2010).

According to the commonly used definition of NUE for grain crops by Moll *et al.* (1982), the NUE is the product of the N uptake efficiency (NUpE) and the N utilization efficiency (NUtE). Under field conditions, the apparent NUpE depends on the N uptake

capacity of the plants and the N losses from the plant-soil system. The key processes affecting the N utilization efficiency, on the other hand, are the assimilation, remobilization and, in the case of seed crops, the conversion of N to grain yield (Good *et al.* 2004; Chardon *et al.* 2010).

Besides breeding and transgenic tools (Good *et al.* 2004), agronomic strategies including optimization of plant nutrition can be used to enhance the NUE in crop production. Nitrogen management practices for improving the NUE include among others, the avoidance of excessive N applications by considering the N demand of the crop and the N availability in the soil, application of N fertilizers in split doses and use of urease and nitrification inhibitors (Cassman *et al.* 2002; Villar and Guillaumes 2010; Dawar *et al.* 2011). An insufficient supply of any essential mineral other than N can also lower the NUE indirectly by reducing the yield potential or directly by impairing the N metabolism as in the case of Mo-deficient plants supplied with nitrate (Hawkesford and Barraclough 2011). Nickel may also be a critical nutrient in this context due to its involvement in N metabolism (Polacco *et al.* 2013), and reduced urea use efficiencies were reported for Ni-deficient plants (Nicoulaud and Bloom 1996; Gerendas *et al.* 1998b).

A.6. Other Roles of Nickel in Plant Physiology

Hydrogenase is a Ni metalloenzyme and plays a critical role in the nutrition of legumes, which are N_2-fixing crops, although it is not synthesized by plants (Stults *et al.* 1984; Kim and Maier 1990). These plants can not fix atmospheric N_2 on their own but symbiotic bacteria (*Rhizobium* and *Bradyrhizobium* spp.) living in their root nodules are able to convert molecular N_2 into NH_3. Hydrogen uptake hydrogenases of these bacterial symbionts are Ni-Fe hydrogenases, where Ni is associated with Fe-S clusters (Li and Zamble 2009). They are required for the recycling of molecular H_2 produced as a result of the nitrogenase reaction and thus for efficient N_2 fixation (Gerendas *et al*. 1999; Marschner 2012). In accordance with this role of Ni in N_2 fixation, Ni applications were shown to increase nodulation and seed yield of legumes substantially under field conditions (Bertrand and DeWolff 1973).

Reportedly, the Ni nutritional status can also affect the amino acid, organic acid and ureide metabolisms of plants (Gerendas *et al.* 1999; Bai *et al.* 2006; Polacco *et al.* 2013). A distinct accumulation of arginine (Arg) was shown in several Ni-deficient crops (Shimada *et al.* 1980; Walker *et al.* 1985; Bai *et al.* 2006) and explained by feedback inhibition of arginase due to urea accumulation (details described in section A.5) (Gerendas *et al.* 1999). Total free amino acids decreased (Gerendas and Sattelmacher 1997; Gerendas *et al.* 1999) or increased (Brown *et al.* 1990; Bai *et al.* 2006) significantly in response to Ni deficiency, depending on the source of N and the crop species. Apart from the amino acid metabolism, disruption of the organic acid metabolism was reported in Ni-deficient barley (Brown *et al.* 1990) and pecan nut (Bai *et al.* 2006), and the symptoms of Ni deficiency observed in pecan were attributed to the toxic accumulation of lactic and oxalic acids. Although the role of Ni in the catabolism of ureides in ureide-transporting plants is still debated, significantly altered ureide levels due to Ni deficiency were documented for pecan (Gerendas *et al.* 1999; Bai *et al.* 2006; Polacco *et al.* 2013).

Foliar or soil applications of Ni were also documented to enhance plant resistance against fungal diseases, particularly rust, in different species including wheat (Forsyth and Peturson 1959), sugarcane (Bachchhav *et al.* 1978), cowpea (Graham *et al.* 1985), pine (Ahonen-Jonnarth *et al.* 2004), peanut and daylily (Wood and Reilly 2007). This beneficial effect of Ni on plant growth can be attributed to the direct toxicity of Ni to fungi and/or the toxic properties of plant urease to pathogens and/or Ni-induced production of some secondary metabolites involved in plant defense (Mishra and Kar 1974; Gerendas *et al.* 1999; Polacco *et al.* 2013).

Moreover, Ni is known to be an inhibitor of ethylene biosynthesis, along with Co (Lau and Yang 1976; Roustan *et al.* 1989; Polacco *et al.* 2013). Ethylene is a gaseous phytohormone involved in developmental processes such as fruit ripening, leaf and flower senescence, leaf and fruit abscission and root hair formation (Taiz and Zeiger 2006). It is also known as a stress hormone because it mediates stress and defense responses and its production is induced under abiotic and biotic stress conditions. Ethylene is synthesized from S-adenosyl-L-methionine (SAM) in two enzymatic steps. The first and rate-limiting step is catalyzed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which converts SAM to ACC. In the second step, ACC oxidase, also known as the ethylene-forming enzyme (EFE) uses ACC and O_2 to produce ethylene.

Nickel depresses ACC oxidase activity, possibly replacing its Fe cofactor, and thus inhibits ethylene formation (McGarvey and Christoffersen 1992; Pennazio and Roggero 1992). Studies on Japanese persimmon fruit revealed that Ni could effectively inhibit ACC oxidase both *in vitro* (Zheng *et al.* 2005) and *in planta* (Itamura *et al.* 1997), and pre-harvest applications of Ni could significantly prolong the shelf-life of the fruit (Zheng *et al.* 2006).

Moreover, a recent study showed that glyphosate had detrimental effects on the performance of glyphosate-resistant (GR) transgenic soybean and significantly reduced the Ni concentration in leaves (Zobiole *et al.* 2010). The growth impairments observed in that study were attributed to reduced Ni availability to symbiotic bacteria as a result of chelation of Ni by glyphosate.

A.7. Glyphosate Drift and Interactions of Glyphosate with Divalent Minerals

Glyphosate (N-[phosphonomethyl] glycine) is a systemic and non-selective post-emergence herbicide. Due to its high effectiveness and low cost as well as the widespread adoption of GR transgenic crops and no-tillage cropping system, glyphosate has become the most commonly applied herbicide worldwide (Baylis 2000; Cerdeira and Duke 2006; Duke and Powles 2008). It acts by specifically inhibiting a critical enzyme in the shikimate pathway.

Glyphosate drift to non-target crops is a growing practical concern. Herbicide drift rates to susceptible plants may be as high as 10% of the recommended application rates (Al-Khatib and Peterson 1999). In numerous economically important crops, including soybean (*Glycine max*) (Al-Khatib and Peterson 1999; Ellis and Griffin 2002; Cakmak *et al.* 2009), sunflower (*Helianthus annuus*) (Eker *et al.* 2006), potato (*Solanum tuberosum*) (Felix *et al.* 2011), wheat (*Triticum aestivum*) (Baur *et al.* 1977; Deeds *et al.* 2006; Roider *et al.* 2007), sorghum (*Sorghum bicolor*) (Baur *et al.* 1977; Al-Khatib *et al.* 2003), rice (*Oryza sativa*) and corn (*Zea mays*) (Ellis *et al.* 2003; Reddy *et al.* 2010), glyphosate drift simulation studies demonstrated significant growth aberrations and yield reductions.

Apart from the main herbicidal mode of action of glyphosate, the literature reports side effects of this herbicide due to its interactions with divalent mineral nutrients (Duke *et al.* 1983, 1985; Eker *et al.* 2006; Cakmak *et al.* 2009). As a chelating agent, glyphosate can form complexes of varying stability with divalent metal cations including Ca, Mg, Fe, Mn, Zn and Ni (Motekaitis and Martell 1985; Duke *et al.* 2012). The formation of poorly soluble glyphosate-metal complexes *in planta* or in the rhizosphere may be responsible for reduced uptake, translocation and bioavailability of these nutrients as a result of glyphosate (Duke *et al.* 1985; Eker *et al.* 2006; Cakmak *et al.* 2009; Zobiole *et al.* 2010). On the other hand, such glyphosate-metal interactions were also reported to occur in spray solutions and reduce the herbicidal efficacy of glyphosate as well as the bioavailability of foliar fertilizers (Thelen *et al.* 1995; Bernards *et al.* 2005; Chahal *et al.* 2012).

A.8. What was this PhD Thesis Project about?

This PhD thesis project investigated the functions and benefits of Ni as a plant micronutrient in two major crops, namely soybean (Chapters 1 & 2) and wheat (Chapters 3 & 4). Soybean is the most commonly produced grain legume in the world (Gowda *et al.* 2009), and wheat is the most widely cultivated cereal and also the most important staple crop in many regions of the world, accounting for 20% of the global daily calorie intake and over 50% of the calorie intake in many developing countries (Cakmak 2008). Even minor increases in the yield, quality and NUE of these crop species could have a great impact on the global crop production, food safety and environment.

In order to investigate the importance of seed Ni reserves in the Ni nutrition of plants, soybean seeds with different Ni concentrations were obtained by growing soybean plants in nutrient solutions containing different levels of Ni, as described in Chapter 1. Clear effects of the Ni supply on the seed yield and seed urease activity are documented. In another solution culture experiment, it is shown that adequate Ni nutrition provided by seed reserves and/or external supply is critical for efficient utilization of foliar-applied urea, alleviation of foliar urea damage and improved remobilization of N.

As the second step, soybean plants were grown with either nitrate or urea as the sole N source in the nutrient solution, by using previously produced seeds with different Ni concentrations. Chapter 2 deals with how the Ni nutritional status affects the uptake, assimilation and accumulation of the different N sources and the NUE of soybean as well as various growth parameters. Various analyses were performed on different plant organs, leaves of different ages and even samples of depleted nutrient solutions in order to get an insight into the physiology behind the impaired growth and NUE of urea-fed soybean in the absence of any Ni source.

Chapter 3 reports and discusses the results of greenhouse experiments conducted to investigate the beneficial effects of Ni on the growth, yield and NUE of soil-grown wheat. In these experiments, soil and/or foliar applications of Ni were tested in wheat plants grown with NO_3^- as the principle N source supplied at different levels. Foliar urea treatment was also included in the factorial design. Nickel applications to presumably Ni-sufficient wheat can apparently provide significant benefits if the N supply is ample, and the productivity of tillers is probably a key determinant in this context.

A completely novel beneficial effect of Ni is reported in Chapter 4. Due to the chemical properties of Ni ions and glyphosate and the possible involvement of Ni in plant stress responses, it was a tempting hypothesis that Ni applications could alleviate sublethal glyphosate damage. Several experiments were carried out under greenhouse conditions to study the effects of soil and/or foliar Ni applications on the vegetative growth, development, grain yield and seed quality of soil-grown wheat plants subjected to sublethal doses of glyphosate at different developmental stages. The final chapter of this thesis discusses the potential of foliar Ni treatments in the alleviation of glyphosate drift injury to wheat.

(B) GENERAL MATERIALS AND METHODS

Soybean (*Glycine max* cv. Nova) and durum wheat (*Triticum durum* cv. Balcali2000) are the experimental plant species in the first two and last two chapters of this thesis, respectively.

B.1. Plant Growth Facilities

B.1.1. Growth Chamber

Solution culture experiments were conducted in a growth chamber under controlled climatic conditions (light / dark periods: 16 / 8 h; temperature (light / dark): 27 / 23°C; relative humidity (light / dark): 60 / 70%; photosynthetic flux density: 400 μmol m^{-2} s^{-1}).

B.1.2. Greenhouse

Soil experiments were carried out under natural daylight in a computer-controlled greenhouse. With the help of a heating and an evaporative cooling system, the daytime temperature was kept at 25±4°C and the night time temperature at 20±4°C in the greenhouse located at the geographic coordinates: 40° 53′ 25″ N, 29° 22′ 47″ E.

B.2. Solution Culture

Solution culture experiments have been conducted only with soybean. Seeds were germinated in moistened perlite containing 2mM $CaSO_4$ for 5 d at room temperature before being transferred to nutrient solution. Each pot contained 3 or 5 L of nutrient solution, depending on the experiment. The nutrient solution consisted of 0.85 mM K_2SO_4, 0.2 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 0.1 mM KCl, 100 µM Fe in form of FeEDTA, 10 µM H_3BO_3, 3 µM $MnSO_4.H_2O$, 1µM $ZnSO_4.7H_2O$, 0.2 µM $CuSO_4.5H_20$, 0.14 µM $(NH_4)_6Mo_7O_{24}.4H_2O$. Depending on the experiment and treatment group, N was supplied in the form of $Ca(NO_3)_2.4H_2O$ or urea, and Ni was added to the nutrient solution as $NiCl_2.6H_2O$. The final Ca concentration was kept at 2 mM by supplementing the solution with $CaSO_4.2H_2O$, if necessary. Nutrient solutions were continuously aerated and refreshed every 2-3 d.

B.3. Soil Culture

The experimental soil of used in greenhouse studies is a calcareous (18% $CaCO_3$) and alkaline (pH 8.0 in dH_2O) soil transported from Eskisehir, Central Anatolia. It has a clayey-loam texture and is poor in organic matter (1.5%). The total mineral N concentration of the unfertilized soil is 20 mg kg^{-1}, and the diethylenetriamine pentaacetic acid (DTPA)-extractable micronutrient concentrations are as follows: 1.22 mg kg^{-1} Ni, 0.13 mg kg^{-1} Zn, 2.73 mg kg^{-1} Fe.

Each pot was filled with 2 (Chapter 3) or 3 (Chapter 4) kg air-dry soil. The following mineral nutrients were added to each pot as concentrated solutions and mixed with the soil thoroughly before seeds were sown: 100 mg kg^{-1} P as KH_2PO_4, 25 mg kg^{-1} S as K_2SO_4 and 5 mg kg^{-1} Zn as $ZnSO_4.7H_2O$. With the same method, N in the form of $Ca(NO_3)_2.4H_2O$ and Ni in the form of $NiCl_2.6H_2O$ were incorporated into the soil, depending on the experimental design. Plants were watered with deionized water (dH_2O) regularly throughout the experiment.

B.4. Element Analysis

In order to minimize surface contamination, all shoot samples for element analysis were washed thoroughly with deionized water. Root samples for element analysis were washed with 1 mM $CaCl_2$, then 1 mM EDTA and finally deionized water. All these samples were dried at 70°C for 2 days. All dry plant samples were finely ground in an agate vibrating cup mill (Pulverisette 9; Fritsch GmbH; Germany). Ground samples were acid-digested (ca. 0.2 g sample in 2 ml 30% H_2O_2 and 5 ml 65% HNO_3) in a closed vessel microwave system (MarsExpress; CEM Corp., Matthews, NC, USA). After digestion, the total sample volume was finalized to 20 ml by adding double-deionized water. Inductively coupled plasma optical emission spectrometry (ICP-OES) (Vista-Pro Axial, Varian, Australia) was used to determine the concentrations of mineral nutrients, including Fe, Ni, P and Zn, in both digested plant samples and nutrient solution samples. Readings below 1 $\mu g\ kg^{-1}$ Ni were considered negligible and not used for further calculations. The total N concentrations in the samples were measured by using LECO TruSpec C/N Analyzer (Leco Corp., St Joseph, MI, USA). The accuracy of element analyses was checked by using certified standard reference materials obtained from the National Institute of Standards and Technology (Gaithersburg, MD, USA).

B.5. Preparation of Crude Plant Extracts

By mixing 1 mM KH_2PO_4 and 1 mM K_2HPO_4 in a 1:5.5 volume ratio, a potassium phosphate (K-P) buffer with a pH of 7.6 was obtained. The extraction buffer was prepared daily by adding 2.5 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid trisodium salt (Na_3EDTA) to this K-P buffer and kept on ice. Crude extracts were obtained from leaf, root and seed samples.

Germinated seeds were homogenized in 5 ml of extraction buffer. The homogenates were centrifuged at 4000 g for 15 min at 4°C, and the supernatants were transferred to fresh tubes, which were centrifuged at 20,000 g for 20 min at 4°C. This time, a lipid phase appeared on top of the aqueous phase above the solid pellet. The

aqueous supernatants were transferred to fresh tubes and centrifuged once again at 20,000 g for 10 min at 4°C. The aqueous phase collected after this final centrifugation step was called crude extract and used for urease and catalase analyses in Chapter 1.

Leaf and root samples for analyses to be conducted in fresh tissues were stored at -80°C. Frozen fresh samples were ground in liquid N and homogenized in 5 ml of extraction buffer. The homogenates were centrifuged at 5000 g for 20 min at 4°C, and the supernatants were then centrifuged again at 20,000 g for 20 min at 4°C. These supernatants were used for the colorimetric analyses of total proteins, total free amino acids, nitrate, urea and ammonium in Chapter 2.

B.6. Total Protein Analysis

Protein concentrations in the crude extracts were measured by using the linearized Bradford assay described by Zor and Selinger (1996). The Bradford reagent was prepared as follows: 0.1 g Coomassie Brilliant Blue G-250 was dissolved in 50 ml ethanol and this solution was mixed with 100 ml 85% ortho-phosphoric acid. The mixture was filtered. 100 ml glycerin was added to the reagent and the volume was brought to 1000 ml with deionized water. The reagent was kept at 4°C for 24 h and then used for the assay. Protein standards were prepared by dissolving bovine serum albumin in K-P buffer. 5 ml of Bradford reagent was added to 0.1 ml sample (or standard) and vortexed. After 5 minutes, the ratio of the absorbance at 595 nm to that at 450 nm was used as the measure of protein concentration.

B.7. Calculations

For vegetative plant parts, the mineral (*e.g.* Ni, N) contents were calculated by multiplying the mineral concentrations by the dry weights. Similarly, the grain mineral yields were determined by multiplying the grain mineral concentrations by the grain yield.

The harvest index (HI) is defined as the ratio of the grain yield to the sum of the grain yield and the dry straw biomass.

The translocation index for an element is calculated by dividing the shoot content by the total plant content.

In Chapter 2, the nitrogen uptake (NUpE), utilization (NUtE) and use efficiencies (NUE) were calculated according to the definitions given by Moll *et al.* (1982), but the seed yield in these definitions was replaced by the shoot dry weight as the plants in this study were harvested at the vegetative stage. So, the following formulas were used:

- NUpE = (Shoot N content) / (*Amount of N available)
- NUtE = (Shoot dry weight) / (Shoot N content)
- NUE = NUpE x NUtE = (Shoot dry weight) / (*Amount of N available)

* Amount of N available = Amount of N supplied per plant + Seed N content – N content of 2 abscised cotyledons

The uptake efficiency formula for N was also adapted to P in order to calculate the P uptake efficiency.

In Chapter 3, the NUE was calculated according to the original definition:

- NUE = (Grain yield) / (†Amount of N available)

† Amount of N available = Amount of N available per plant in unfertilized soil + Amount of N supplied per plant via soil N fertilization + Amount of N supplied per plant via foliar urea application

B.8. Statistical Analysis

The JMP software was used for statistical analysis. The significance of the effects of the treatments and their interactions on the reported traits was evaluated by analysis of variance (ANOVA). Where ANOVA revealed a significant effect, post-hoc tests at 5% significance were used to determine significant differences between means. When there was a single source of variation, Fisher's protected least significant difference (LSD) test was used, whereas Tukey's honestly significant difference (HSD) test was applied, when there were more than one sources of variation.

CHAPTER 1

NICKEL-ENRICHED SEED OR EXTERNAL NICKEL SUPPLY IMPROVES GROWTH AND ALLEVIATES FOLIAR UREA DAMAGE IN SOYBEAN

1.1. Introduction

Since the discovery that Ni is the cofactor of urease (Dixon *et al.* 1975), most reports about Ni as a plant mineral nutrient focused on urea metabolism and urease activity (Shimada and Ando 1980; Gerendas and Sattelmacher 1997; Arkoun *et al.* 2013). The accumulation of urea and the resulting toxicity symptoms in Ni-deficient plants both in the presence and in the absence of external urea supply were interpreted as evidence for the unconditional Ni requirement of higher plants (Eskew *et al.* 1984; Walker *et al.* 1985; Gerendas *et al.* 1998b). In plants grown with urea as the sole N source in the nutrient solution, deficiency of Ni was reported to impair growth significantly, result in N-deficient phenotypes, reduce the tissue amino acid levels and also lower the rate of urea uptake (Gerendas and Sattelmacher 1997; Arkoun *et al.* 2013). Despite the fact that Ni is essential for all higher plants, hydroponics experiments with several crop species including rice, tomato, canola and wheat documented that Ni addition to the nutrient solution was critical for urea-fed plants but had little or no effect on plants grown with mineral N (ammonium and/or nitrate) (Gerendas *et al.* 1998b; Tan *et al.* 2000; Bybordi and Gheibi 2009; Gheibi *et al.* 2009).

Foliar urea was also used as an N source in nutrient solution studies on the effects of Ni nutrition on urease activity and urea toxicity (Krogmeier *et al.* 1991; Nicoulaud and Bloom 1998). Soybean and tomato were the experimental species in these studies. Reportedly, these two crops differ greatly in their leaf urease activities, and soybean has

much higher urease activity than tomato (Hogan *et al.* 1983). Leaves of Ni-deficient soybean had significantly reduced urease activity and were more prone to foliar urea damage (Krogmeier *et al.* 1991). The authors concluded from these results and the findings of a previous study (Krogmeier *et al.* 1989) that the accumulation of urea and not ammonia was the reason behind the leaf burn symptoms caused by foliar urea. In the tomato study, Ni deficiency impaired the growth of plants that received foliar urea as the only N source (Nicoulaud and Bloom 1998). Interestingly, no differences could be detected between the urease activities of Ni-deficient and –sufficient plants; however, the distribution of foliar urea within the plant was affected by Ni nutrition.

Legumes have a particularly high urease activity in their seeds (Holland *et al.* 1987). Soybean possesses two urease isozymes: a highly expressed "embryo-specific urease" encoded by the *Eu1* gene and a "ubiquitous urease" synthesized in all tissues as a housekeeping enzyme and encoded by the *Eu4* gene (Polacco and Winkler 1984; Stebbins *et al.* 1991; Follmer *et al.* 2004). Two accessory proteins encoded by the *Eu2* and *Eu3* genes are required for the insertion of Ni and activation of apo-ureases (Gerendas *et al.* 1999). Although the embryo-specific soybean urease has at least 100-fold more activity than the ubiquitous urease in mature seeds, only the ubiquitous urease is responsible for the re-assimilation of urea N, whereas the embryo-specific urease does not have an assimilatory function (Polacco and Winkler 1984; Stebbins *et al.* 1991; Witte 2011). The embryo-specific urease appears to enhance plant resistance against insects and pathogens by its toxic properties which may not be related to its ureolytic activity (Follmer *et al.* 2004; Carlini and Polacco 2008).

In addition to external Ni availability, seed Ni reserves are also important for the Ni nutrition of plants, as shown for soybean (Eskew *et al.* 1984) and barley (Brown *et al.* 1987a). Nickel-poor seeds were associated with leaf tip necrosis due to toxic accumulation of urea in soybean (Eskew *et al.* 1984) and severely impaired germination in barley (Brown *et al.* 1987a). A common distinguishing feature of these studies is that plants were hydroponically grown to maturity for obtaining seeds with low Ni concentrations. Although the seed yields tended to decrease in both studies, the yield responses to Ni were statistically not significant. The obtained Ni-poor seeds were not tested for their performance under growth conditions where urea was applied as an N source.

To our knowledge, this is the first study investigating the effects of seed Ni reserves, along with external Ni supply, on the growth of soybean, the utilization of foliar urea and the occurrence of foliar urea toxicity. Here, soybean seeds with different Ni concentrations were produced in a nutrient solution experiment, where the effect of Ni supply on the seed yield was also investigated. Then, these seeds were used to study the effect of seed Ni concentration on the urease activity during germination and to find out if external Ni can compensate for seed Ni in this context. Seeds with different Ni contents were also used for studying how seed Ni reserves and external Ni supply interactively affect the N-nutritional status of soybean plants in the absence or presence of foliar urea treatment. In this experiment, special attention was paid to the growth and nutrient concentrations of the youngest part of the shoot.

1.2. Materials and Methods

1.2.1. Plant Growth and Experimental Design

Soybean (*Glycine max* cv. Nova) seeds containing 5.3 µg Ni per g were germinated in perlite and grown in nutrient solution under growth chamber conditions as described in "General Materials and Methods". For producing soybean seeds with different Ni concentrations, the plants were grown with 4 mM N in the form of $Ca(NO_3)_2.4H_2O$ at 4 different Ni supply levels (0, 10^{-8}, 10^{-7} and 10^{-6} M $NiCl_2.6H_2O$) to full maturity. The experiment was designed as a 5-replicate experiment with 4 plants grown in each 5-L pot. When seedlings were transferred from perlite to nutrient solution, the cotyledons were cut off to minimize the utilization of seed Ni reserves. Pods were harvested when plants senesced completely. Seed yield was measured, and the seed concentrations of Ni, Zn and Fe were determined as described in "General Materials and Methods".

From the seeds produced, low-Ni (0.04 mg kg^{-1}), medium-Ni (0.62 mg kg^{-1}) and high-Ni (8.32 mg kg^{-1}) seeds were selected for use in another nutrient solution experiment, where the plants were grown with or without Ni supply (2 x 10^{-7} M Ni). Nitrogen was provided at a sub-optimal level (1.5 mM N as $Ca(NO_3)_2.4H_2O$), and no nodulation was observed throughout the experiment. After 25 days of growth, one half

of the pots were sprayed with urea (2% w/v urea + 0.02% w/v Tween20) to the point of run-off, while the other half were sprayed with equal volume of deionized water containing only 0.02% (w/v) Tween20. In this experiment with a full factorial design and 3 pot replicates for each treatment, 3 plants were grown in each 3-L pot.

After foliar urea application, the plants were grown for one week. Chlorotic leaves abscised from urea-damaged plants during this time were collected. Three, five and seven days after urea application, SPAD readings (by using SPAD-502, Konica Minolta, Japan) were taken on the 4th trifoliate leaves. On the same days, the length between the 6th trifoliate leaf and the shoot apex was measured, and these data were used to calculate the average shoot elongation rate per day. When the plants were 32 days old, different plant parts were harvested separately: youngest parts (6th trifoliate leaf and all parts above), leaves, stem and roots. All harvested parts were washed in deionized water and dried for 2 days at 70°C for biomass and mineral analysis as described in "General Materials and Methods".

1.2.2. Seed Germination for Enzyme Analysis

Thirty seeds were randomly selected from each of the 4 seed batches produced in the first experiment. Six Petri dishes each containing 5 seeds were prepared for each seed type by putting the seeds between filter papers. Three of them were wetted with equal volumes of deionized H_2O, whereas the others were wetted with equal volumes of 2 x 10^{-6} M Ni solution. The seeds were germinated for 18 h at 26°C. Crude extracts were prepared from the germinated seeds and used for total protein assay as described in "General Materials and Methods" and also for urease and catalase assays as described below.

1.2.3. Urease Assay

Urease activity was determined based on the methods described by Kaltwasser and Schlegel (1966) and Bai *et al.* (2006), but with slight modifications. In this method, the reaction catalyzed by urease is coupled to a reaction catalyzed by glutamate dehydrogenase (GLUD):

$$(NH_2)_2CO\ (Urea) + H_2O \xrightarrow{UREASE} CO_2 + 2\ NH_3$$

$$NH_3 + \alpha\text{-KG} + NAD(P)H/H^+ \xrightarrow{GLUD} Glu + NAD(P)^+ + H_2O$$

Overall: $$Urea + 2\ \alpha\text{-KG} + 2\ NAD(P)H/H^+ \longrightarrow 2\ Glu + 2\ NAD(P)^+ + H_2O + CO_2$$

The assay mix contained 0.56 ml K-P buffer, 0.1 ml 25 mM adenosine diphosphate (ADP – acts as an activator on GLUD), 0.1 ml 25 mM α-ketoglutarate (α-KG), 0.1 ml 50 Unit/ml GLUD, 20 µl sample (crude extract), 0.1 ml 1.8 M urea and 20 µl 4 mM NADPH. All reagents used in this assay were dissolved in K-P buffer. For samples with high urease activity, the crude extracts were diluted as required. The change in the absorbance of this assay mix at 340 nm was followed for 3 min to calculate the average rate of NADPH consumption. Jack bean (*Canavalia ensiformis*) urease was used as a positive control.

1.2.4. Catalase Assay

The catalase activity was also determined spectrophotometrically. 0.8 ml of K-P buffer was mixed with 0.1 ml of 100 mM H_2O_2 (also prepared in K-P buffer) and 0.1 ml of crude extract. The change in the absorbance of this mixture at 240 nm was followed for 2 min to calculate the average rate of H_2O_2 breakdown.

1.3. Results

There was a significant effect of Ni nutrition on seed yield as revealed by analysis of variance (Table 1.1A). The lowest seed yield was obtained from plants grown without any Ni supply, whereas plants provided with 10^{-7} or 10^{-6} M Ni produced the highest yield (Fig. 1.1A). The average seed size was not affected by the Ni supply, while the number of seeds produced increased progressively from 75 to 97 per plant, and this increase accounted for the yield response. Increasing Ni supply in the nutrient solution resulted in the production of soybean seeds with significantly higher Ni concentrations (Table 1.1A; Fig. 1.1B). The Ni concentration of seeds obtained from plants grown in Ni-deficient nutrient solution was barely detectable (40 µg kg^{-1}), while higher Ni supply levels in growth medium increased the seed Ni concentration by up to

200-fold (Fig. 1.1B). Seed levels of other essential micronutrients like Zn and Fe were not affected by Ni treatment (Table 1.1A; Fig. 1.1C, D).

Table 1.1: (A) One-way ANOVA of the effect of solution Ni supply on seed yield and selected mineral concentrations of soybean (*Glycine max* cv. Nova) grown in hydroponics in the first experiment; **(B)** Two-way ANOVA of the effects of seed Ni concentration and solution Ni supply on urease and catalase activities of soybean grown in hydroponics in the second experiment

(A)	Source of Variation	Seed Yield	Seed Ni Conc.[a]	Seed Zn Conc.	Seed Fe Conc.
	Solution Ni Supply	*	***	*n.s.*	*n.s.*

(B)	Source of Variation	Urease Activity	Sp.[b] Urease Activity	Catalase Activity	Sp. Catalase Activity
	Seed Ni Conc.	***	***	*n.s.*	*n.s.*
	Solution Ni Supply	*	*n.s.*	*n.s.*	*n.s.*
	Seed Ni x Solution Ni	*n.s.*	*n.s.*	*n.s.*	*n.s.*

n.s. Not significant; * $0.01 \leq F\ Pr. < 0.05$; ** $0.001 \leq F\ Pr. < 0.01$; *** $F\ Pr. < 0.001$

[a] Concentration; [b] Specific

Table 1.2: Urease and catalase activities of soybean (*Glycine max* cv. Nova) seeds with different Ni concentrations, germinating in the absence and presence of external Ni ($2x10^{-6}$ M)

Seed Ni (mg kg^{-1})	Urease Activity (µmol NH_3 g^{-1} FW min^{-1})		Specific Urease Activity (µmol NH_3 g^{-1} protein min^{-1})	
	- Ni	+ Ni	- Ni	+ Ni
0.04	[a]0.7 ± 0.2[b]	1.3 ± 0.7	14 ± 4	23 ± 13
0.11	4.8 ± 0.6	8.9 ± 1.5	84 ± 14	182 ± 48
0.62	69 ± 9	74 ± 13	1307 ± 39	1366 ± 147
8.32	110 ± 8	130 ± 2	2519 ± 200	2654 ± 372

Seed Ni (mg kg^{-1})	Catalase Activity (- µmol H_2O_2 g^{-1} FW min^{-1})		Specific Catalase Activity (- µmol H_2O_2 mg^{-1} protein min^{-1})	
	- Ni	+ Ni	- Ni	+ Ni
0.04	285 ± 71	348 ± 143	5.5 ± 0.6	6.2 ± 2.4
0.11	325 ± 16	301 ± 44	5.8 ± 0.6	6.1 ± 1.1
0.62	329 ± 71	286 ± 12	6.4 ± 2.1	5.4 ± 0.6
8.32	364 ± 13	377 ± 16	8.3 ± 0.4	7.7 ± 0.8

Values are [a]means and [b]standard deviations of 3 replicates, each composed of 5 seeds.

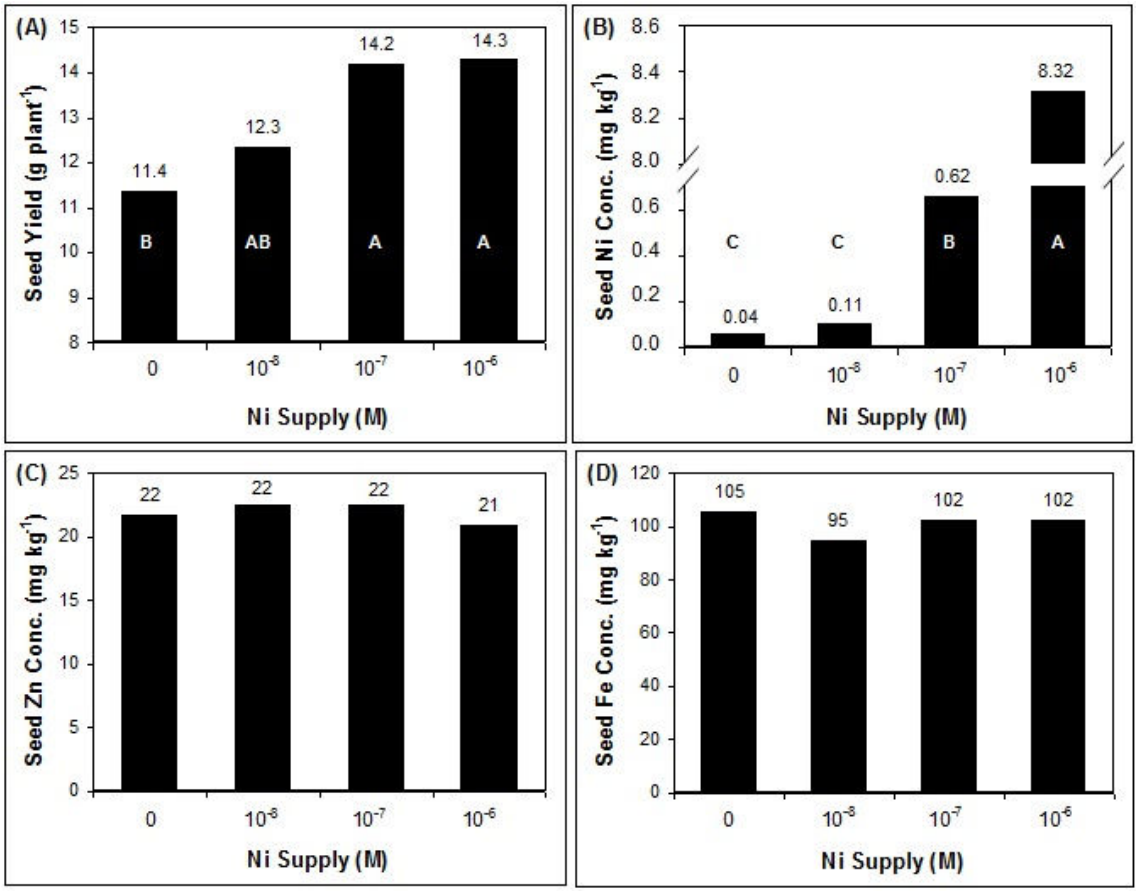

Fig. 1.1. Effect of solution Ni supply on seed **(A)** yield, **(B)** Ni concentration, **(C)** Zn concentration, and **(D)** Fe concentration of soybean (*Glycine max* cv. Nova) grown in hydroponics under growth chamber conditions. Values are means of 5 independent pot replicates, each containing 4 plants. Different uppercase letters indicate significant differences between means according to Fisher's protected LSD test.

In seeds with different Ni concentrations germinating with or without external Ni treatment, the urease activity was determined. Seed Ni concentration had a highly significant effect on urease activity (Table 1.1B). The urease activity was progressively enhanced by higher levels of seed Ni. Seeds with the highest Ni concentration exhibited urease activities over 100-times as high as seeds with the lowest Ni concentration (Table 1.2). External Ni treatment appeared to have a barely significant positive effect on urease activity, but it could certainly not substitute for seed Ni (Table 1.2). As the protein levels of the analyzed seed samples were not affected by Ni level (data not shown), the response of specific urease activity (per g protein) to seed Ni concentration was similar to that of urease activity per g sample (Table 1.1B, 1.2). In the same seeds, catalase activities were also measured, and it was demonstrated that there was no significant effect of seed or external Ni on catalase activity.

Table 1.3: Three-way ANOVA of the effects of seed Ni concentration, solution Ni supply and foliar urea treatment on reported traits of soybean (*Glycine max* cv. Nova) grown in hydroponics in the third experiment

Source of Variation	Shoot Biomass	Root Biomass	Number of Lost Leaves	Leaf Ni Conc.
Seed Ni Conc.[a] (A)	*n.s.*	*n.s.*	***	*n.s.*
Solution Ni Supply (B)	***	*n.s.*	***	***
Foliar Urea (C)	***	***	***	***
A x B	*n.s.*	*n.s.*	***	*n.s.*
A x C	*n.s.*	*n.s.*	***	*n.s.*
B x C	***	*n.s.*	***	***
A x B x C	*n.s.*	*n.s.*	***	*n.s.*

Source of Variation	Root Ni Conc.	Plant Ni Content	Ni Transloc.[b] Index	Youngest Part N Conc.
Seed Ni Conc. (A)	***	*	*n.s.*	*n.s.*
Solution Ni Supply (B)	***	***	*n.d.*	**
Foliar Urea (C)	***	*n.s.*	***	***
A x B	**	*n.s.*	*n.d.*	*
A x C	*n.s.*	*n.s.*	*n.s.*	***
B x C	***	*n.s.*	*n.d.*	*n.s.*
A x B x C	*n.s.*	*n.s.*	*n.d.*	*n.s.*

Source of Variation	Stem N Conc.	Root N Conc.	Plant N Content	Elongation Rate
Seed Ni Conc. (A)	*n.s.*	*n.s.*	*n.s.*	*
Solution Ni Supply (B)	***	*	***	**
Foliar Urea (C)	***	*	***	*
A x B	*n.s.*	*n.s.*	*n.s.*	*n.s.*
A x C	***	*n.s.*	*	*n.s.*
B x C	***	*	**	*n.s.*
A x B x C	*n.s.*	*n.s.*	*n.s.*	*n.s.*

[a] Concentration; [b] Translocation

n.s. Not significant; * $0.01 \leq$ F Pr. < 0.05; ** $0.001 \leq$ F Pr. < 0.01; *** F Pr. < 0.001

n.d. Not determined (Ni transloc. indices could be calculated only for Ni-fed plants.)

Out of the 4 seed groups produced, 3 groups of seeds with statistically significant Ni concentrations (Fig. 1.1B) were selected. Low-Ni (0.04 mg kg^{-1}), medium-Ni (0.62 mg kg^{-1}) and high-Ni (8.32 mg kg^{-1}) seeds were grown in solution culture with or without Ni addition, and one half of the plants were sprayed with urea to examine the role of seed Ni in the context of foliar urea application. Seed Ni concentrations did not affect germination rate or seedling development. Foliar urea reduced the shoot biomass of plants grown without Ni supply significantly, but it did not affect the shoot biomass when Ni was added to the nutrient solution (Table 1.3, Fig. 1.2A). There was no

statistically significant effect of seed Ni reserves on the shoot biomass (Table 1.3), although high seed Ni tended to alleviate the negative effect of foliar urea on the shoot biomass (Fig. 1.2A). Neither solution Ni nor seed Ni affected the root biomass; however, the urea-treated plants produced lower root biomass than the non-sprayed plants (Table 1.3, Fig. 1.2B).

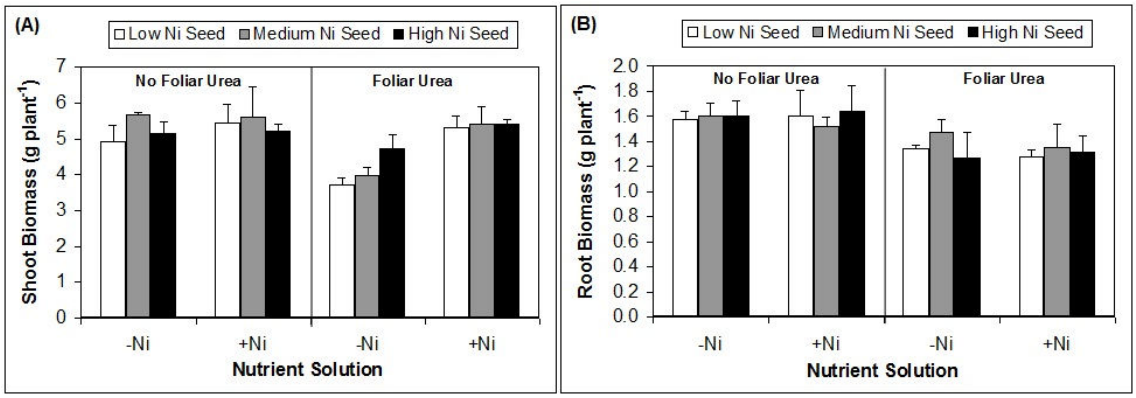

Fig. 1.2. Effects of seed Ni concentration, solution Ni supply and foliar urea application on **(A)** shoot and **(B)** root biomass of 32-day-old soybean (*Glycine max* cv. Nova) plants grown in hydroponics with marginal N supply under growth chamber conditions. Values are means of 3 independent pot replicates, each containing 3 plants. Bars represent standard deviations.

In the absence of foliar urea application, neither seed nor solution Ni had visible effects on soybean plants (Fig. 1.3). Toxic effects of foliar urea included necrotic lesions concentrated near leaf margins and whole leaf chlorosis that was followed by leaf abscission. These symptoms were severe in plants raised from low-Ni seeds and not supplied with external Ni. Both seed Ni and solution Ni distinctly mitigated the urea toxicity symptoms. Irrespective of seed Ni, plants grown with external Ni supply exhibited only milder necrosis and no whole-leaf chlorosis associated with foliar urea supply. The protective effect of seed Ni reserves against foliar urea damage were marked when plants were not externally supplied with Ni. Urea-sprayed plants grown from high-Ni seeds without external Ni looked as vigorous as plants grown in Ni-containing nutrient solution. In the absence of external Ni supply, the number of leaves lost due to whole blade chlorosis followed by leaf abscission was significantly lowered by seed Ni (Table 1.3; Fig. 1.4).

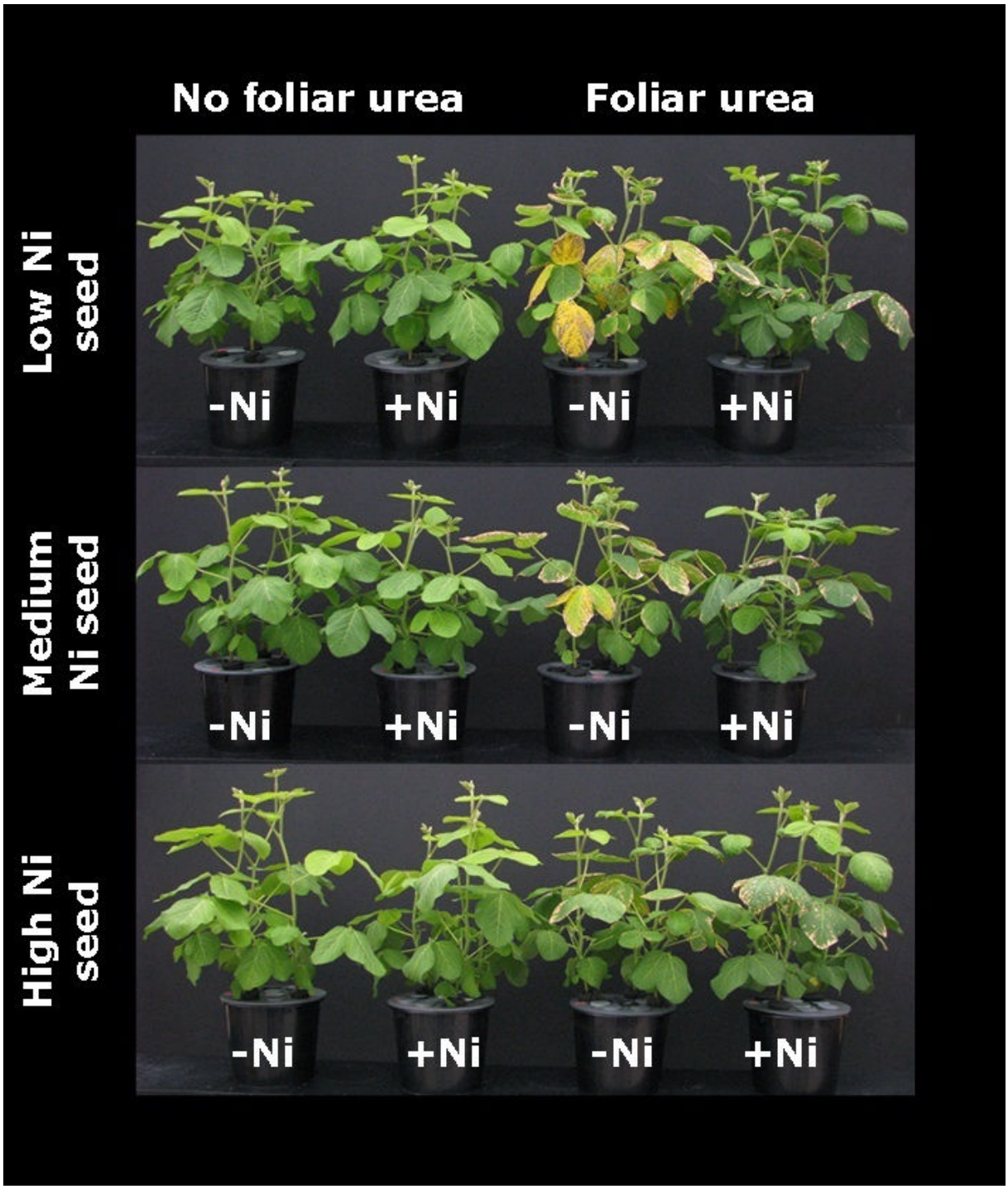

Fig. 1.3. 29-day-old soybean (*Glycine max* cv. Nova) plants grown from low-Ni, medium-Ni or high-Ni seeds with or without Ni ($2x10^{-7}$ M) in nutrient solution under growth chamber conditions. Half of the plants were sprayed with 2% (w/v) urea 25 days after sowing.

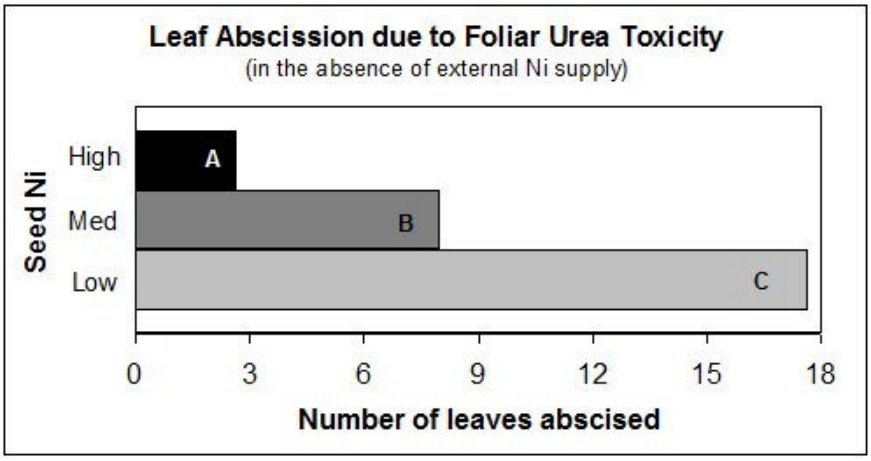

Fig. 1.4. Role of seed Ni concentration on the number of chlorotic leaves abscised from soybean (*Glycine max* cv. Nova) plants due to foliar urea toxicity (during 7 days after application) in the absence of solution Ni supply. Values are means of 3 independent pot replicates, each containing 3 plants. Different uppercase letters indicate significant differences between means according to Tukey's protected HSD test.

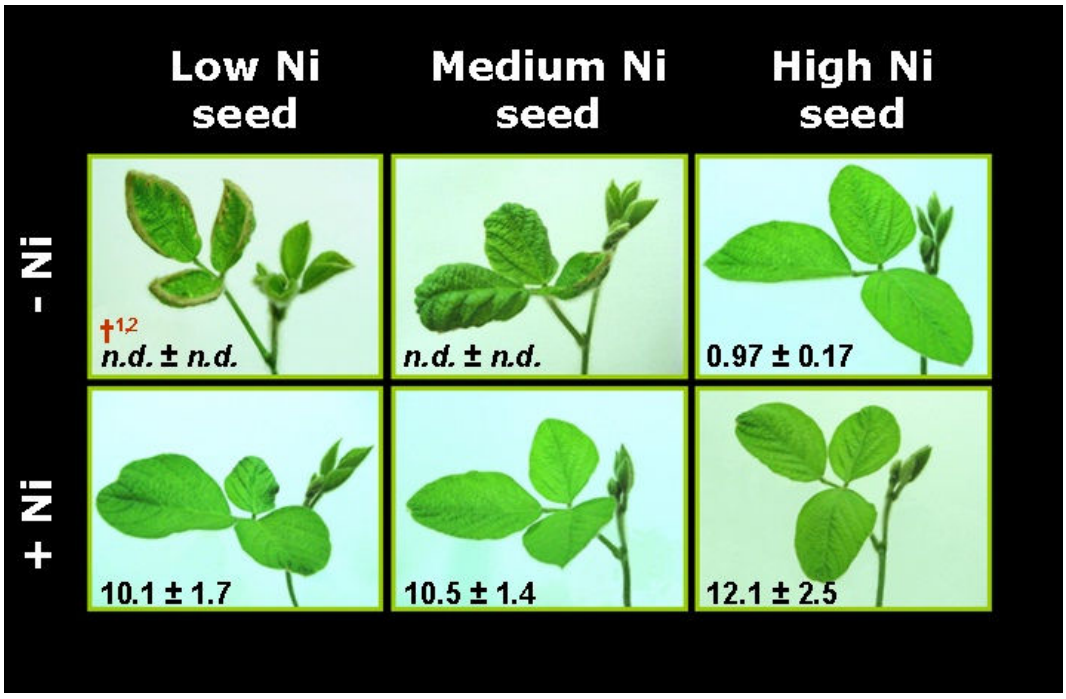

Fig. 1.5. Toxicity symptoms in youngest parts of 28-day-old urea-sprayed (2%) soybean (*Glycine max* cv. Nova) plants 3 days after foliar urea application. Plants were raised from seeds with low, medium or high Ni concentrations with or without solution Ni supply under growth chamber conditions.

†[1] Nickel concentrations of youngest parts. Values are means and standard deviations of 3 independent pot replicates, each containing 3 plants.

†[2] *n.d.* not detectable (< 1 µg kg^{-1})

Close-up photos and Ni concentrations of the youngest parts of urea-sprayed plants are shown in Fig. 1.5. In addition to necrosis along leaf margins, urea toxicity caused wrinkling and curling of young leaves, only when Ni levels in these tissues were not sufficient. Youngest parts of plants externally supplied with Ni had Ni concentrations over 10 mg kg^{-1}, and did not exhibit any visual urea toxicity symptoms. Even at non-detectable Ni concentrations, youngest parts of plants raised from medium-Ni seeds looked healthier than those of plants raised from low-Ni seeds. Remarkably, youngest parts of plants raised from high-Ni seeds and not externally supplied with Ni had only ca. 1 mg kg^{-1} Ni, which appeared to be sufficient to completely prevent visible symptoms of urea toxicity.

Table 1.4: Effects of seed Ni (SN), external Ni (2 x 10^{-7} M) supply (EN) and foliar urea (2% w/v) treatment (FU) on **(A)** leaf Ni concentration, **(B)** root Ni concentration, **(C)** plant Ni content, and **(D)** Ni translocation index of 32-day-old soybean (*Glycine max* cv. Nova) plants grown in nutrient solution

(A)	**Leaf Ni Concentration (µg kg^{-1})**			
Seed Ni	No Urea		Foliar Urea	
	- Ni	+ Ni	- Ni	+ Ni
Low	*n.d.*	866	*n.d.*	1297
Medium	60	818	*n.d.*	1211
High	87	938	158	1425

(B)	**Root Ni Concentration (µg kg^{-1})**			
Seed Ni	No Urea		Foliar Urea	
	- Ni	+ Ni	- Ni	+ Ni
Low	*n.d.*	3578	*n.d.*	3077
Medium	*n.d.*	4933	54	3102
High	182	5579	115	3871

(C)	**Plant Ni Content (µg $plant^{-1}$)**			
Seed Ni	No Urea		Foliar Urea	
	- Ni	+ Ni	- Ni	+ Ni
Low	*n.d.*	12.7	*n.d.*	13.0
Medium	*n.d.*	14.6	*n.d.*	13.1
High	0.8	15.0	0.9	14.8

(D)	**Ni Translocation Index (%)**			
Seed Ni	No Urea		Foliar Urea	
	- Ni	+ Ni	- Ni	+ Ni
Low	*n.d.*	55	*n.d.*	70
Medium	*n.d.*	49	*n.d.*	67
High	65	43	80	65

Values are means of 3 pot replicates, each containing 3 plants.

Leaf Ni Concentration:

$HSD_{0.05}$ (SN; EN; FU; SNxEN; SNxFU; ENxFU; SNxENxFU) = *n.s.*; 108; 108; *n.s.*; *n.s.*; 205; *n.s.*

Root Ni Concentration:

$HSD_{0.05}$ (SN; EN; FU; SNxEN; SNxFU; ENxFU; SNxENxFU) = 405; 274; 274; 709; *n.s.*; 517; *n.s.*

Plant Ni Content:

$HSD_{0.05}$ (SN; EN; FU; SNxEN; SNxFU; ENxFU; SNxENxFU) = 1.0; 0.7; *n.s.*; *n.s.*; *n.s.*; *n.s.*; *n.s.*

Ni Translocation Index:

$HSD_{0.05}$ (SN; EN; FU; SNxEN; SNxFU; ENxFU; SNxENxFU) = *n.s.*; *n.d.*; 7; *n.d.*; *n.s.*; *n.d.*; *n.d.*

n.d. Not determined; *n.s.* Not significant

In leaves and roots of plants grown without external Ni supply, the Ni concentration was not detectable when seed Ni was low (Table 1.4A, B). Low levels of Ni could be detected in leaves and roots of plants grown from high-Ni seeds, but even these levels were markedly lower than the levels detected in leaves and roots of plants externally supplied with Ni. Roots of high seed-Ni plants reached higher concentrations of Ni when Ni was available in the nutrient solution (Table 1.4B). In comparison to leaf Ni concentrations, the Ni concentrations measured in roots of Ni-fed plants were 3-5 times higher (Table 1.4A, B). However, it is noteworthy that the youngest part of the shoot was richer in Ni than both leaves and roots (Fig. 1.5; Table 1.4A, B). The Ni concentrations measured in youngest parts of Ni-fed and urea-sprayed plants were at least 2.5 times as high as the root Ni concentrations of the same group of plants. Foliar urea application elevated the leaf Ni concentrations of Ni-fed plants by 50%, whereas it reduced the root Ni concentrations of the same plants by 30% (Table 1.3; Table 1.4A, B). The total Ni content of plants grown from high-Ni seeds without external Ni supply could be fully explained by the Ni content of high-Ni seeds (Table 1.4C, data not shown). Seed Ni had a significant but slight positive effect on the total Ni content of Ni-fed plants (Table 1.3; Table 1.4C). The total Ni content of plants was not affected by foliar urea treatment (Table 1.3; Table 1.4C). Foliar application of urea enhanced the Ni translocation index on average by 35% (Table 1.3, Table 1.4D), while its effect was less pronounced in the case of other micronutrients such as Fe (20%) and Zn (12%).

Both seed and solution Ni improved the N concentrations in youngest parts of non-sprayed plants by up to 30% (Table 1.3; Table 1.5A). Spraying the plants with urea increased the N concentrations in youngest parts significantly. In terms of the N concentrations of youngest parts, the positive effect of solution Ni was still observed in urea-sprayed plants, but the effect of seed Ni disappeared. In contrast to the N concentration, the concentrations of other macronutrients (e.g. K, P, S) were not affected by seed or solution Ni in youngest parts (data not shown). In the stem, foliar urea application raised the N concentrations by 40% only when Ni was available in the nutrient solution (Table 1.3; Table 1.5B). A similar but weaker positive effect of solution Ni was also observed on the root N concentrations of urea-sprayed plants (Table 1.3; Table 1.5C). The total plant N content was significantly (30%) improved by foliar urea when plants were externally supplied with Ni (Table 1.3; Table 1.5D). In the absence of external Ni supply, a similar improvement of the total plant N content

(excluding abscised leaves) by foliar urea was only observed in plants grown from high-Ni seeds.

Table 1.5: Effects of seed Ni (SN), external Ni (2 x 10^{-7} M) supply (EN) and foliar urea (2% w/v) treatment (FU) on **(A)** youngest part N concentration, **(B)** stem N concentration, **(C)** root N concentration, and **(D)** plant N content of 32-day-old soybean (*Glycine max* cv. Nova) plants grown in nutrient solution

(A)	**Youngest Part N Concentration (%)**			
Seed Ni	No Foliar Urea		Foliar Urea	
	- Ni	+ Ni	- Ni	+ Ni
Low	2.14	2.49	2.97	3.34
Medium	2.58	2.71	3.20	2.95
High	2.61	2.81	2.67	2.99

(B)	**Stem N Concentration (%)**			
Seed Ni	No Foliar Urea		Foliar Urea	
	- Ni	+ Ni	- Ni	+ Ni
Low	0.75	0.70	0.67	0.98
Medium	0.72	0.71	0.67	0.95
High	0.58	0.69	0.87	0.99

(C)	**Root N Concentration (%)**			
Seed Ni	No Foliar Urea		Foliar Urea	
	- Ni	+ Ni	- Ni	+ Ni
Low	2.08	1.92	1.86	2.12
Medium	2.03	2.06	1.95	2.30
High	1.89	1.95	2.12	2.28

(D)	**Plant N Content (mg plant^{-1})**			
Seed Ni	No Foliar Urea		Foliar Urea	
	- Ni	+ Ni	- Ni	+ Ni
Low	102	105	99	137
Medium	112	111	115	138
High	95	96	125	142

Values are means of 3 pot replicates, each containing 3 plants.

Youngest Part N Concentration:

$HSD_{0.05}$ (SN; EN; FU; SNxEN; SNxFU; ENxFU; SNxENxFU) = *n.s.*; 0.12; 0.12; 0.32; 0.32; *n.s.*; *n.s.*

Stem N Concentration:

$HSD_{0.05}$ (SN; EN; FU; SNxEN; SNxFU; ENxFU; SNxENxFU) = *n.s.*; 0.04; 0.04; *n.s.*; 0.11; 0.08; *n.s.*

Root N Concentration:

$HSD_{0.05}$ (SN; EN; FU; SNxEN; SNxFU; ENxFU; SNxENxFU) = *n.s.*; 0.11; 0.11; *n.s.*; *n.s.*; 0.20; *n.s.*

Plant N Content:

$HSD_{0.05}$ (SN; EN; FU; SNxEN; SNxFU; ENxFU; SNxENxFU) = *n.s.*; 7; 7; *n.s.*; 19; 13; *n.s.*

n.d. Not determined; *n.s.* Not significant

As a measure of plant growth rate, the elongation rate of the shoot was determined. In general, the shoot elongation rate responded positively to foliar urea treatment, seed Ni concentration and Ni availability in the nutrient solution (Fig. 1.6). Analysis of variance revealed that all these responses were significant (Table 1.3). Under all conditions, plants raised from low-Ni seeds exhibited the lowest elongation rates (Fig. 1.6). Urea-sprayed plants grown with external Ni supply had the highest average elongation rate, whereas non-sprayed plants without external Ni supply had the lowest average. The positive effects of solution Ni on the shoot elongation were

especially pronounced when seed Ni was low. Moreover, these effects of solution Ni were not only observed in urea-sprayed plants but also in non-sprayed ones.

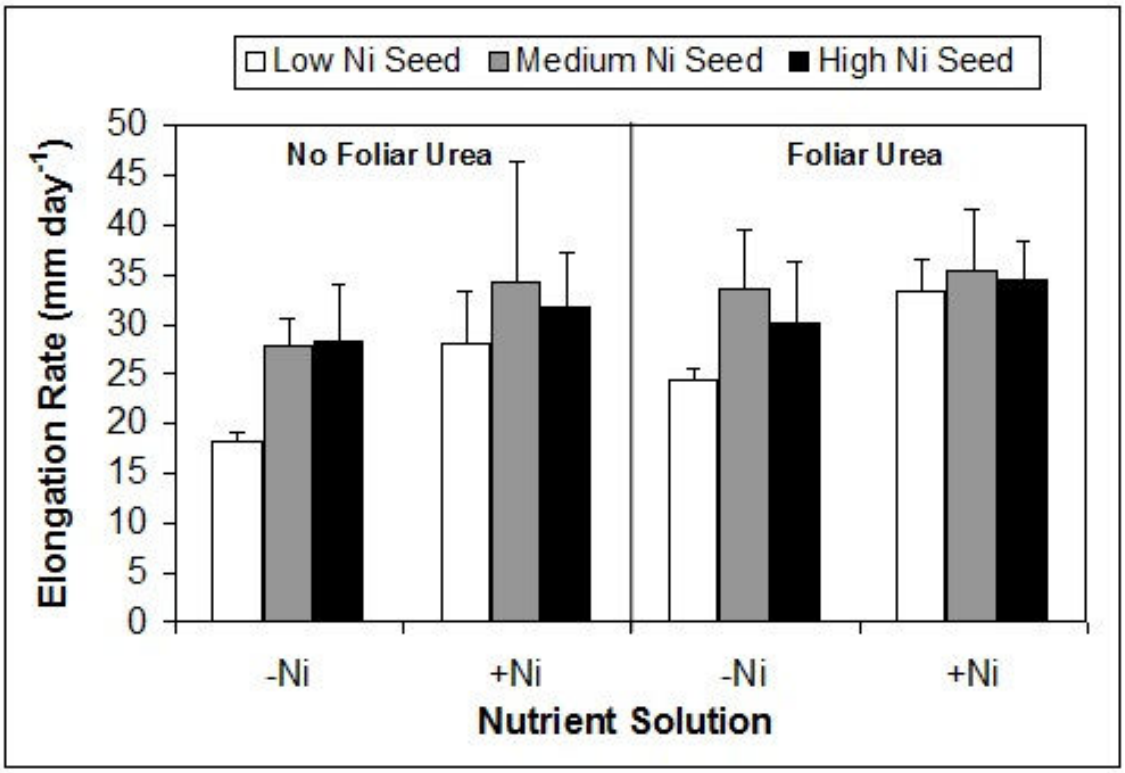

Fig. 1.6. Effects of seed Ni concentration, solution Ni supply and foliar urea application on shoot elongation rate of soybean (*Glycine max* cv. Nova) plants grown in hydroponics with marginal N supply under growth chamber conditions. Values are means of 3 independent pot replicates, each containing 3 plants. Bars represent standard deviations.

SPAD readings taken on the 4th oldest trifoliate leaf 3, 5 and 7 days after foliar urea application (DAA) were not significantly affected by seed Ni level (data not shown). Therefore, SPAD measurements were averaged over different seed Ni levels and plotted in Fig. 1.7. Foliar urea treatment, solution Ni supply and DAA had significant positive effects on SPAD value. On all three days of measurement, plants grown without external Ni supply and not sprayed with urea showed the lowest SPAD readings, whereas urea-sprayed plants grown in Ni-containing solution had the highest scores. Solution Ni supply tended to increase the SPAD scores even in the absence of foliar urea application. The greening effect of foliar urea treatment was gradual when Ni was not supplied externally. On the contrary, Ni-fed plants sprayed with urea reached the final SPAD level just within 3 DAA.

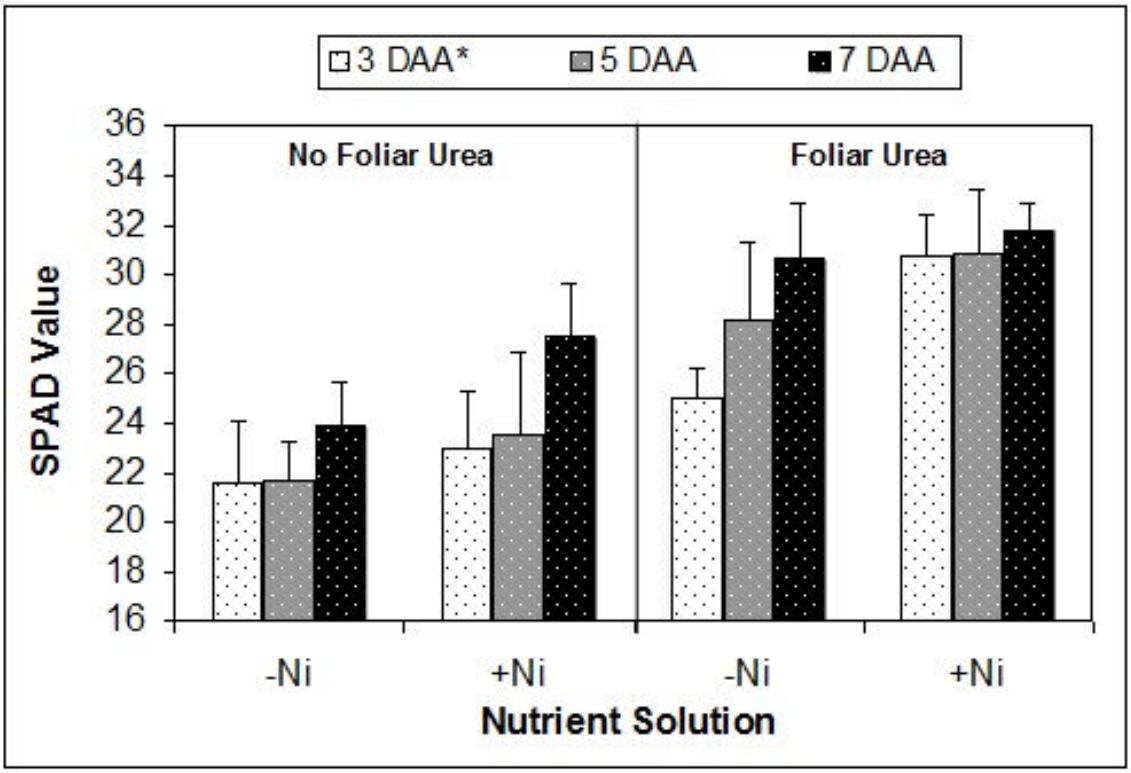

Fig. 1.7. Effects of solution Ni supply, foliar urea application and the time after foliar application on SPAD values of fourth trifoliate leaves of soybean (*Glycine max* cv. Nova) plants grown in hydroponics with marginal N supply under growth chamber conditions. Values are means of 9 independent replicates. (As seed Ni concentration did not have a significant effect on the measured SPAD values, readings were averaged over different seed Ni levels.) Bars represent standard deviations.

* DAA: days after foliar application

1.4. Discussion

Soybean plants grown from seeds containing 5.35 mg kg^{-1} Ni showed a significant yield increase up to 25% in response to external Ni supply, in spite of the fact that these plants were neither dependent on N_2-fixation nor treated with urea (Table 1.1A; Fig. 1.1A). To our knowledge, a statistically significant seed yield response to Ni supply in nutrient solution is reported for the first time for a crop plant in the present study. Previously, Eskew *et al*. (1984) and Brown *et al*. (1987a) also observed a positive trend for seed yield in response to Ni availability in soybean and barley, respectively; but differences between treatments were not statistically significant. In the present study, Ni treatment affected not the average seed size but the seed number per plant. This observation suggests that the yield response to Ni was most probably related to seed set, not to seed filling. Negative impacts of Ni deficiency on seed development were also reported by Brown *et al*. (1987a) in barley.

Seed Ni concentrations were significantly enhanced by more than two orders of magnitude in response to external Ni supply (Table 1.1A; Fig. 1.1B). Only the seeds produced by plants supplied with the highest Ni level had higher Ni concentrations (8.32 mg kg^{-1}) than the soybean seeds used in this experiment (5.35 mg kg^{-1}). Flyvholm *et al*. (1984) also reported an average Ni concentration of 5.2 mg kg^{-1} for soybeans consumed in human diet. Nickel is an essential trace element for not only plants but also animals (Nielsen 1984; Spears 1984), although physiological functions of Ni in animal systems are still debated. High levels of Ni supplied in this experiment did not have any effect on the Zn, Fe (Table 1.1A; Fig. 1.1C-D), N and protein concentrations (data not shown) of the seeds.

When the ureolytic activities of germinating seeds with different Ni concentrations were measured, a logarithmic positive correlation was observed between the urease activities and Ni concentrations (Table 1.1B, 1.2). These results, which are in agreement with the urease activities reported by Winkler *et al*. (1983) for soybean seeds with three different Ni concentrations, also show how the need of soybean seeds for Ni is saturated at higher Ni levels, at least with regard to urease activity. It can be assumed that both the ubiquitous and embryo-specific urease activities responded to seed Ni. As a sufficiently high ubiquitous urease activity is required for the re-assimilation of N in

urea produced during germination from Arg (Micallef and Shelp 1989; Stebbins *et al*. 1991), a low seed Ni content associated with a low seed ureolytic activity may imply an impaired utilization of seed N reserves. On the other hand, a great share of the measured ureolytic activity is probably attributable to the activity of embryo-specific urease, implicated in defense against insects and pathogens (Polacco and Winkler 1984; Stebbins *et al*. 1991; Follmer *et al*. 2004; Carlini and Polacco 2008). The lack of a significant response of catalase activity to seed Ni shows the specificity of the Ni effect on urease activity (Table 1.1B, 1.2).

It was also obvious that availability of Ni in the germination environment could not compensate for the lack of sufficient Ni in seeds (Table 1.2). Partial recovery of urease activity in low-Ni seeds by imbibition in Ni-containing solution was previously reported by Winkler *et al*. (1983), although the Ni concentration used in that study was about two orders of magnitude higher then DTPA-extractable Ni concentrations reported for Ni-sufficient soils (Rahmatullah *et al*. 2001; Penney 2004) and can only represent Ni-toxic soils (L'Huillier and Edighoffer 1996). These facts together with the results presented here indicate that availability of Ni in the environment at non-toxic concentrations cannot substitute for sufficient seed Ni during germination. The apo-protein concentration of embryo-specific urease in mature seeds is not significantly reduced under Ni deficiency (Winkler *et al*. 1983). However, mature seeds may contain lower concentrations of accessory proteins than developing embryos, as reported for Eu3 by Freyermuth *et al*. (2000). This may explain why external Ni can only to a limited extent increase the ureolytic activity of germinating Ni-deficient seeds.

For improving the N nutritional status and enhancing the seed protein content in crop plants, foliar application of urea is a commonly applied method, which may be preferable to soil N fertilization, especially when root activity is low (Gooding and Davies 1992; Nicoulaud and Bloom 1996; Varga and Svecnjak 2006; Kutman *et al*. 2010). However, leaf damage due to foliar urea treatments has been frequently reported (Krogmeier *et al*. 1991; Gooding and Davies 1992; Peltonen 1993; Khemira *et al*. 2000). For soybean, the reason behind leaf burn caused by foliar urea treatment (2-4% w/v) was shown to be the accumulation of not NH_4^+ but urea itself (Krogmeier *et al*. 1991). In the third experiment of this study, plants expected to have low urease activity due to low Ni status did not only exhibit typical leaf burn symptoms but also whole leaf chlorosis followed by abscission (Fig. 1.3; Fig. 1.4), when they were foliarly treated

with 2% (w/v) urea. Furthermore, these plants had significantly lower shoot biomass than the non-sprayed plants seven days after foliar urea treatment (Fig. 1.2A). It was interesting to notice that high seed Ni was almost as effective as Ni availability in the nutrient solution in the alleviation of these symptoms (Fig. 1.2A; Fig. 1.3; Fig. 1.4). SPAD measurements revealed that the N nutritional status of plants was improved by foliar urea application (Fig. 1.7). However, in plants not supplied with Ni, this correction took 4 more days than in Ni-fed plants, indicating significantly quicker assimilation of urea N by Ni-fed plants.

Nickel has high phloem mobility in wheat and soybean (Cataldo *et al*. 1978; Page and Feller 2005; Riesen and Feller 2005). Notably, the youngest part of the shoot appeared to be a very strong sink for Ni in this study (Fig. 1.5), indicating a high requirement for Ni in metabolically highly active, meristematic tissues. For several crops, including cowpea (Walker *et al*. 1985), barley (Brown *et al*. 1990) and pecan (Bai *et al*. 2006), Ni deficiency was shown to disturb amino acid metabolism. It can be speculated that the high requirement of the youngest part for Ni might be related to the high protein metabolism in these tissues. Zinc, known to be required for protein synthesis, is also preferentially allocated to meristematic tissues, and its deficiency is associated with impaired protein synthesis (Kitagishi and Obata 1986; Kitagishi *et al*. 1987; Cakmak *et al*. 1989). Even plants grown from high-Ni seeds without external Ni supply could accumulate nearly 1 mg kg^{-1} Ni in the youngest part, which was sufficient to prevent visual symptoms of foliar urea toxicity (Fig. 1.5).

Foliar urea application affected the distribution of Ni within the plant without causing any effect on the plant Ni content: More Ni was transported from the root to the shoot and allocated to leaves and growing tissues (Table 1.3; Table 1.4). In Ni-sufficient plants, urea application is known to significantly increase the concentrations of free amino acids, especially glutamine and asparagine, which are involved in N storage and transport (Gerendas *et al*. 1998b; Witte 2011). Amino acids and organic acids are implicated in long-distance transport of transition metals (Gerendas *et al*. 1999; Grusak *et al*. 1999, Cakmak *et al.* 2010). Kerkeb and Krämer (2003) showed that histidine is required for xylem loading of Ni. The involvement of histidine and the non-proteinogenic amino acid nicotianamine in Ni homeostasis was also reported by Callahan *et al*. (2007). Foliar urea application may increase the abundance of amino acids facilitating the root-to-shoot translocation of Ni. Why the root-to-shoot

translocation of Ni is enhanced by foliar urea more than the translocation of other micronutrients like Fe and Zn remains to be elucidated.

Fully expanded leaves are main sites of absorption of foliar-applied urea, whereas the roots, stem and youngest part of the shoot are principally dependent on source leaves in this respect. In plants treated with foliar urea, the N concentrations but not the concentrations of other macronutrients (K, P or S) in these sink tissues were enhanced by improved Ni status (Table 1.3; Table 1.5A-C). The transport of urea-N to sink tissues in the form of amino acids is only possible after the degradation of urea by urease and the re-assimilation of NH_3-N into amino acids (Youssefi *et al*. 2000; Witte 2011). This explains the critical role of Ni in the distribution of foliar-applied urea-N. However, the Ni status is not expected to affect the absorption of foliar-applied urea. The apparent lack of a positive effect of foliar urea application on the total N content of Ni-deficient plants is due to the abscission of severely-damaged leaves (Table 1.5D; Fig. 1.4). An efficient and quick (Fig. 1.7) utilization of foliar-applied urea-N was only possible when sufficient Ni, supplied by seed reserves and/or externally, was available to the plant.

Plants not sprayed with urea also benefited from Ni nutrition, possibly owing to improved utilization of internal N. The N concentrations measured in the youngest parts of non-sprayed plants were enhanced by up to 30% by both seed and external Ni (Table 1.3; Table 1.5A), indicating better N remobilization from older leaves. Degradation of proteins in senescing source tissues to remobilize N and thus sustain growth in sinks results in urea production (Gerendas *et al*. 1999; Wang *et al*. 2008). Nickel is expected to play a role in N remobilization because of its functions in urea and amino acid metabolism. Higher SPAD values of relatively younger expanded leaves (Fig. 1.7) and higher elongation rates (Fig. 1.6) measured in non-sprayed plants with higher Ni levels also support the idea that Ni improves internal N utilization efficiency. These improved growth parameters could also lead to a positive response at the level of vegetative biomass, which was, however, not the case at the time of harvest (Fig. 1.2). Possibly, such an effect on biomass could be observed if the plants were harvested later; but the experiment could not be continued for a longer time because the urea-treated low-Ni plants suffered severe damage and lost many of their fully expanded leaves (Fig. 1.4). The role of Ni nutritional status in internal N utilization of soybean represents an important research topic that needs to be studied in future experiments.

1.5. Conclusions

Supporting the evidence for the essentiality of Ni for higher plants, yield of soybean plants was reduced by Ni deficiency in the absence of urea nutrition. Lower Ni availability in the growth medium also resulted in the production of seeds with extremely low Ni concentrations and urease activities. With respect to urease activity, external Ni supply could not substitute for seed Ni reserves during germination, which might impair the utilization of seed N reserves. On the other hand, at later stages of development, the positive effects of seed and externally supplied Ni on soybean plants were additive. These effects included prevention of visible symptoms of foliar urea toxicity (leaf burn and whole-leaf chlorosis followed by abscission) and quicker and more efficient utilization of foliar urea as well as better N mobilization within shoot tissue in the absence of foliar urea. All these results together with the proven and proposed roles of Ni in urea and amino acid metabolism indicate that Ni has a high potential to improve the utilization of N fertilizers by soybean and possibly other crops, which represents an important future research topic.

CHAPTER 2

EFFECTS OF SEED NICKEL RESERVES AND EXTERNALLY SUPPLIED NICKEL ON THE GROWTH, NITROGEN METABOLITES AND NITROGEN USE EFFICIENCY OF UREA- OR NITRATE-FED SOYBEAN

2.1. Introduction

Chapter 1 showed the critical role of Ni nutrition in the utilization of foliar-applied-urea and alleviation of the leaf burn symptoms associated with foliar urea toxicity in soybean plants grown with nitrate as the N source in the nutrient solution. In this respect, seed Ni reserves could effectively compensate for the lack of adequate Ni availability in the growth medium. However, the impact of seed Ni reserves on the growth and N metabolism of soybean was not investigated under conditions where urea (or nitrate) was supplied via the growth medium as the sole N source, which is the main focus of this chapter.

Soybean meets on average 50-60% of its total N requirement via biological N fixation, although this percentage varies a lot depending on the yield potential and soil conditions (Salvagiotti *et al.* 2008). Low soil pH is known to significantly impair N fixation, and soil acidity is a common problem in major soybean production regions of the world (Lin *et al.* 2012). More than 1 million tonnes of N fertilizer per annum are applied to soybean fields (IFA), and significant yield improvements are observed particularly if conditions are not favorable for nodulation (Salvagiotti *et al.* 2008). Starter applications of N can also enhance early plant vigor and thus the yield of soybean (Osborne and Riedell 2006). For soybean, which accumulates high levels of Ni

in its seeds known for their high urease activities, adequate Ni nutrition is critical not only for biological N fixation but probably also for efficient utilization of urea fertilizers (Chapter 1; Holland *et al.* 1987; Polacco *et al.* 2013). In several studies where plants were grown with urea as the only N source in the nutrient solution, impairment of urea metabolism under Ni-deficient conditions caused urea accumulation in plant tissues and visual toxicity symptoms such as brown discolorations and necrosis in tips and margins of older leaves (Gerendas and Sattelmacher 1997; Gerendas *et al.* 1998b; Tan *et al.* 2000)

The importance of seed micronutrient reserves for the seed quality and mineral nutrition of crops is well documented in the literature (Welch 1999; Cakmak 2008). Seed micronutrient concentrations below critical levels can reduce seed viability and germination efficiency as shown for boron (B)-deficient soybean (Rerkasem *et al.* 1997), manganese (Mn)-deficient lupin (Longnecker *et al.* 1996) and Ni-deficient barley (Brown *et al.* 1987a) seeds. Low seed concentrations of micronutrients can also impair seedling vigor, especially under stress conditions and disease pressure (Welch 1999; Cakmak 2008). Reportedly, sufficiently high seed concentrations of micronutrients can improve not only the early vegetative growth but also the micronutrient uptake and seed yield of crops, particularly in nutrient-poor soils (Grewal and Graham 1997; Rerkasem *et al.* 1997; Cakmak 2008). Moreover, externally supplied nutrients may not completely compensate for the lack of nutrients in seeds (Longnecker *et al.* 1996). In Chapter 1, it was also reported that external Ni could not replace seed Ni in terms of urease activity during germination.

In this study, for the first time in the literature, the impact of seed Ni on the growth and N nutritional status of soybean was investigated in the absence or presence of Ni in the growth medium containing either nitrate or urea as the sole N source. The chlorophyll concentrations of old and young leaves and the depletion of N sources from the nutrient solution were followed. In different shoot organs and roots, the concentrations of Ni, total N and N metabolites including protein, total free amino acids, nitrate, urea and ammonium were measured. The effect of Ni on the NUE and its components was evaluated in urea- or nitrate-fed soybean plants.

2.2. Materials and Methods

2.2.1. Experimental design

This study was conducted with soybean (*Glycine max* cv. Nova) plants grown from either low-Ni (0.05 mg kg^{-1} Ni) or high-Ni (10 mg kg^{-1} Ni) seeds. The N concentration of both seeds was 4.7%. (How these seeds were produced is described in detail in Chapter 1.) Plants were grown hydroponically in 3 L pots under growth chamber conditions as described in "General Materials and Methods".

The experiment had a factorial design with 3 pot replicates each containing 9 plants. There were three variables: seed Ni, external Ni supply and N form. Plants were raised from either low-Ni (0.05 μg g^{-1} Ni) or high-Ni (10 μg g^{-1} Ni) seeds and grown either with or without 0.2 μM Ni as $NiCl_2.6H_2O$. For the first 8 days in the nutrient solution, all the plants were fed with 2 mM N in the form of $Ca(NO_3)_2.4H_2O$. During the last 9 days of the growth period, half of the plants received 2 mM N in the form of urea as the sole N source, while the remaining half continued to grow with the same N concentration in the form of $Ca(NO_3)_2.4H_2O$. Throughout this chapter, the plants are referred to as urea-fed and nitrate-fed plants, depending on the sole N source in the latter half of the growth period. The urea-fed plants were supplied with additional 1 mM Ca in the form of $CaSO_4.2H_2O$ in order to keep the total Ca concentration at 2 mM as in nitrate-containing pots. Fresh nutrient solution had a pH of 6.0. The pH in each pot was checked daily and adjusted to 6.0 by using 1 M KOH if a pH decrease by at least 0.5 was observed.

2.2.2. SPAD Measurements

Chlorophyll concentrations of primary and 2nd oldest trifoliate leaves were measured by using a chlorophyll meter (SPAD-502, Konica Minolta, Japan). The reported values are means of 6-replicate measurements (2 plant replicates per pot x 3 independent pot replicates). For primary leaves, the chlorophyll measurements were started when half of the plants were transferred to urea-containing solution and conducted every day or every other day till the end of the experiment. The SPAD values

of the 2nd oldest trifoliate leaves were started when the leaves were sufficiently expanded for SPAD measurement and followed for five days until harvest.

2.2.3. Nutrient Solution Sampling

The nutrient solution was refreshed for the last time two days before the harvest. Samples were taken from the nutrient solutions of all pots immediately after refreshment, 24 h later and finally 48 h later in order to follow the changes in the nutrient solution composition. They were stored at -80°C. The concentrations of nitrate, urea, ammonium and P were determined as described below.

2.2.4. Harvest

The experiment was terminated 22 days after sowing. Plant samples were split into 4 pieces:

i. second and third oldest trifoliate leaves + shoot tip (referred to as young leaves)
ii. primary leaves + oldest trifoliate leaves (referred to as old leaves)
iii. stem
iv. root

During the experiment, the abscised cotyledons had also been collected. All shoot samples were washed with deionized water. The root samples were washed with 1 mM $CaCl_2$, then 1 mM EDTA and finally deionized water. All these plant samples were dried at 70°C for 2 days, and weighed. By using an agate vibrating cup mill (Pulverisette 9; Fritsch GmbH; Germany), the dried samples were ground to fine powders and used for Ni, P and total N analyses as described below.

For the tests conducted on fresh tissues, approximately 1 g samples were taken from the:

i. second oldest trifoliate leaves
ii. primary leaves
iii. young roots

The fresh samples were immediately weighed for fresh biomass determination, dipped in liquid N and stored at -80°C. These samples were used for protein, free amino acid, nitrate, urea and ammonium measurements as described below.

2.2.5. Colorimetric Measurements

All colorimetric measurements were conducted by using a UV-visible spectrophotometer (Cary 300 Bio, Varian, Australia). Protein measurements were conducted as described in "General Materials and Methods".

2.2.5.1. Total free amino acids

The free amino acid concentrations in the samples were determined by using a modified version of the ninhydrin-based assay described by Sadasivam and Manickam (2005). 0.2 M citrate buffer was prepared from citric acid, and its pH was adjusted to 5.0 with 10 M NaOH. 0.5% (w/v) ninhydrin was dissolved in ethanol. Equal volumes of the ninhydrin solution and the citrate buffer were mixed. Stannous chloride ($SnCl_2.2H_2O$) was dissolved in the mixture at a concentration of 0.05% (w/v). The reagent was immediately used for the assay. Amino acid standards were prepared from glutamic acid which was dissolved in K-P buffer. 1 ml of the ninhydrin reagent was added to 0.1 ml of crude extract (or standard), and the reaction mixture was incubated in a water bath at 90°C for 10 min. The absorbance was read at 570 nm.

2.2.5.2. Nitrate

The nitrate analysis was performed after Cataldo *et al.* (1975). 5% (w/v) salicylic acid was prepared in concentrated sulfuric acid. This reagent was mixed with samples (or standards) in a volume ratio of 4:1. For nitrate standards, KNO_3 was dissolved in K-P buffer. After incubating the reaction mixtures in the dark at room temperature for 20 min, 2 M NaOH was added to the reaction mixture in a volume ratio of 19:1. When the samples cooled down to room temperature, the absorbance at 410 nm was measured.

2.2.5.3. Urea

The urea concentrations in the samples were determined by using a slightly modified version of the assay described by Merigout *et al.* (2008a, b), which was based

on the original method by Kyllingsbæk (1975). The assay reagent consisted of the following 2 reagents and deionized water in a volume ratio of 1:1:2. Reagent 1 was prepared by dissolving 14 mM diacetylmonoxime (2,3-butanedione 3-monoxime) and 3.5 mM thiosemicarbazide in deionized water. Reagent 2 contained 0.06% (v/v) ferric chloride solution (74 mM $FeCl_3.6H_2O$ in 9% ortho-phosphoric acid) and 20% (v/v) sulfuric acid in water. 5 ml of the assay reagent was added to 0.1 ml of sample (or urea standard). The reaction mixture was incubated in a water bath at 90°C for 30 min and then put on ice. When the samples cooled down to room temperature, the absorbance at 540 nm was measured.

2.2.5.3. Ammonium

For ammonium assay, the improved Berthelot reaction method by Rhine *et al.* (1998) was slightly modified. Three reagents were prepared for this assay. Reagent 1 was 0.2 M trisodium citrate in deionized water containing 0.05 mM sodium nitroprusside dihydrate. As reagent 2, 0.15 M 2-phenylphenol (biphenyl-2-ol) was dissolved in ethanol. For preparing reagent 3, commercial household bleach was diluted by 1:10 (v/v) with deionized water, and 0.2 M NaOH was dissolved in it. For this analysis, ammonium standards were prepared by using $(NH_4)_2SO_4$. To 0.25 ml sample (or standard), 1 ml reagent 1, 1 ml reagent 2 and 0.5 ml reagent 3 were sequentially added, and the final volume was brought up to 5 ml with deionized water. The mixture was kept in the dark at room temperature for 2 h before the absorbance was measured at 660 nm.

2.3. Results

Urea supply significantly impaired the growth of soybean plants (Fig. 2.1; Table 2.1). With a loss in dry matter by 35%, the young leaves exhibited the most dramatic response to urea (Table 2.1). The stem growth was also significantly inhibited by urea supply, whereas the old leaves were totally unaffected. In total, the shoot biomass of the urea-fed group decreased by 20% when compared to that of the nitrate-fed group. However, the root growth was only slightly affected by the N form. Nevertheless, the reduction of the root biomass by 8% in response to urea was significant.

Fig. 2.1: Effect of seed Ni content and external Ni supply ($2x10^{-7}$ M) on 22-day-old soybean (*Glycine max* cv. Nova) plants grown in nutrient solution containing $2x10^{-3}$ M N in the form of either **(A)** nitrate or **(B)** urea under growth chamber conditions

Table 2.1: Dry weights of different organs and root-to-shoot ratios of 22-day-old soybean (*Glycine max* cv. Nova) plants grown hydroponically from low- or high-Ni seeds, with or without external Ni supply, and with $2x10^{-3}$ M N in the form of nitrate or urea

Ext. Ni (M)	Seed Ni (µg g^{-1})	Young Leaves DW (mg $plant^{-1}$)		Old Leaves DW (mg $plant^{-1}$)		Stem DW (mg $plant^{-1}$)	
		Nitrate	Urea	Nitrate	Urea	Nitrate	Urea
0	0.05	662 ± 35	302 ± 54	424 ± 25	380 ± 36	406 ± 3	263 ± 27
	10	624 ± 4	427 ± 31	414 ± 10	469 ± 24	400 ± 6	338 ± 21
2×10^{-7}	0.05	617 ± 26	426 ± 86	493 ± 48	494 ± 112	396 ± 16	316 ± 12
	10	596 ± 55	464 ± 31	425 ± 11	436 ± 35	365 ± 27	318 ± 28

Ext. Ni (M)	Seed Ni (µg g^{-1})	Root DW (mg $plant^{-1}$)		Shoot DW (mg $plant^{-1}$)		Root / Shoot (%)	
		Nitrate	Urea	Nitrate	Urea	Nitrate	Urea
0	0.05	528 ± 18	483 ± 56	1492 ± 8	945 ± 48	35 ± 1	51 ± 3
	10	479 ± 19	494 ± 14	1439 ± 12	1234 ± 66	33 ± 1	40 ± 3
2×10^{-7}	0.05	540 ± 69	441 ± 14	1506 ± 89	1236 ± 45	36 ± 3	36 ± 0
	10	455 ± 22	427 ± 19	1386 ± 85	1217 ± 90	33 ± 0	35 ± 1

Values are means and standard deviations of 3 pot replicates, each containing 7 plants.

$HSD_{0.05}$ values:	N Source (A); Ext. Ni (B); Seed Ni (C); AxB; AxC; BxC; AxBxC
Young Leaves DW:	40; *n.s.*; *n.s.*; 77; 77; *n.s.*; *n.s.*
Old Leaves DW:	*n.s.*; *n.s.*; *n.s.*; *n.s.*; *n.s.*; 80; *n.s.*
Stem DW:	17; *n.s.*; *n.s.*; 33; 33; 33; *n.s.*
Root DW:	30; 30; 30; *n.s.*; 58; *n.s.*; *n.s.*
Shoot DW:	55; 55; *n.s.*; 105; 105; 105; 179
Root / Shoot:	2; 2; 2; 3; *n.s.*; 3; 6

n.s. Not significant

Among nitrate-fed plants, no differences were observed according to seed Ni content or external Ni supply (Fig. 2.1A). In contrast, among urea-fed plants, the Ni-deprived ones appeared stunted, weak and chlorotic (Fig 2.1B). Neither seed nor externally supplied Ni had consistent effects on the shoot and root dry weights in the nitrate group (Table 2.1). But all shoot organs of the plants which were grown from low-Ni seeds and not supplied with external Ni had the lowest biomass within the urea group. The availability of Ni, either in the seed or nutrient solution, improved the dry weights of the shoot organs by up to 50%. Overall, Ni deprivation resulted in a 25% loss in total shoot biomass of urea-fed plants, whereas it did not have any negative impact on the root growth. Even the root dry weights of plants grown with urea tended to decrease upon external Ni supply. As a consequence of this contrasting effect of Ni on the root and shoot growth, the root-to-shoot ratio of Ni-poor plants supplied with urea was significantly higher than the root-to-shoot ratios of Ni-rich or nitrate-fed plants.

Close-up photographs of both primary and 2nd oldest trifoliate leaves show that the leaf area was smaller in urea-fed plants when compared to nitrate-fed plants (Fig. 2.2). In both leaf types, the smallest leaves were observed in Ni-deficient plants grown with urea. Over the latter half of the experimental period where either nitrate or urea was used as the sole nitrogen source, the SPAD values of primary leaves did not change considerably except in urea-fed plants grown from low-Ni seeds without external Ni supply (Fig. 2.3A, B). The chlorophyll content of the primary leaves of these plants started to decrease two days after the start of urea treatment and was about 20% lower than others (Fig. 2.3A, B) at the time of harvest in agreement with the chlorotic appearance of these leaves in Fig. 2.2A. The SPAD measurements of the 2nd oldest trifoliate leaves were started once they were sufficiently expanded. Till the end of the experiment, the chlorophyll contents of these leaves increased continuously in all treatment groups (Fig. 2.3C, D). However, in the urea-fed group, the rates of this increase were clearly dependent on Ni availability (Fig. 2.3D). At the end, the trifoliate leaves of Ni-deprived plants contained 35% less chlorophyll than their Ni-rich counterparts (Fig. 2.3D), as can also be seen in Fig. 2.2B. It is also noteworthy that external Ni supply was slightly more effective than using high-Ni seeds in increasing the chlorophyll levels of the trifoliate leaves of urea-fed plants.

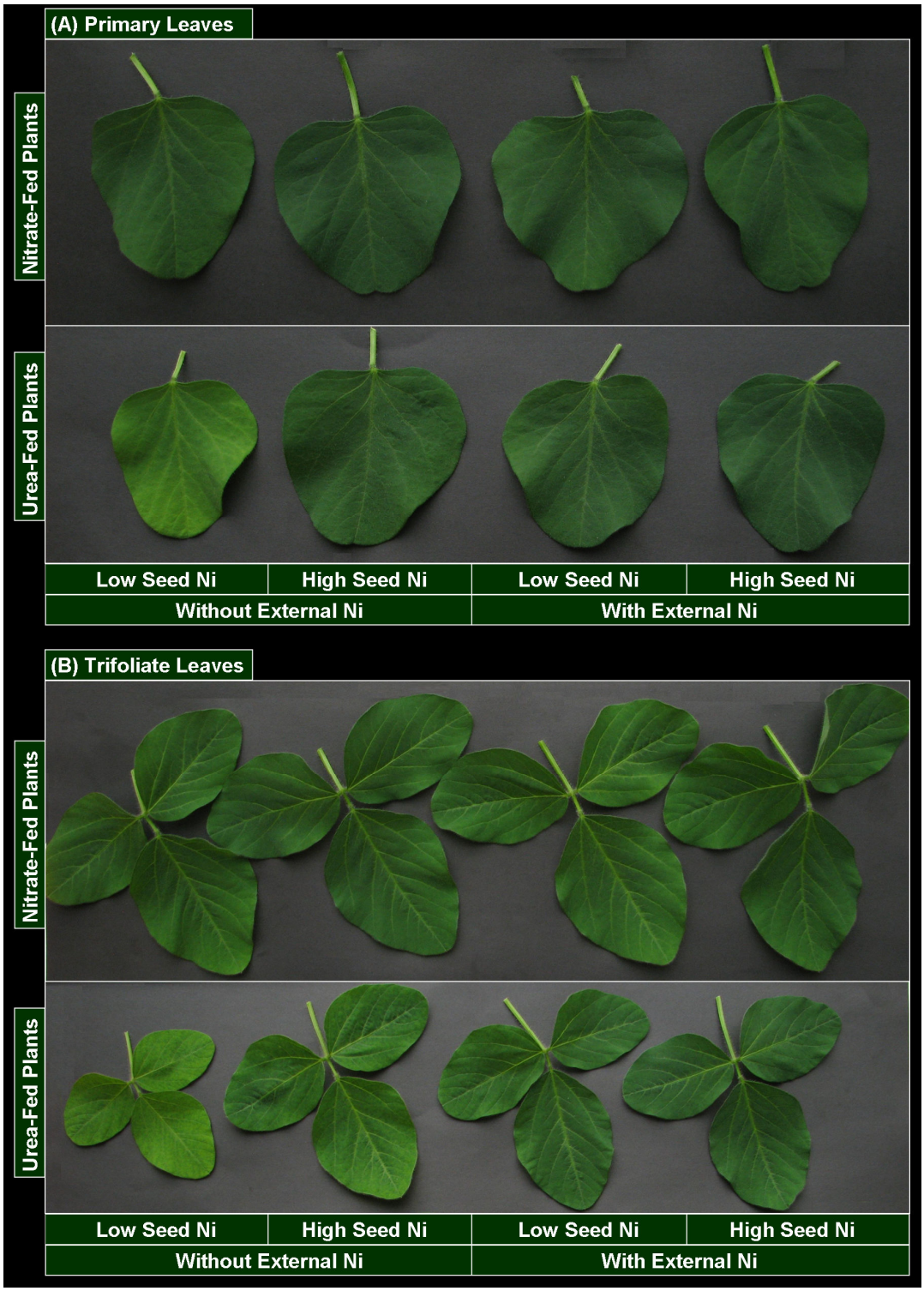

Fig. 2.2: Close-up photographs of **(A)** primary and **(B)** 2nd oldest trifoliate leaves of 22-day-old soybean (*Glycine max* cv. Nova) plants hydroponically grown from low-Ni or high-Ni seeds with or without external Ni ($2x10^{-7}$ M) and with $2x10^{-3}$ M N supply in the form of either nitrate or urea under growth chamber conditions

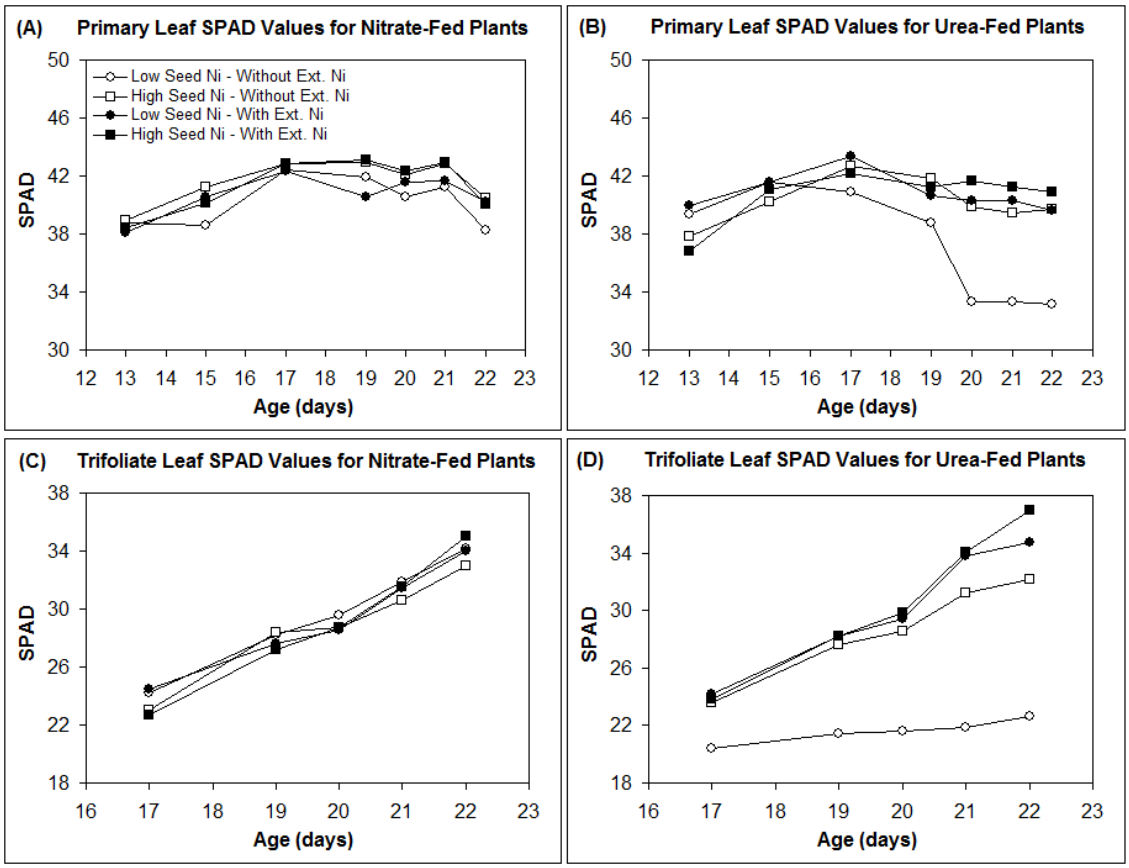

Fig. 2.3: Changes in SPAD values of primary **(A&B)** and 2nd oldest trifoliate **(C&D)** leaves of nitrate-fed **(A&C)** and urea-fed **(B&D)** soybean (*Glycine max* cv. Nova) plants depending on seed Ni content (circles for low seed Ni, squares for high seed Ni) and external Ni supply (open symbols for without external Ni, filled symbols for with external Ni)

Nickel was not detectable in any shoot organ or roots of plants grown from low-Ni seeds without external Ni; irrespective of the N source (Table 2.2). In the case of high seed Ni without external Ni, the young leaves had the highest Ni concentration among all plant organs, whereas the Ni concentration of the old leaves were below detection limits as in totally Ni-deprived plants. Markedly higher Ni concentrations were measured in all parts of plants grown with external Ni. The young leaves were again the Ni-richest shoot organs, having Ni concentrations 6-12 times higher than those of the old leaves. When Ni was supplied via nutrient solution, nitrate-fed plants had higher Ni concentrations than urea-fed plants in all plant organs except the old leaves. Accordingly, nitrate-fed plants accumulated significantly higher amounts of Ni in their shoots and roots than urea-fed plants. As expected, the shoot and root Ni contents of

plants grown with external Ni were dramatically higher than those of plants which depend on their seed reserves as their sole Ni source.

Table 2.2: Nickel concentrations of different organs and shoot and root Ni contents of 22-day-old soybean (*Glycine max* cv. Nova) plants grown hydroponically from low- or high-Ni seeds, with or without external Ni supply, and with $2x10^{-3}$ M N in the form of nitrate or urea

Ext. Ni (M)	Seed Ni (µg g^{-1})	Young Leaves Ni Conc. (µg g^{-1})		Old Leaves Ni Conc. (µg g^{-1})	
		Nitrate	Urea	Nitrate	Urea
0	0.05	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*
	10	1.03 ± 0.03	1.02 ± 0.05	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*
2 x 10^{-7}	0.05	6.76 ± 0.42	3.53 ± 0.61	0.53 ± 0.10	0.50 ± 0.43
	10	7.26 ± 0.31	4.40 ± 0.31	0.56 ± 0.05	0.66 ± 0.12

Ext. Ni (M)	Seed Ni (µg g^{-1})	Stem Ni Conc. (µg g^{-1})		Root Ni Conc. (µg g^{-1})	
		Nitrate	Urea	Nitrate	Urea
0	0.05	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*
	10	0.43 ± 0.07	0.26 ± 0.02	0.56 ± 0.11	0.83 ± 0.08
2 x 10^{-7}	0.05	2.83 ± 0.12	0.82 ± 0.09	6.52 ± 0.65	2.77 ± 0.27
	10	3.64 ± 0.10	1.08 ± 0.10	8.24 ± 0.48	3.35 ± 0.11

Ext. Ni (M)	Seed Ni (µg g^{-1})	Shoot Ni Content (µg $plant^{-1}$)		Root Ni Content (µg $plant^{-1}$)	
		Nitrate	Urea	Nitrate	Urea
0	0.05	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*	*n.d.* ± *n.d.*
	10	0.82 ± 0.02	0.52 ± 0.05	0.27 ± 0.04	0.41 ± 0.04
2 x 10^{-7}	0.05	7.93 ± 0.97	4.52 ± 2.91	3.50 ± 0.32	1.22 ± 0.16
	10	8.04 ± 0.36	5.22 ± 0.24	3.74 ± 0.23	1.43 ± 0.11

Values are means and standard deviations of 3 pot replicates, each containing 7 plants.

$HSD_{0.05}$ values:	N Source (A); Ext. Ni (B); Seed Ni (C); AxB; AxC; BxC; AxBxC
Young L. Ni Conc.:	0.26; 0.26; 0.26; 0.50; *n.s.*; *n.s.*; *n.s.*
Old L. Ni Conc.:	*n.s.*; 0.14; *n.s.*; *n.s.*; *n.s.*; *n.s.*; *n.s.*
Stem Ni Conc.:	0.07; 0.07; 0.07; 0.13; 0.13; 0.13; 0.22
Root Ni Conc.:	0.27; 0.27; 0.27; 0.51; *n.s.*; *n.s.*; 0.87
Shoot Ni Content:	0.95; 0.95; *n.s.*; 1.81; *n.s.*; *n.s.*; *n.s.*
Root Ni Content:	0.14; 0.14; 0.14; 0.26; *n.s.*; *n.s.*; *n.s.*

n.d. Not detectable *n.s.* Not significant

Table 2.3: Nitrogen concentrations of different organs and shoot and root N contents of 22-day-old soybean (*Glycine max* cv. Nova) plants grown hydroponically from low- or high-Ni seeds, with or without external Ni supply, and with $2x10^{-3}$ M N in the form of nitrate or urea

Ext. Ni (M)	Seed Ni (µg g^{-1})	Young Leaves N Conc. (%)		Old Leaves N Conc. (%)	
		Nitrate	Urea	Nitrate	Urea
0	0.05	3.8 ± 0.1	2.9 ± 0.1	3.9 ± 0.2	2.7 ± 0.0
	10	4.4 ± 0.0	3.2 ± 0.1	4.2 ± 0.1	2.9 ± 0.1
2×10^{-7}	0.05	4.0 ± 0.0	3.9 ± 0.1	3.5 ± 0.0	3.4 ± 0.0
	10	4.3 ± 0.2	3.9 ± 0.2	4.1 ± 0.2	3.5 ± 0.2

Ext. Ni (M)	Seed Ni (µg g^{-1})	Stem N Conc. (%)		Root N Conc. (%)	
		Nitrate	Urea	Nitrate	Urea
0	0.05	1.5 ± 0.0	1.0 ± 0.0	2.5 ± 0.1	2.1 ± 0.0
	10	1.8 ± 0.0	1.2 ± 0.1	2.7 ± 0.1	2.7 ± 0.1
2×10^{-7}	0.05	1.5 ± 0.1	1.3 ± 0.1	2.7 ± 0.1	2.6 ± 0.1
	10	1.7 ± 0.1	1.3 ± 0.1	2.8 ± 0.1	2.7 ± 0.1

Ext. Ni (M)	Seed Ni (µg g^{-1})	Shoot N Content (mg $plant^{-1}$)		Root N Content (mg $plant^{-1}$)	
		Nitrate	Urea	Nitrate	Urea
0	0.05	48 ± 1	22 ± 1	13 ± 1	10 ± 1
	10	52 ± 0	31 ± 1	13 ± 0	13 ± 0
2×10^{-7}	0.05	48 ± 3	37 ± 0	15 ± 1	12 ± 0
	10	49 ± 2	37 ± 1	13 ± 1	11 ± 0

Values are means and standard deviations of 3 pot replicates, each containing 7 plants.

$HSD_{0.05}$ values:	N Source (A); Ext. Ni (B); Seed Ni (C); AxB; AxC; BxC; AxBxC
Young L. N Conc.:	0.1; 0.1; 0.1; 0.2; 0.2; *n.s.*; *n.s.*
Old L. N Conc.:	0.1; 0.1; 0.1; 0.2; 0.2; *n.s.*; *n.s.*
Stem N Conc.:	0.1; 0.1; 0.1; 0.1; 0.1; *n.s.*; *n.s.*
Root N Conc.:	0.1; 0.1; 0.1; *n.s.*; *n.s.*; 0.1; 0.3
Shoot N Content:	1; 1; 1; 2; *n.s.*; 2; 4
Root N Content:	1; *n.s.*; *n.s.*; *n.s.*; 1; 1; *n.s.*

n.s. Not significant

In general, nitrate-fed plants had significantly higher N concentrations than urea-fed plants in all organs, but the extent of this difference was very much dependent on the Ni availability (Table 2.3). The lowest N concentrations for all plant organs were measured in Ni-deprived plants grown with urea. In the case of urea nutrition, external Ni supply improved the N concentrations of different plants organs by about 30% so that the N concentrations reached similar levels as in nitrate-fed plants. Although not as effectively as external Ni supply, high seed Ni also provided significant improvements in N concentrations measured in urea-fed plants, particularly in their stems and roots. When nitrate was the sole N source, Ni coming from the seed reserves or the nutrient solution did not have any significant effect on the shoot N content. Remarkably, Ni deprived plants could accumulate about 55% less N when supplied with urea instead of nitrate. This loss in shoot N accumulation due to urea nutrition was reduced to 40% by using high Ni seeds and further reduced to less than 25% by adding Ni to the nutrient solution. As far as the root N content is concerned, the effects of both N supply form and Ni availability were less pronounced than in the case of the shoot N content. Yet, urea-fed soybean plants grown from low Ni seeds without external Ni had again the lowest root N content.

When the protein concentrations of the 2nd oldest trifoliate leaves were measured, similar results were obtained for all treatment groups, except the Ni-deprived, urea-fed group where the protein concentration was 30% lower (Table 2.4A). The protein concentrations of the primary leaves were on average 60% lower than those of trifoliate leaves. All plants grown with nitrate as well as plants supplied with urea and external Ni had comparable levels of protein in their primary leaves. In the absence of Ni from the nutrient solution, urea as the sole N source resulted in markedly reduced protein concentrations in the primary leaves, particularly in those of plants grown from low-Ni seeds. The root protein concentrations were much lower when compared to both leaf types. With respect to the root protein concentration, Ni-deficient plants grown with urea were comparable to nitrate-fed plants. In urea-fed plants Ni from the seed reserves or from the nutrient solution increased the root protein concentration significantly by up to 100%.

Table 2.4: (A) Protein and **(B)** free amino acid concentrations of different organs of 22-day-old soybean (*Glycine max* cv. Nova) plants grown hydroponically from low- or high-Ni seeds, with or without external Ni supply, and with $2x10^{-3}$ M N in the form of nitrate or urea

(A)		Protein Concentration (mg g^{-1} FW)					
Ext. Ni (M)	Seed Ni (µg g^{-1})	Trifoliate Leaves		Primary Leaves		Root	
		Nitrate	Urea	Nitrate	Urea	Nitrate	Urea
0	0.05	21 ± 3	15 ± 1	9.2 ± 0.9	5.9 ± 1.0	2.3 ± 0.1	2.3 ± 0.2
	10	22 ± 1	21 ± 5	9.2 ± 1.3	6.9 ± 0.4	2.2 ± 0.1	4.7 ± 0.3
2×10^{-7}	0.05	22 ± 1	22 ± 1	8.8 ± 1.1	8.9 ± 1.0	2.2 ± 0.1	3.3 ± 0.3
	10	22 ± 1	24 ± 1	10.3 ± 1.9	8.9 ± 0.3	1.8 ± 0.3	3.6 ± 0.3

(B)		Free Amino Acid Concentration (mg g^{-1} FW)					
Ext. Ni (M)	Seed Ni (µg g^{-1})	Trifoliate Leaves		Primary Leaves		Root	
		Nitrate	Urea	Nitrate	Urea	Nitrate	Urea
0	0.05	2.44 ± 0.15	2.02 ± 0.36	1.08 ± 0.23	0.95 ± 0.05	0.17 ± 0.01	0.17 ± 0.02
	10	2.32 ± 0.30	2.62 ± 0.51	1.48 ± 0.21	1.05 ± 0.05	0.17 ± 0.01	0.30 ± 0.03
2×10^{-7}	0.05	3.31 ± 0.40	2.37 ± 0.37	0.87 ± 0.14	1.21 ± 0.13	0.17 ± 0.00	0.26 ± 0.03
	10	3.40 ± 0.41	3.43 ± 0.46	1.64 ± 0.24	0.66 ± 0.03	0.15 ± 0.00	0.26 ± 0.01

Values are means and standard deviations of 3 pot replicates, each containing 7 plants.

$HSD_{0.05}$ values: N Source (A); Ext. Ni (B); Seed Ni (C); AxB; AxC; BxC; AxBxC

Protein:		Free Amino Acids:	
Trifoliate Leaves:	*n.s.*; 2; 2; 3; *n.s.*; *n.s.*; *n.s.*	Trifoliate Leaves:	*n.s.*; 0.33; 0.33; *n.s.*; 0.64; *n.s.*; *n.s.*
Primary Leaves:	1.0; 1.0; *n.s.*; 1.8; *n.s.*; *n.s.*; *n.s.*	Primary Leaves:	0.14; *n.s.*; 0.14; *n.s.*; 0.26; *n.s.*; 0.45
Root:	0.2; *n.s.*; 0.2; *n.s.*; 0.4; 0.4; 0.7	Root:	0.02; *n.s.*; 0.02; 0.03; 0.03; 0.03; 0.05

n.s. Not significant

The free amino acid concentrations of the 2nd oldest trifoliate leaves were enhanced by external Ni supply (Table 2.4B). In the case of urea nutrition, soybean plants grown from high-Ni seeds had up to 45% higher amino acid concentrations in their trifoliate leaves. In accordance with protein results, Ni-deficient plants fed with urea had the lowest free amino acid concentration among all treatment groups (Table 2.4). When compared to the primary leaves and roots, the trifoliate leaves were richer in free amino acids as in proteins. Neither the form of N supply nor the Ni availability had any consistent effect on the free amino acid concentrations of the primary leaves (Table 2.4B). In the roots, however, the responses of the free amino acid concentration to the treatments were parallel to those of the protein concentration (Table 2.4). Nickel from any source elevated the free amino acid concentration in the roots of urea-fed plants by up to 80% (Table 2.4B).

The nitrate analysis revealed that nitrate-fed plants had lower nitrate concentrations in both their trifoliate and primary leaves under Ni-deficient conditions (Table 2.5A). In contrast, the nitrate levels in the root were unaffected by the Ni availability. As expected, the nitrate concentrations measured in urea-fed plants were negligible. With respect to the urea concentration in the trifoliate leaves, plants in different treatment groups exhibited no significant differences (Table 2.5B). A significant urea accumulation in the primary leaves was only observed in the case of urea nutrition under Ni-deficient conditions, whereas low background levels of urea were detected in the primary leaves of all other plants. Using urea as the sole N source caused a significant increase in the urea concentrations detected in the roots, although the measured values were very low in general. The effects of the N form and Ni availability on the ammonium concentrations of the trifoliate leaves were inconsistent (Table 2.5C). Negligibly low ammonium levels were detected in the primary leaves. The ammonium concentrations of the roots were on average quadrupled, when the plants were fed with urea instead of nitrate.

Table 2.5: (A) Nitrate, **(B)** urea and **(C)** ammonium concentrations of different organs of 22-day-old soybean (*Glycine max* cv. Nova) plants grown hydroponically from low- or high-Ni seeds, with or without external Ni supply, and with $2x10^{-3}$ M N in the form of nitrate or urea

(A)		Nitrate Concentration (µg g^{-1} FW)					
Ext. Ni (M)	Seed Ni (µg g^{-1})	Trifoliate Leaves		Primary Leaves		Root	
		Nitrate	Urea	Nitrate	Urea	Nitrate	Urea
0	0.05	169 ± 43	1 ± 2	69 ± 33	5 ± 8	348 ± 48	38 ± 13
	10	499 ± 188	0 ± 0	162 ± 14	0 ± 0	365 ± 82	34 ± 9
2×10^{-7}	0.05	327 ± 75	6 ± 9	155 ± 91	39 ± 14	383 ± 70	21 ± 2
	10	743 ± 23	2 ± 3	237 ± 18	28 ± 11	352 ± 75	47 ± 23

(B)		Urea Concentration (µg g^{-1} FW)					
Ext. Ni (M)	Seed Ni (µg g^{-1})	Trifoliate Leaves		Primary Leaves		Root	
		Nitrate	Urea	Nitrate	Urea	Nitrate	Urea
0	0.05	55 ± 33	63 ± 8	49 ± 5	244 ± 16	16 ± 3	23 ± 4
	10	54 ± 36	82 ± 9	62 ± 13	51 ± 3	11 ± 1	23 ± 1
2×10^{-7}	0.05	67 ± 7	45 ± 11	58 ± 3	53 ± 12	11 ± 1	19 ± 3
	10	71 ± 20	101 ± 10	63 ± 13	49 ± 4	14 ± 3	26 ± 12

(C)		Ammonium Concentration (µg g^{-1} FW)					
Ext. Ni (M)	Seed Ni (µg g^{-1})	Trifoliate Leaves		Primary Leaves		Root	
		Nitrate	Urea	Nitrate	Urea	Nitrate	Urea
0	0.05	5.4 ± 1.3	2.6 ± 1.0	0.35 ± 0.13	0.42 ± 0.18	4.9 ± 0.2	11.4 ± 2.4
	10	3.2 ± 1.4	3.1 ± 0.2	0.46 ± 0.45	0.25 ± 0.23	2.8 ± 0.6	18.0 ± 0.8
2×10^{-7}	0.05	2.7 ± 0.5	3.4 ± 0.3	0.13 ± 0.22	0.43 ± 0.30	3.2 ± 0.5	11.0 ± 1.6
	10	3.0 ± 0.1	2.7 ± 0.1	0.60 ± 0.35	0.06 ± 0.11	2.1 ± 0.1	11.5 ± 0.2

Values are means and standard deviations of 3 pot replicates, each containing 7 plants.

$HSD_{0.05}$ values: N Source (A); Ext. Ni (B); Seed Ni (C); AxB; AxC; BxC; AxBxC

	Nitrate:	Urea:	Ammonium:
Trifoliate Leaves:	64; 64; 64; 122; 122; *n.s.*; *n.s.*	40; *n.s.*; *n.s.*; 77; 77; *n.s.*; *n.s.*	*n.s.*; *n.s.*; *n.s.*; 1.3; *n.s.*; *n.s.*; 2.2
Primary Leaves:	31; 31; 31; *n.s.*; 59; *n.s.*; *n.s.*	9; 9; 9; 17; 17; 17; 28	*n.s.*; *n.s.*; *n.s.*; *n.s.*; 0.44; *n.s.*; *n.s.*
Root:	44; *n.s.*; *n.s.*; *n.s.*; *n.s.*; *n.s.*	4; *n.s.*; *n.s.*; *n.s.*; *n.s.*; *n.s.*; *n.s.*	1.0; 1.0; 1.0; 1.8; 1.8; 1.8; 3.1

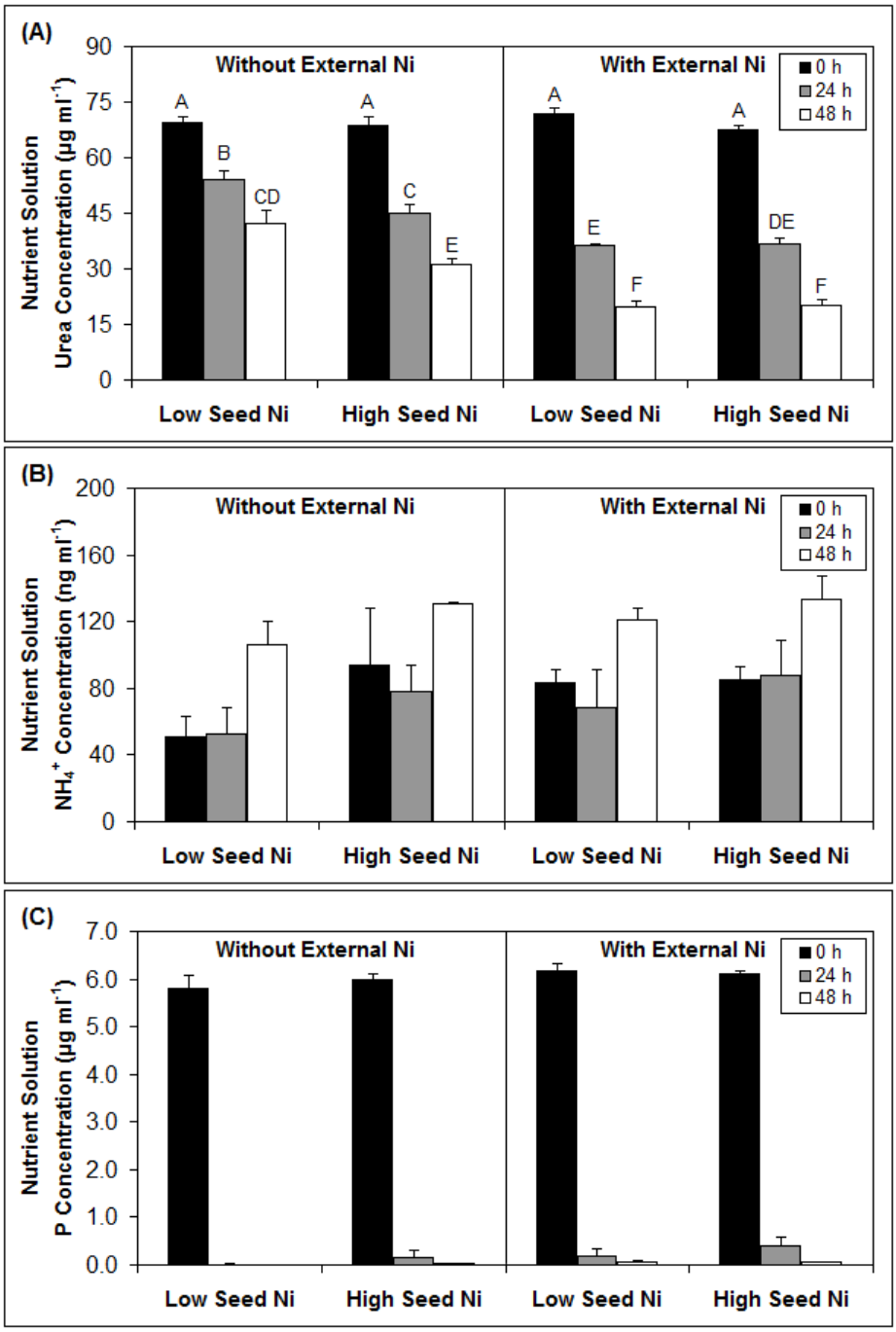

Fig. 2.4: Effect of seed Ni content and external Ni supply on (A) urea, (B) ammonium and (C) P concentrations of nutrient solutions, where 20-day-old soybean (*Glycine max* cv. Nova) plants were grown with urea as the sole N source, at 0 (black bars), 24 (grey bars) and 48 (white bars) h after refreshment. Different letters above bars indicate significant differences according to Tukey's HSD test ($p<0.05$). Letters are available only if the interaction of the variables (seed Ni x external Ni x sampling time) has a significant effect.

The changes in the urea concentrations of urea-containing nutrient solutions over 48 h after refreshment are shown in Fig. 2.4A. Both seed and external Ni affected the rate of urea depletion from the nutrient solution. In the first 24 h, the Ni-deprived plants absorbed only about 20% of urea from the nutrient solution. This ratio was 35% for plants grown from high-Ni seeds without external Ni and 50% for plants externally supplied with Ni. The same trend was also observed at the end of 48 h. Nickel-deficient plants took up about 40% of urea in this time interval. High seed Ni increased this ratio to 55%, and external Ni supply further increased it to 70%. The ammonium concentrations measured in the same nutrient solutions were by three orders of magnitude lower than the urea concentrations (Fig. 2.4A, B). Nevertheless, slight increases in ammonium concentrations were noted at 48 h, irrespective of the Ni availability (Table 2.4B). In contrast to urea depletion, P depletion from the nutrient solution was not affected by seed or external Ni (Fig 2.4A, C). Almost all the P was consumed within just 24 h (Fig. 2.4C).

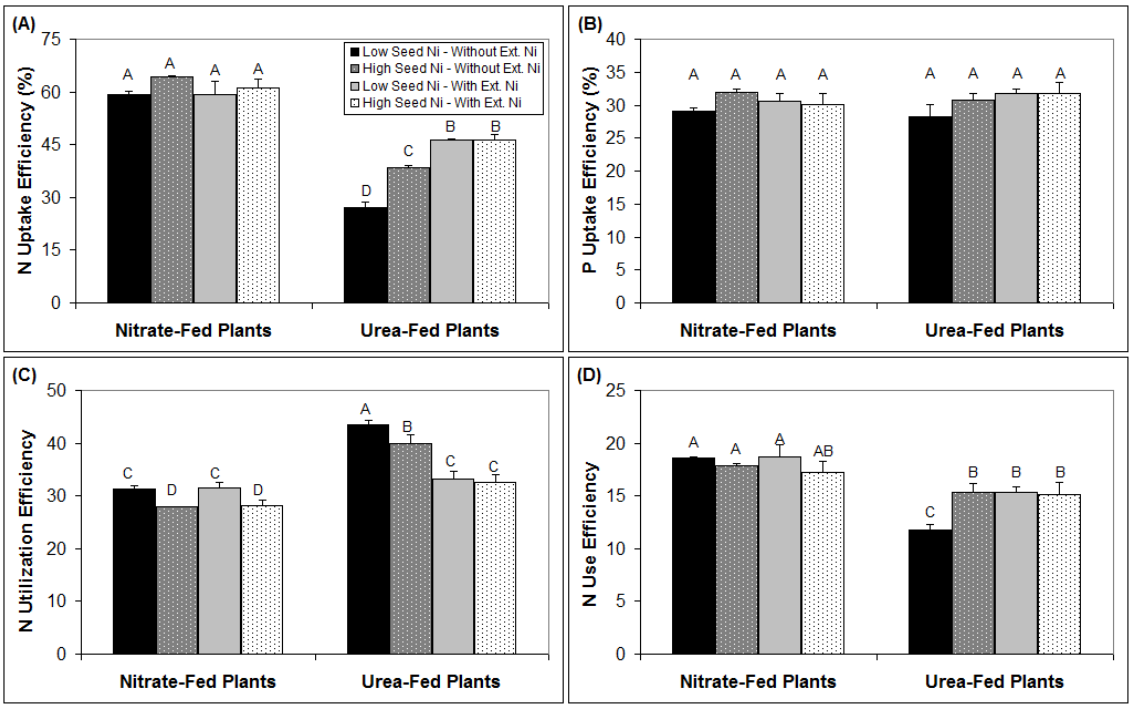

Fig. 2.5: Effect of seed Ni content and external Ni supply on the shoot **(A)** N uptake efficiency, **(B)** P uptake efficiency, **(C)** N utilization efficiency, and **(D)** N use efficiency of nitrate- or urea-fed soybean (*Glycine max* cv. Nova) plants grown hydroponically for 22 days

Nitrogen uptake efficiency calculations revealed that the nitrate-fed plants absorbed about 60% of the available N over the whole growth period, irrespective of the Ni availability (Fig. 2.5A). Generally, the N uptake efficiency was reduced by urea supply. When urea was the sole N source, the Ni-deficient plants had an N uptake efficiency of only 27%. High seed Ni reserves increased the N uptake efficiency of urea-fed plants to 38%, and external Ni supply further enhanced it to 46%. In contrast, P uptake efficiency was 30% for all treatment groups (Fig. 2.5B). Urea-fed plants grown in the absence of external Ni supply appeared to have significantly higher N utilization efficiencies than the rest of the plants (Fig. 2.5C). The N utilization efficiencies of urea-fed plants supplied with external Ni were comparable to those of nitrate-fed plants. Nitrate-fed plants did not differ significantly from each other in their N use efficiencies (Fig. 2.5D). The lowest N use efficiency was observed in urea-fed plants which were deprived of Ni. Nickel from any source significantly increased the N use efficiency of urea-fed plants, but this increase was not sufficient to reach the levels observed in nitrate-fed plants.

2.4. Discussion

Plants depending on urea as the sole N source in solution culture studies were reported to exhibit a reduced growth rate when compared to plants growing with nitrate and/or ammonium (Gerendas *et al.* 1999; Merigout *et al.* 2008a, b). Several possible explanations for this observation were discussed in the literature, including the relatively slow uptake of urea (Bradley *et al.* 1989; Watson and Miller 1996), toxicity problems associated with urea (Gerendas *et al.* 1998b; Tan *et al.* 2000) and the growth-stimulating effects of nitrate as a signaling molecule (Rahayu *et al.* 2005). The results presented here (Figs. 2.1 and 2.2; Table 2.1) are in agreement with previous reports where the adverse effects of urea on plant growth were alleviated by a sufficiently high Ni supply (Gerendas and Sattelmacher 1997; Gerendas *et al.* 1998b; Tan *et al.* 2000; Gheibi *et al.* 2009). Only the old leaves were not significantly affected by the N form, as they had completed their growth before half of the plants were transferred to urea-containing nutrient solution (Table 2.1). However, it should be noted that even for old leaves, the lowest dry weights were measured in urea-grown plants under Ni deficiency,

possibly indicating net remobilization from these source tissues. Previous reports on rice (Gerendas *et al.* 1998b) and tomato (Tan *et al.* 2000) revealed that the root growth was affected less than the shoot growth by urea nutrition in Ni-deficient plants. In accordance with these results, urea nutrition significantly increased the root-to-shoot ratio of Ni-deprived soybean plants (Table 2.1), which may be interpreted as an indicator of physiological N deficiency (Gerendas *et al.* 1998b; Merigout *et al.* 2008a; Erenoglu *et al.* 2011). When the plants could acquire sufficient Ni from the seed reserves or the nutrient solution, the root-to-shoot ratios of urea-fed plants remained at the same level as those of nitrate-fed plants (Table 2.1).

Leaf chlorophyll readings are commonly used to detect N deficiency in otherwise healthy plants (Minotti *et al.* 1994; Blackmer and Schepers 1995). In various experiments where urea was the sole N source, the chlorophyll concentrations of Ni-deficient plants were lower than those of Ni-sufficient plants (Gerendas and Sattelmacher 1997; Tan *et al.* 2000; Gheibi *et al.* 2009). Here, the observed loss of chlorophyll from the primary leaves of Ni-deprived plants supplied with urea can be explained by the retranslocation of N from these leaves to sink tissues in order to meet their N demand under N-deficient conditions (Figs. 2.2A and 2.3B). Nickel starvation also severely impaired the greening of the developing, 2nd oldest trifoliate leaves of urea-fed plants, probably by limiting their N supply (Figs. 2.2B and 2.3D). In addition to the chlorotic appearance of leaves, the total N and protein analyses also indicate Ni deficiency-induced N deficiency (Tables 2.3 and 2.4A). Among all experimental plants, those which were grown from Ni-poor seeds without external Ni supply and supplied with urea as the only N source had the lowest total N and protein concentrations.

Nickel was not detectable in any part of plants grown from low-Ni seeds without external Ni supply, indicating that there was no significant Ni contamination in the nutrient solution (Table 2.2). Both Ni concentration and content data show that nitrate-fed plants accumulated markedly higher levels of Ni than urea-fed plants. Hu *et al.* (2013) reported stimulation of Ni uptake by nitrate and explained this phenomenon by the nitrate-induced expression of iron-regulated transporter 1 (IRT1), which mediates not only ferrous iron (Vert *et al.* 2002) but also Ni (Nishida *et al.* 2011) uptake into root cells. In conformity with the results presented in Chapter 1, the young leaves had much higher Ni concentrations than the old leaves (Table 2.2). The difference was so marked that in plants depending only on the seed reserves for Ni, young leaves accumulated

around 1 mg Ni per g dry weight, whereas the concentration was below detection limits in old leaves. These findings can be explained by the high phloem mobility of Ni and its remobilization from non-senescent source leaves to sink tissues (Neumann and Chamel 1986; Page and Feller 2005).

In the absence of urea supply, free amino acids were reported to accumulate in Ni-deficient barley (Brown *et al.* 1990) and pecan (Bai *et al.* 2006), whereas in various urea-grown species, Ni availability in the nutrient solution enhanced free amino acid levels (Gerendas and Sattelmacher 1997, 1999). Here, the total free amino acid concentration was not affected by Ni availability in nitrate-fed soybean, but significantly elevated in both trifoliate leaves and roots of urea-fed plants (Table 2.4B). Using urea as the only N source instead of nitrate resulted in markedly reduced protein concentrations in leaves of Ni-deficient plants (Table 2.4A). This could, in theory, be explained by either disruption of protein synthesis or inadequate N supply. Specific impairment of protein synthesis due to a stress factor would cause an accumulation of free amino acids, as shown in Zn-deficient common bean (Cakmak *et al.* 1989). In this study, the lack of such an accumulation and the tendency of free amino acid levels to also decrease under Ni deficiency in response to urea exclude Ni deficiency-induced impairment of protein synthesis as a possible explanation for lower protein levels (Table 2.4). As the total N results indicate (Table 2.3), the main reason behind the low protein concentrations measured in leaves of urea-fed plants under Ni deficiency (Table 2.4A) appears to be the physiological deficiency of N raw material.

Generally, the nitrate levels detected in nitrate-fed plants were much higher than the urea levels detected in urea-fed plants (Table 2.5A, B), indicating that the uptake rate was higher than the assimilation rate for nitrate but not for urea. Both seed and external Ni increased the nitrate concentrations of leaves (Table 2.5A). In wheat, excess Ni was documented to reduce the activity of nitrate reductase (Gajewska and Sklodowska 2009), but in this study there was no indication of Ni toxicity due to high seed Ni or external Ni supply. The observed differences in leaf nitrate levels (Table 2.5A) did not have a significant influence on the growth and N nutritional status of nitrate-fed plants (Fig 2.1; Tables 2.1, 2.3 and 2.4). A noteworthy accumulation of urea was observed only in the primary leaves of Ni-deficient plants grown with urea (Table 2.4B), but this level was not high enough to cause any visual toxicity symptom like leaf burn and leaf-tip necrosis (Fig 2.2). In studies where obvious urea toxicity symptoms

were observed in Ni-deficient plants, urea was either applied foliarly or added to nutrient solutions at higher concentrations and caused higher urea accumulations in plant tissues than in this experiment (Chapter 1; Krogmeier *et al.* 1991; Gerendas and Sattelmacher 1997; Tan *et al.* 2000). Both nitrate and urea are converted to ammonium which is an important intermediate in N assimilation and incorporated into amino acids by glutamine synthetase (Miflin and Habash 2002). Reportedly, neither Ni deficiency nor toxicity affects the activity of glutamine synthetase (Gerendas *et al.* 1998b; Gajewska and Sklodowska 2009; Arkoun *et al.* 2013). The ammonium concentrations of plant tissues were very low in this study and not consistently affected by seed or external Ni (Table 2.5C), suggesting that the ammonium assimilation is not a limiting step in N metabolism of soybean, irrespective of the Ni nutritional status.

Under field conditions, soil-applied urea is either directly absorbed by plant roots as the intact molecule or first converted into ammonium and even nitrate by soil microbiota and then taken up in these forms (Witte 2011). Although urea hydrolysis is typically rapid in soils, soil properties as well as environmental conditions can substantially alter the rate of this process (Zantua *et al.* 1977; Kumar and Wagenet 1984), and **intact** urea uptake may be generally underestimated (Witte 2011). Direct absorption of urea from the soil is particularly favored by the application of urease inhibitors in order to retard urea hydrolysis in the soil and minimize ammonia volatilization (Watson and Miller 1996; Dawar *et al.* 2011). In the present study, the extremely low ammonium concentrations measured in the nutrient solutions 24 h and 48 h after refreshment suggest that urea hydrolysis in the growth medium was negligible (Fig. 2.4B). The better the Ni nutritional status of soybean, the faster was the urea uptake (Fig. 2.4A; Table 2.2), in agreement with Arkoun *et al.* (2013), who showed that Ni deficiency reduced ^{15}N uptake from urea in oilseed rape. Since there was no effect of Ni on the observed P uptake rates, the impaired urea uptake of Ni-deficient plants can not be simply a consequence of limited plant growth and root activity (Fig. 2.4C). Depending on the Ni treatments, 30-60% of urea was still in the growth medium after 48 h (Fig. 2.4A), whereas nitrate completely vanished within 24 h, irrespective of the Ni nutrition (data not shown). The significantly higher uptake rate of nitrate than that of urea is in agreement with the previous reports about the relative absorption rates of urea and inorganic N fertilizers (Bradley *et al.* 1989; Watson and Miller 1996; Merigout *et al.* 2008a). The relatively slow uptake of urea is also reflected in the shoot N contents,

which were in all cases higher for nitrate-fed plants (Table 2.3). It is important to note that the impact of Ni nutrition on the rate of urea uptake and thus the N contents of urea-fed plants was stronger than its impact on plant growth (Fig. 2.4A; Tables 2.1 and 2.3). Consequently, Ni starvation was associated with significantly reduced N concentrations in all parts of urea-fed plants (Table 2.3), in conformity with the literature reporting reduced shoot N concentrations in urea-grown plants as a result of Ni deprivation (Gerendas and Sattelmacher 1997; Tan *et al.* 2000).

Although urea is a small and neutral molecule, its root uptake is not based on simple diffusion as it was thought for a long time, but protein-mediated mechanisms (Kojima *et al.* 2006; Witte 2011). These mechanisms include secondary active urea uptake mediated by a high-affinity urea-proton symporter designated as DUR3 in *Arabidopsis thaliana* and passive urea uptake facilitated by certain aquaporins localized at the plasma membrane (Liu *et al.* 2003a, b; Merigout *et al.* 2008b, Witte 2011). Protein-mediated uptake mechanisms enable the regulation of urea uptake. Accordingly, N deficiency was shown to up-regulate DUR3 and induce urea uptake in various species (Bradley *et al.* 1989; Liu *et al.* 2003a; Arkoun *et al.* 2013). The results of the present study demonstrate that urea uptake is also regulated by the Ni nutritional status in soybean (Fig. 2.4A). Despite the fact that Ni starvation caused physiological N deficiency, the urea uptake was not enhanced but on the contrary impaired by Ni deficiency, which suggests a dominant negative effect of Ni starvation on the activities of urea transporters.

In addition to the urea depletion results, the NUpE calculations also demonstrate a profound positive effect of adequate Ni availability (Fig. 2.5A). The lack of any Ni effect on the P uptake efficiency supports the specificity of the Ni effect on NUpE (Fig. 2.5A, B). Due to the well documented role of Ni as the cofactor of urease (Polacco *et al.* 2013), a negative effect of Ni starvation on urea assimilation and therefore the NUtE of urea-fed plants would be expected, but the NUtE of urea-fed plants was not reduced by Ni deficiency in this study (Fig. 2.5C). It appears that the assimilation of absorbed urea was not the main problem of Ni-deprived plants. The decrease in the NUpE of urea-fed plants under Ni deficiency was the reason behind the decrease in their NUE (Fig. 2.5). It is well known that the relatively slow uptake of urea results in lower NUpEs and NUEs in urea-fed plants when compared to plants fed with ammonium or nitrate even in model environments where no N losses via volatilization, leaching, etc.

are observed (Bradley *et al.* 1989; Watson and Miller 1996; Merigout *et al.* 2008a). In the present study, using Ni-rich seeds as well as the presence of adequate Ni in the growth medium minimized the difference between the NUpEs and thus NUEs of urea- and nitrate-fed plants (Fig. 2.5A, D). Under field conditions, where N losses can be substantial as urea is eventually converted into other N forms even if inhibitors are used (Rawluk *et al.* 2001; Dawar *et al.* 2011), assuring the fastest possible urea uptake by adequate Ni nutrition may also reduce such losses and thus contribute to NUE.

2.5. Conclusions

In plants depending on urea as N source, Ni deficiency can lead to physiological N deficiency without causing any urea toxicity symptoms. The negative effect of Ni starvation on the urea-N uptake is apparently stronger than its effect on the urea assimilation. So, the N uptake can be the major limitation for the NUE of Ni-deficient plants supplied with urea. Understanding the mechanism behind the Ni deficiency-induced impairment of urea uptake may be important for the efforts to enhance the NUE. Seed Ni reserves can be almost as effective as external Ni supply in improving the N nutritional status, as reflected by the leaf chlorophyll, total N, amino acid and protein levels, and thus the growth of urea-fed soybean. In addition to the application of urease inhibitors and other agronomic practices, considering the Ni nutrition of crops and using Ni-rich seeds may contribute to the efficient use of urea fertilizers.

CHAPTER 3

SOIL AND FOLIAR NICKEL APPLICATIONS IMPROVE GRAIN YIELD OF WHEAT UNDER AMPLE NITROGEN SUPPLY BY ENHANCING THE TILLER PRODUCTIVITY

3.1. Introduction

The first two chapters focused on the functions of Ni as an essential element in the N and specifically urea metabolism. In these studies, soybean known to have a relatively high Ni requirement was used as a model plant species and grown hydroponically in order to create a Ni deficient environment. Chapter 3 investigates the potential beneficial effects of Ni nutrition on the yield of soil-grown wheat.

Wheat is the most important staple food for humans (Curtis 2002). The area dedicated to wheat cultivation is over 240 million ha and so, larger than to any other crop species. Although this area did not change considerably, world wheat production increased dramatically in the second half of the 20th century as a result of breeding efforts and improved cultural practices including higher use of production inputs, mainly N fertilizers and irrigation. Since additional arable land and water resources are limited and the world population continues to grow rapidly, further yield increases per ha cultivated land must be achieved in cereal production for ensuring the food safety (Cakmak 2002).

On a global scale, more than half of the total N fertilizers are used for cereal production and almost one third of this half is applied just to wheat fields (Heffer 2009).

The promotion of tiller production by higher N supply can significantly contribute to grain yield of wheat (Marschner 2012). However, not all tillers survive to produce grains and particularly higher-order tillers are very susceptible to environmental stresses such as drought and salinity (Maas *et al.* 1996; Acevedo *et al.* 2002; Duggan *et al.* 2005). Besides maximizing the yield, N fertilization is also critical for enhancing the grain protein content of wheat, which is one of the most important quality parameters for both bread and durum wheat (Liu *et al.* 1996; Pena 2002; Kong *et al.* 2013). Soil N and foliar urea applications at booting or later developmental stages were reported to be particularly effective for improving the grain protein content (Gooding and Davies 1992; Kutman *et al.* 2010; Kong *et al.* 2013).

In spite of the importance of wheat as a food crop, there are only a few published studies on the effects of Ni nutrition in wheat production. As early as 1946, Roach and Barclay reported significant yield responses to soil Ni applications for wheat under field conditions. Nickel salts were shown to have both eradicative and protective effects on rust pathogens and applied to wheat for disease control (Forsyth and Peturson 1959; Hoffman *et al.* 1962). In two solution culture studies, the growth of wheat plants supplied with urea as the sole N source was significantly improved by Ni applications (Gerendas and Sattelmacher 1997; Gheibi et al 2009). Positive growth responses of urea-supplied wheat to soil Ni applications were also reported under greenhouse conditions (Singh *et al.* 1990). However, none of these hydroponics or greenhouse studies investigated the effects of Ni on the grain yield of wheat (Singh *et al.* 1990; Gerendas and Sattelmacher 1997; Gheibi et al 2009).

Improving the yield and NUE of wheat has great implications for food safety, environmental protection and the economy of crop production. In order to investigate the potential roles of Ni fertilization in wheat production in the context of N nutrition, greenhouse studies were conducted where soil and foliar applications of Ni were tested for their benefits on the yield and NUE of durum wheat plants grown with different soil and foliar N supplies. The main stem and tiller yields were considered separately to find out their relative contributions to the total grain yield, depending on the N and Ni nutrition.

3.2. Materials and Methods

In this chapter 2 soil experiments are reported, both conducted with durum wheat (*Triticum durum* cv. Balcali2000). The soil culture and greenhouse conditions were described in "General Materials and Methods. All soil experiments had completely randomized and full factorial designs. Each treatment group consisted of 4 independent pot replicates.

3.2.1. First Experiment

In the first experiment 8 plants were grown in each pot. At the beginning, all pots were fertilized with either 150 (low) or 450 (high) mg N per kg soil as $Ca(NO_3)_2.4H_2O$ and half of them were supplied with 2 mg kg^{-1} Ni in the form of $NiCl_2.6H_2O$. Then, half of the plants were sprayed with foliar urea (1% (w/v) urea + 0.01% (w/v) Tween-20; 20 ml per pot), once at booting (44 days after sowing) and again at inflorescence emergence (50 days after sowing). From each unsprayed pot, the leaves of a single plant were harvested 50 days after sowing as follows:

i. the two youngest leaves of the main stem including the flag leaf (refereed to as young leaves)
ii. the third and fourth leaves from the top (referred to as middle leaves)
iii. the fifth and sixth leaves from the top (referred to as old leaves)

These leaf samples were used for chlorophyll analysis as described below.

When the plants reached maturity, the spikes and the straw were harvested separately. The grains were separated from the husks by using a thresher and weighed to determine the grain yield. The straw samples were weighed and ground to fine powder. Both grain and straw samples were used for N and micronutrient analyses as described in "General Materials and Methods".

3.2.2. Second Experiment

In the second experiment, 10 plants were grown in each pot. Prior to seeding, all pots were fertilized with 50 (very low), 100 (low), 300 (medium) or 600 (high) mg N

per kg soil as $Ca(NO_3)_2.4H_2O$ and half of them were supplied with 2 mg kg^{-1} Ni in the form of $NiCl_2.6H_2O$. Foliar Ni and foliar urea treatments were included in this full factorial design. When the plants were at booting (48 days after sowing), foliar Ni (0.01% (w/v) $NiCl_2.6H_2O$ + 0.01% (w/v) Tween-20; 20 ml per pot) was applied to half of the pots. One day later, half of the plants were sprayed with foliar urea (1% (w/v) urea + 0.01% (w/v) Tween-20; 20 ml per pot). This foliar urea application was repeated five days later at inflorescence emergence.

At maturity, the main spikes, the tiller spikes and the straw were harvested separately. Both grain and ground straw samples were used for N and micronutrient analyses as described in "General Materials and Methods". The harvest indices and NUEs of all treatment groups were calculated according to the formulas given in "General Materials and Methods".

3.2.3. Chlorophyll Analysis

Fresh leaf samples were homogenized in 80% (v/v) acetone. The homogenates were centrifuged at 5000 g for 20 min at 4°C, and the supernatants were then centrifuged again at 20,000 g for 20 min at 4°C. These supernatants were used for the spectrophotometric analysis of total chlorophyll (Harborne 1998). The absorbance was read at 652 nm, and the extinction coefficient was used as 27.8 μg cm ml^{-1}.

3.3. Results

At the booting stage, plants grown with high N had more tillers and apparently a higher biomass than plants grown with low N (Fig. 3.1). There was no visible effect of soil Ni application on the high-N plants. Low-N plants appeared chlorotic in the absence of soil Ni treatment but remained green when grown on Ni-fertilized soil. In agreement with these visual symptoms, spectroscopic measurements revealed that the chlorophyll concentrations of the middle and old leaves of low-N plants were significantly lower in the absence of soil Ni application than in its presence, and Ni amendment increased their chlorophyll levels to those measured in high-N plants (Fig.

3.2). Even at high N supply, the chlorophyll levels of Ni-fertilized plants were slightly higher than those of control plants, although these differences were statistically not significant.

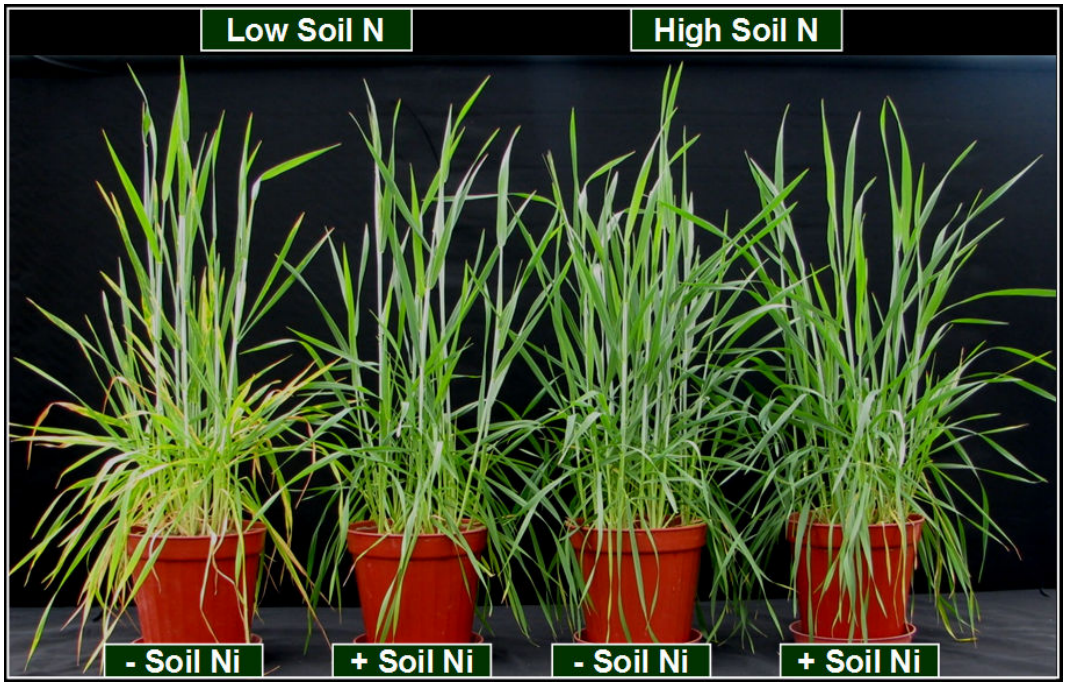

Fig. 3.1: Effect of soil Ni application (2 mg Ni kg^{-1} soil) on the growth and leaf color of 50-day-old durum wheat (*Triticum durum* cv. Balcali2000) plants grown at low (150 mg N kg^{-1} soil) and high (450 mg N kg^{-1} soil) N supply under greenhouse conditions

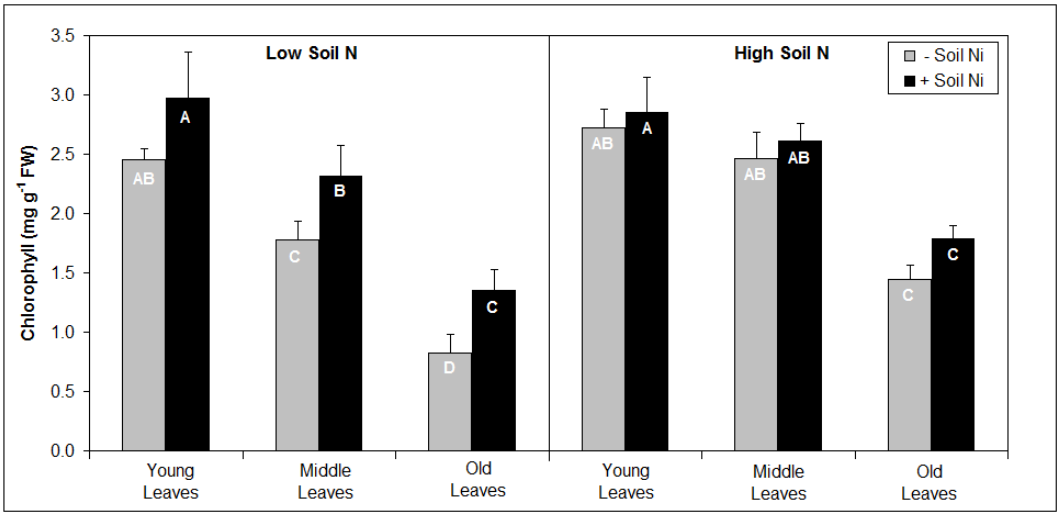

Fig. 3.2: Effect of soil Ni application (2 mg Ni kg^{-1} soil) on the chlorophyll concentrations of the young (the 2 youngest leaves of the main stem including the flag leaf), middle (the next 2 leaves) and old (the next 2 leaves) leaves of 50-day-old durum wheat (*Triticum durum* cv. Balcali2000) plants grown at low (150 mg N kg^{-1} soil) and high (450 mg N kg^{-1} soil) N supply under greenhouse conditions

Table 3.1: Analysis of variance (ANOVA) of the effects of soil N, foliar urea and soil Ni treatments as well as their interactions on reported traits of mature durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions: degrees of freedom, *F* value probabilities and Tukey's $HSD_{0.05}$ test scores.

Source of Variation	DF	Grain Yield		Straw DW		Grain Ni Conc.		Straw Ni Conc.		Shoot Ni Content	
		F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$
Soil N (A)	1	<0.001	0.2	<0.001	0.2	0.003	0.3	0.020	0.18	<0.001	1.2
Foliar N (B)	1	0.004	0.2	0.229	*n.s.*	0.076	*n.s.*	0.093	*n.s.*	0.145	*n.s.*
Soil Ni (C)	1	<0.001	0.2	0.029	0.2	0.059	*n.s.*	0.040	0.18	<0.001	1.2
AxB	1	0.925	*n.s.*	0.654	0.3	0.561	*n.s.*	0.267	*n.s.*	0.830	*n.s.*
AxC	1	0.018	0.3	0.234	*n.s.*	0.072	*n.s.*	0.493	*n.s.*	0.023	2.3
BxC	1	0.025	0.3	0.002	0.3	0.760	*n.s.*	0.207	*n.s.*	0.065	*n.s.*
AxBxC	1	0.470	*n.s.*	0.140	*n.s.*	0.209	*n.s.*	0.581	*n.s.*	0.248	*n.s.*
Source of Variation	**DF**	**Grain Fe Conc.**		**Grain Zn Conc.**		**Grain N Conc.**		**Straw N Conc.**		**Shoot N Content**	
		F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$
Soil N (A)	1	<0.001	3	<0.001	4	<0.001	0.1	<0.001	0.07	<0.001	5
Foliar N (B)	1	0.359	*n.s.*	0.110	*n.s.*	0.152	*n.s.*	<0.001	0.07	<0.001	5
Soil Ni (C)	1	0.261	*n.s.*	0.110	*n.s.*	0.019	0.1	0.078	*n.s.*	0.049	5
AxB	1	0.022	5	0.150	*n.s.*	0.001	0.3	0.482	*n.s.*	0.681	*n.s.*
AxC	1	0.498	*n.s.*	0.572	*n.s.*	0.975	*n.s.*	0.007	0.14	0.096	*n.s.*
BxC	1	0.291	*n.s.*	0.797	*n.s.*	0.562	*n.s.*	0.624	*n.s.*	0.012	10
AxBxC	1	0.568	*n.s.*	0.408	*n.s.*	0.723	*n.s.*	0.457	*n.s.*	0.061	*n.s.*

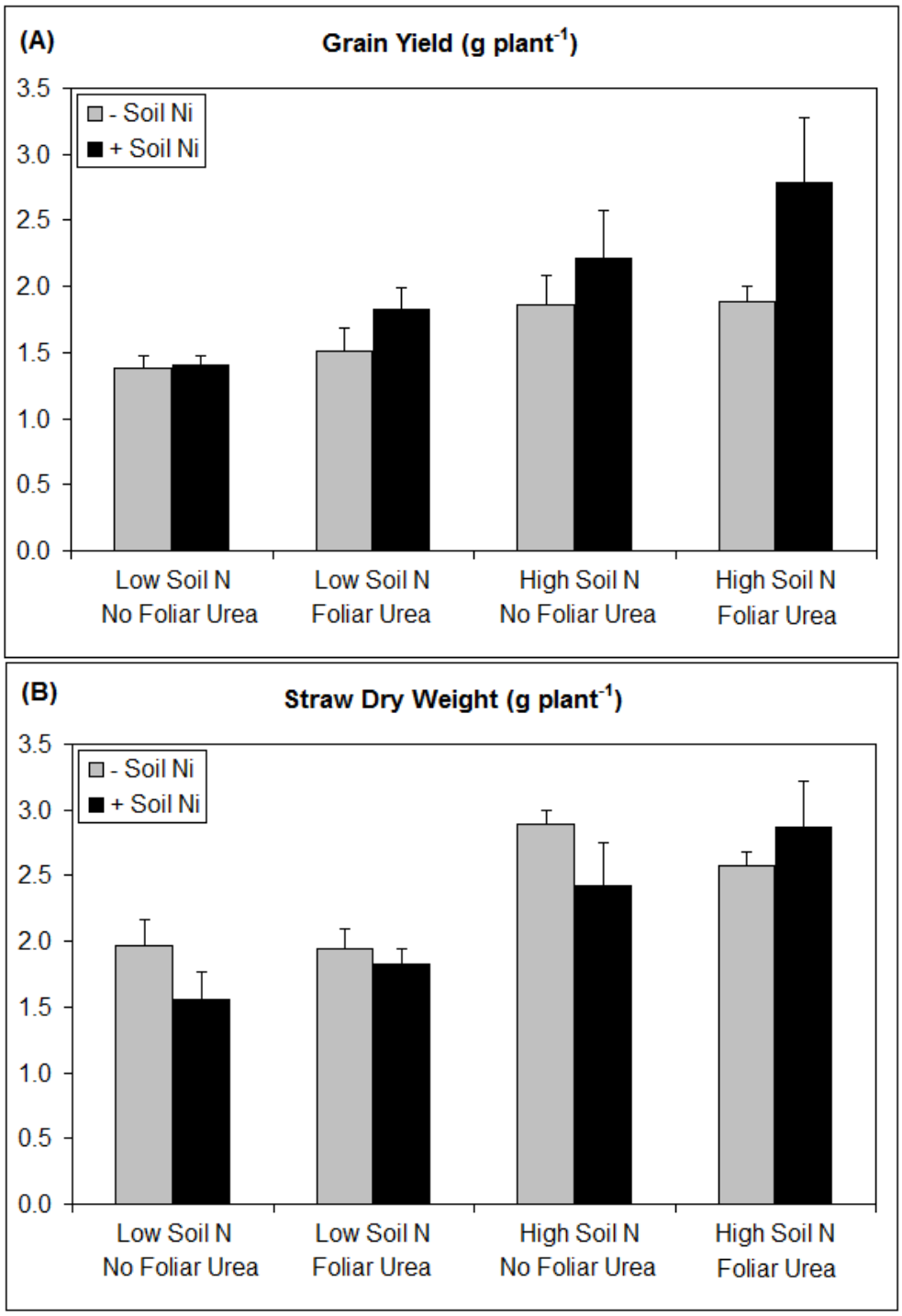

Fig. 3.3: Effect of soil Ni application (2 mg Ni kg^{-1} soil) on the **(A)** grain yield and **(B)** straw dry weight of mature durum wheat (*Triticum durum* cv. Balcali2000) plants under greenhouse conditions. The plants were grown with low (150 mg N kg^{-1} soil) or high (450 mg N kg^{-1} soil) N supply, and half of them were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

Analysis of variance showed that the grain yield was significantly affected by the soil N level, foliar urea application and soil Ni treatment as well as the double interactions of the soil Ni treatment with the other treatments (Table 3.1). On average, higher soil N application increased the grain yield by 45%, but the positive response of wheat yield to higher N supply was dependent on the soil Ni (Fig. 3.3A). The soil Ni application was totally ineffective when the plants were supplied with low soil N and not sprayed with urea. However, significant yield enhancements were observed in response to Ni application in plants supplied with higher levels of N either via soil or foliar treatments. When the high soil N application was combined with urea spray, a yield increase by 50% was achieved by Ni fertilization, which was the highest yield response observed in this experiment. The straw dry weight of mature plants also increased by 50% in response to high soil N supply but did not respond consistently to Ni and foliar urea applications (Fig. 3.3B; Table 3.1).

Table 3.2: Grain Ni concentration, straw Ni concentration and shoot Ni content of mature durum wheat (*Triticum durum* cv. Balcali2000) plants as affected by soil-applied Ni (2 mg Ni kg^{-1} soil). The plants were grown with low (150 mg N kg^{-1} soil) or high (450 mg N kg^{-1} soil) N supply, and half of them were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

Grain Ni Concentration (mg kg^{-1})				
Soil Ni App.	**Low Soil N**		**High Soil N**	
	No Foliar Urea	**Foliar Urea**	**No Foliar Urea**	**Foliar Urea**
-	*3.2 ± 0.3	3.1 ± 0.6	3.5 ± 0.3	3.2 ± 0.6
+	3.4 ± 0.1	2.8 ± 0.4	3.9 ± 0.3	3.9 ± 0.4
Straw Ni Concentration (mg kg^{-1})				
Soil Ni App.	**Low Soil N**		**High Soil N**	
	No Foliar Urea	**Foliar Urea**	**No Foliar Urea**	**Foliar Urea**
-	0.30 ± 0.05	0.39 ± 0.07	0.63 ± 0.21	0.62 ± 0.16
+	0.39 ± 0.09	0.80 ± 0.42	0.70 ± 0.20	0.81 ± 0.43
Shoot Ni Content (µg $plant^{-1}$)				
Soil Ni App.	**Low Soil N**		**High Soil N**	
	No Foliar Urea	**Foliar Urea**	**No Foliar Urea**	**Foliar Urea**
-	5.0 ± 0.7	5.3 ± 0.6	8.4 ± 1.5	7.6 ± 1.2
+	5.4 ± 0.4	6.6 ± 0.7	10.3 ± 1.7	13.2 ± 3.9

* Values are means and standard deviations of 4 independent replicates. For related statistics, refer to Table 3.1.

High soil N slightly increased the grain Ni concentration, which was not significantly affected by any other treatment (Tables 3.1 and 3.2). The straw Ni concentration was significantly enhanced by both soil Ni and high soil N applications. It is noteworthy that the grain Ni concentrations were much higher than the straw Ni concentrations in all treatment groups (Table 3.2). When the shoot (grain + straw) Ni content was considered, the effect of the soil Ni x soil N interaction was significant (Tables 3.1 and 3.2). The shoot Ni content was elevated by Ni fertilization in all cases; however, the extent of this effect was low (17%) when the N supply was low and high (48%) when the N supply was high (Table 3.2). The grain concentrations of Fe and Zn were also increased by high soil N supply, but not affected by Ni or foliar urea applications (Tables 3.1 and 3.3).

Table 3.3: Grain Fe and Zn concentrations of durum wheat (*Triticum durum* cv. Balcali2000) plants as affected by soil-applied Ni (2 mg Ni kg^{-1} soil). The plants were grown with low (150 mg N kg^{-1} soil) or high (450 mg N kg^{-1} soil) N supply, and half of them were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

Grain Fe Concentration (mg kg^{-1})				
Soil Ni App.	Low Soil N		High Soil N	
	No Foliar Urea	Foliar Urea	No Foliar Urea	Foliar Urea
-	*26 ± 1	27 ± 3	41 ± 7	35 ± 4
+	25 ± 1	27 ± 3	36 ± 4	34 ± 1
Grain Zn Concentration (mg kg^{-1})				
Soil Ni App.	Low Soil N		High Soil N	
	No Foliar Urea	Foliar Urea	No Foliar Urea	Foliar Urea
-	34 ± 2	35 ± 5	48 ± 9	42 ± 6
+	34 ± 3	32 ± 3	43 ± 6	39 ± 4

* Values are means and standard deviations of 4 independent replicates. For related statistics, refer to Table 3.1.

The grain N concentration increased on average by 40% in response to high soil N supply (Table 3.4). Spraying the plants with urea provided an increase of 20% in the grain N concentration at low soil N, but had no significant effect at high soil N level (Tables 3.1 and 3.4). Plants grown on Ni-fertilized soil produced grains with slightly lower N concentrations. The straw N concentration was not only nearly doubled by the high soil N application but also markedly enhanced by the foliar urea application. Upon the soil Ni treatment, a slight decrease in the straw N concentration was observed only

under high soil N conditions. As expected, the total N content of the shoot was also significantly increased by both the foliar urea and high soil N applications. A positive effect of soil Ni on the shoot N content was observed only when the high soil N application was combined with urea spray. Under all the other N conditions, the shoot N content was independent of the soil Ni treatment.

Table 3.4: Effect of soil Ni application (2 mg Ni kg^{-1} soil) on the grain N concentration, straw N concentration and shoot N content of mature durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions with low (150 mg N kg^{-1} soil) or high (450 mg N kg^{-1} soil) N supply. Half of the plants were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

Grain N Concentration (%)				
Soil Ni App.	Low Soil N		High Soil N	
	No Foliar Urea	Foliar Urea	No Foliar Urea	Foliar Urea
-	*2.0 ± 0.2	2.4 ± 0.2	3.1 ± 0.2	2.9 ± 0.1
+	1.9 ± 0.1	2.2 ± 0.1	2.9 ± 0.3	2.8 ± 0.2
Straw N Concentration (%)				
Soil Ni App.	Low Soil N		High Soil N	
	No Foliar Urea	Foliar Urea	No Foliar Urea	Foliar Urea
-	0.36 ± 0.03	0.75 ± 0.07	0.97 ± 0.16	1.36 ± 0.15
+	0.44 ± 0.03	0.74 ± 0.09	0.80 ± 0.02	1.20 ± 0.12
Shoot N Content (mg $plant^{-1}$)				
Soil Ni App.	Low Soil N		High Soil N	
	No Foliar Urea	Foliar Urea	No Foliar Urea	Foliar Urea
-	34 ± 2	51 ± 3	86 ± 5	90 ± 6
+	33 ± 3	53 ± 2	84 ± 5	111 ± 17

* Values are means and standard deviations of 4 independent replicates. For related statistics, refer to Table 3.1.

In the next experiment, the effects of soil and foliar Ni applications as well as foliar urea treatment were investigated at 4 different levels of soil N. The main stem (MS) grain yield of durum wheat increased by a factor of 2 when the soil N supply increased from very low to medium (Tables 3.5 and 3.6A). A further increase in the soil N level did not provide any additional benefit on the MS grain yield. Although the effect of soil Ni on the MS grain yield appeared to be significant, this effect was conditional and only observed at higher soil N levels in the absence of foliar urea application. Moreover, foliar Ni also had a significant positive effect on the MS grain yield at higher N levels. No tiller grains were harvested from plants grown with very low or low soil N supply, except in the case of the combination of foliar urea and Ni treatments (Table 3.6B). The highest tiller yields were obtained from high-N plants. At both the medium and high soil N levels, soil Ni quadrupled the tiller grain yield. The significant positive effects of foliar Ni and foliar urea treatments on the tiller yield were dependent on each other, i.e. only plants sprayed with both Ni and urea showed marked yield increases (Tables 3.5 and 3.6B). In the case of high soil N, the combined application of soil Ni, foliar Ni and foliar urea led to a 10-fold increase in the tiller yield. This was the only condition where the tiller yield was comparable to the MS yield (Table 3.6). In all the other cases, the contribution of the MS to the total grain yield was much higher than that of the tillers under the experimental conditions of this study. When the soil N level was increased from very low to low, medium and high, the total grain yield was enhanced by 50%, 140% and 175%, respectively (Table 3.6C). Under the very low N condition, soil and foliar Ni applications did not have any effect on total grain yield, whereas foliar urea treatment provided an increase of 15%. The positive impact of soil Ni fertilization on the total grain yield was first observed under the medium N condition and became more pronounced under the high N condition. At all soil N levels except the very low level, foliar urea was effective only in the presence of foliar Ni treatment and *vice versa*. The combination of these foliar treatments resulted in 15-30% increases in the total grain yield.

Table 3.5: ANOVA of the effects of soil and foliar applications of N and Ni as well as their interactions on reported traits of durum wheat (*Triticum durum* cv. Balcali2000) plants: degrees of freedom, *F* value probabilities and Tukey's $HSD_{0.05}$ test scores.

Source of Variation	DF	MS Gr. Yield		Tiller Gr. Yield		Total Gr. Yield		MS Gr. Ni Conc.	
		F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$
Soil N (A)	3	<0.001	0.08	<0.001	0.10	<0.001	0.10	0.004	0.6
Foliar N (B)	1	0.110	*n.s.*	<0.001	0.06	<0.001	0.05	0.165	*n.s.*
Soil Ni (C)	1	0.017	0.04	<0.001	0.06	<0.001	0.05	0.151	*n.s.*
Foliar Ni (D)	1	<0.001	0.04	0.004	0.06	<0.001	0.05	<0.001	0.3
AxB	3	0.130	*n.s.*	0.035	0.18	0.074	0.16	0.027	1.0
AxC	3	0.120	*n.s.*	<0.001	0.18	<0.001	0.16	0.636	*n.s.*
AxD	3	0.030	0.13	0.113	*n.s.*	<0.001	0.16	0.040	1.0
BxC	1	0.023	0.08	0.192	*n.s.*	0.671	*n.s.*	0.181	*n.s.*
BxD	1	0.215	*n.s.*	<0.001	0.10	<0.001	0.10	<0.001	0.6
CxD	1	0.841	*n.s.*	0.779	*n.s.*	0.638	*n.s.*	0.106	*n.s.*
AxBxC	3	0.394	*n.s.*	0.225	*n.s.*	0.841	*n.s.*	0.467	*n.s.*
AxBxD	3	0.472	*n.s.*	0.024	0.28	0.002	0.26	0.657	*n.s.*
AxCxD	3	0.513	*n.s.*	0.974	*n.s.*	0.820	*n.s.*	0.104	*n.s.*
BxCxD	1	0.865	*n.s.*	0.047	0.18	0.041	0.16	0.576	*n.s.*
AxBxCxD	3	0.387	*n.s.*	0.135	*n.s.*	0.130	*n.s.*	0.541	*n.s.*

Source of Variation	DF	Total Gr. Ni Yield		MS Gr. N Conc.		Total Gr. N Yield		Straw DW	
		F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$
Soil N (A)	3	<0.001	0.9	<0.001	0.08	<0.001	3	<0.001	0.08
Foliar N (B)	1	0.177	*n.s.*	<0.001	0.04	<0.001	1	<0.001	0.04
Soil Ni (C)	1	<0.001	0.5	0.039	0.04	<0.001	1	0.109	*n.s.*
Foliar Ni (D)	1	<0.001	0.5	0.310	*n.s.*	<0.001	1	0.146	*n.s.*
AxB	3	0.003	1.5	<0.001	0.14	0.007	4	0.330	*n.s.*
AxC	3	<0.001	1.5	0.077	*n.s.*	<0.001	4	0.044	0.14
AxD	3	<0.001	1.5	0.057	*n.s.*	0.005	4	0.154	*n.s.*
BxC	1	0.333	*n.s.*	<0.001	0.08	0.014	3	0.141	*n.s.*
BxD	1	0.798	*n.s.*	0.002	0.08	<0.001	3	0.404	*n.s.*
CxD	1	0.865	*n.s.*	0.879	*n.s.*	0.932	*n.s.*	0.823	*n.s.*
AxBxC	3	0.444	*n.s.*	0.296	*n.s.*	0.914	*n.s.*	0.905	*n.s.*
AxBxD	3	0.089	*n.s.*	0.005	0.23	0.025	7	0.486	*n.s.*
AxCxD	3	0.899	*n.s.*	0.452	*n.s.*	0.982	*n.s.*	0.777	*n.s.*
BxCxD	1	0.322	*n.s.*	0.664	*n.s.*	0.130	*n.s.*	0.268	*n.s.*
AxBxCxD	3	0.523	*n.s.*	0.009	0.35	0.437	*n.s.*	0.025	0.34

Source of Variation	DF	Straw Ni Conc.		Straw N Conc.		Harvest Index		NUE	
		F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$	F Pr.	$HSD_{0.05}$
Soil N (A)	3	<0.001	0.7	<0.001	0.06	<0.001	2	<0.001	1
Foliar N (B)	1	<0.001	0.4	<0.001	0.03	0.079	*n.s.*	<0.001	1
Soil Ni (C)	1	0.006	0.4	<0.001	0.03	<0.001	1	<0.001	1
Foliar Ni (D)	1	<0.001	0.4	0.884	*n.s.*	<0.001	1	<0.001	1
AxB	3	<0.001	1.2	<0.001	0.10	0.027	3	<0.001	2
AxC	3	0.381	*n.s.*	<0.001	0.10	<0.001	3	<0.001	2
AxD	3	<0.001	1.2	0.181	*n.s.*	0.001	3	0.004	2
BxC	1	0.467	*n.s.*	<0.001	0.06	0.531	*n.s.*	0.031	1
BxD	1	<0.001	0.7	0.193	*n.s.*	0.041	2	<0.001	1
CxD	1	0.038	0.7	0.531	*n.s.*	0.695	*n.s.*	0.424	*n.s.*
AxBxC	3	0.259	*n.s.*	<0.001	0.16	0.923	*n.s.*	0.728	*n.s.*
AxBxD	3	<0.001	2.0	0.435	*n.s.*	0.009	5	0.183	*n.s.*
AxCxD	3	0.029	2.0	0.553	*n.s.*	0.875	*n.s.*	0.559	*n.s.*
BxCxD	1	0.996	*n.s.*	0.231	*n.s.*	0.068	*n.s.*	0.412	*n.s.*
AxBxCxD	3	0.904	*n.s.*	0.723	*n.s.*	0.109	*n.s.*	0.044	6

Table 3.6: Effect of soil (2 mg Ni kg^{-1} soil) and foliar (0.01% w/v $NiCl_2.6H_2O$) Ni applications on the **(A)** main stem grain yield, **(B)** tiller grain yield and **(C)** total grain yield of durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions. The plants were supplied with very low (50 mg N kg^{-1} soil), low (100 mg N kg^{-1} soil), medium (300 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N, and half of them were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

(A)		Main Stem Grain Yield (g $plant^{-1}$)			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	*0.70 ± 0.03	1.01 ± 0.07	1.30 ± 0.16	1.39 ± 0.21
	+	0.69 ± 0.03	1.03 ± 0.10	1.49 ± 0.12	1.55 ± 0.08
Urea	-	0.81 ± 0.05	1.04 ± 0.04	1.37 ± 0.17	1.39 ± 0.07
	+	0.79 ± 0.04	1.01 ± 0.09	1.36 ± 0.17	1.45 ± 0.25
Ni	-	0.65 ± 0.02	1.02 ± 0.03	1.53 ± 0.11	1.36 ± 0.16
	+	0.71 ± 0.06	1.07 ± 0.05	1.54 ± 0.18	1.67 ± 0.10
Urea + Ni	-	0.78 ± 0.12	1.15 ± 0.12	1.53 ± 0.11	1.54 ± 0.18
	+	0.73 ± 0.09	1.23 ± 0.08	1.51 ± 0.12	1.54 ± 0.12
(B)		**Tiller Grain Yield (g $plant^{-1}$)**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	0.13 ± 0.11	0.14 ± 0.09
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	0.48 ± 0.19	0.77 ± 0.33
Urea	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	0.13 ± 0.13	0.17 ± 0.16
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	0.42 ± 0.27	0.73 ± 0.42
Ni	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	0.18 ± 0.12	0.24 ± 0.10
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	0.41 ± 0.15	0.58 ± 0.20
Urea + Ni	-	0.04 ± 0.04	0.17 ± 0.10	0.17 ± 0.20	0.45 ± 0.36
	+	0.04 ± 0.05	0.09 ± 0.05	0.68 ± 0.13	1.35 ± 0.26
(C)		**Total Grain Yield (g $plant^{-1}$)**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	0.70 ± 0.03	1.01 ± 0.07	1.42 ± 0.15	1.54 ± 0.20
	+	0.69 ± 0.03	1.03 ± 0.10	1.96 ± 0.11	2.32 ± 0.25
Urea	-	0.81 ± 0.05	1.10 ± 0.08	1.50 ± 0.08	1.56 ± 0.16
	+	0.79 ± 0.04	1.03 ± 0.10	1.79 ± 0.29	2.18 ± 0.25
Ni	-	0.65 ± 0.02	1.02 ± 0.03	1.71 ± 0.04	1.60 ± 0.08
	+	0.71 ± 0.06	1.07 ± 0.05	1.95 ± 0.13	2.26 ± 0.19
Urea + Ni	-	0.82 ± 0.14	1.32 ± 0.09	1.70 ± 0.24	1.98 ± 0.34
	+	0.77 ± 0.07	1.32 ± 0.07	2.19 ± 0.17	2.89 ± 0.15

* Values are means and standard deviations of 4 independent replicates. For related statistics, refer to Table 3.5.

Foliar Ni application caused a 3-fold increase in the MS grain Ni concentration, whereas soil Ni did not have any significant effect on this trait (Tables 3.5 and 3.7A). Although according to ANOVA, soil N appeared to have a significant effect on the Ni concentration of MS grains, this effect was minimal and inconsistent. In the absence of Ni spray, the MS grain Ni concentration did not respond to foliar urea, but in Ni-sprayed plants a significant decrease was observed in the MS grain Ni concentration upon urea spray. The tiller grain Ni concentration was markedly lower in high-N plants than in medium-N plants (Table 3.7B). Foliar Ni application provided on average an 80% increase in the tiller grain Ni concentration. However, neither soil Ni nor foliar urea applications had a clear effect on the Ni concentration of tiller grains. The total grain Ni yield was significantly affected by not only foliar Ni but also soil Ni application (Table 3.5). Table 3.7C shows that foliar Ni treatment enhanced the grain Ni yield by a factor of 3, whereas soil Ni fertilization caused an increase of 25% on average. When the interaction between the soil applications of N and Ni was considered, it was observed that the positive effect of soil Ni application on the grain Ni yield was pronounced only at higher soil N levels. The mean grain Ni yields at higher soil N levels were distinctly higher than those at lower N levels.

The N concentration of MS grains increased step by step with increasing soil N supply (Tale 3.8A). Foliar urea had also a significant effect on this trait and enhanced the MS grain N concentration by 10% on average (Tables 3.5 and 3.8A). The extent of the effect of foliar urea on the MS grain N concentration changed depending on other variables. Its effect was marked at lower soil N levels, particularly in the presence of foliar Ni application. Moreover, soil Ni application seemed to have a significant negative effect on the N concentration of MS grains, but this effect was limited to only 2%. The tiller grains produced by high-N plants had on average 37% higher N concentrations than those produced by medium-N plants (Table 3.8B). It was observed that soil Ni application tended to reduce the N concentrations of tiller grains. Foliar urea application could enhance the tiller grain N concentration only in the absence of foliar Ni treatments. The soil N supply had a drastic impact on the grain N yield, which was at the high N level 4 times as high as at the very low N level (Tables 3.5 and 3.8C). Foliar N was also effective in increasing the grain N yield but its effect was more pronounced in Ni-sprayed plants and at lower N levels. In contrast, higher N was a prerequisite for a positive response of the grain N yield to soil and foliar Ni applications.

Table 3.7: (A) Main stem grain Ni concentration, **(B)** tiller grain Ni concentration and **(C)** total grain Ni yield of durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions. The plants were supplied with very low (50 mg N kg^{-1} soil), low (100 mg N kg^{-1} soil), medium (300 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N, and half of them were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

(A)		Main Stem Grain Ni Concentration (mg kg^{-1})			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	*1.9 ± 0.2	2.4 ± 0.1	2.3 ± 0.5	2.4 ± 0.4
	+	1.8 ± 0.2	2.6 ± 0.3	2.9 ± 0.8	3.1 ± 0.6
Urea	-	2.8 ± 0.4	2.7 ± 0.3	2.0 ± 0.4	2.3 ± 0.4
	+	3.0 ± 0.5	3.5 ± 0.4	2.8 ± 0.4	3.1 ± 0.7
Ni	-	7.7 ± 1.6	9.3 ± 1.5	7.7 ± 2.1	8.8 ± 1.1
	+	8.4 ± 1.6	8.7 ± 1.1	8.2 ± 1.2	6.9 ± 0.5
Urea + Ni	-	7.7 ± 0.6	7.6 ± 0.7	6.3 ± 0.6	7.4 ± 2.0
	+	8.3 ± 0.9	8.5 ± 1.1	6.1 ± 0.9	7.2 ± 0.8
(B)		**Tiller Grain Ni Concentration (mg kg^{-1})**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	3.2 ± 0.0	3.1 ± 0.8
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	5.4 ± 0.6	3.1 ± 0.6
Urea	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	3.9 ± 0.8	2.5 ± 0.3
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	5.7 ± 2.1	3.7 ± 0.7
Ni	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	9.4 ± 1.3	5.3 ± 0.9
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	8.8 ± 1.0	5.1 ± 0.7
Urea + Ni	-	7.3 ± 3.0	6.7 ± 1.7	9.7 ± 2.8	5.8 ± 0.8
	+	9.1 ± 3.2	5.8 ± 0.5	7.1 ± 2.4	5.1 ± 1.0
(C)		**Total Grain Ni Yield (µg $plant^{-1}$)**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	1.3 ± 0.1	2.4 ± 0.2	3.4 ± 0.8	3.7 ± 0.3
	+	1.3 ± 0.1	2.6 ± 0.5	6.7 ± 1.6	7.2 ± 0.8
Urea	-	2.2 ± 0.3	2.8 ± 0.3	3.2 ± 0.6	3.6 ± 0.9
	+	2.4 ± 0.5	3.5 ± 0.4	5.8 ± 1.5	7.1 ± 1.6
Ni	-	5.0 ± 1.0	9.5 ± 1.6	13.4 ± 3.5	13.3 ± 1.1
	+	5.9 ± 1.0	9.3 ± 1.5	16.1 ± 2.0	14.5 ± 0.9
Urea + Ni	-	6.2 ± 0.9	9.9 ± 0.7	11.2 ± 2.3	13.5 ± 2.8
	+	6.4 ± 0.7	10.9 ± 1.1	14.0 ± 2.3	18.0 ± 1.9

* Values are means and standard deviations of 4 independent replicates. For related statistics, refer to Table 3.5.

Table 3.8: Effect of soil (2 mg Ni kg^{-1} soil) and foliar (0.01% w/v $NiCl_2.6H_2O$) Ni applications on the **(A)** main stem grain N concentration, **(B)** tiller grain N concentration and **(C)** total grain N yield of durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions. The plants were supplied with very low (50 mg N kg^{-1} soil), low (100 mg N kg^{-1} soil), medium (300 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N, and half of them were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

(A)		Main Stem Grain N Concentration (%)			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	*1.5 ± 0.0	1.7 ± 0.0	2.7 ± 0.1	2.7 ± 0.0
	+	1.7 ± 0.1	1.8 ± 0.1	2.5 ± 0.2	2.8 ± 0.1
Urea	-	2.2 ± 0.2	2.2 ± 0.1	2.5 ± 0.1	2.8 ± 0.1
	+	1.9 ± 0.1	2.0 ± 0.1	2.3 ± 0.1	2.7 ± 0.1
Ni	-	1.5 ± 0.1	1.7 ± 0.1	2.4 ± 0.3	2.9 ± 0.1
	+	1.5 ± 0.1	1.8 ± 0.1	2.5 ± 0.1	2.8 ± 0.1
Urea + Ni	-	2.4 ± 0.3	2.2 ± 0.2	2.6 ± 0.1	2.8 ± 0.1
	+	2.3 ± 0.2	2.0 ± 0.0	2.4 ± 0.1	2.7 ± 0.1
(B)		**Tiller Grain N Concentration (%)**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	2.4 ± 0.4	3.5 ± 0.5
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	2.2 ± 0.2	3.2 ± 0.3
Urea	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	3.2 ± 0.5	3.8 ± 0.3
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	2.2 ± 0.3	3.3 ± 0.3
Ni	-	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	2.1 ± 0.3	3.3 ± 0.5
	+	*n.a.* ± *n.a.*	*n.a.* ± *n.a.*	2.2 ± 0.3	3.2 ± 0.5
Urea + Ni	-	1.3 ± 0.1	1.5 ± 0.2	2.8 ± 0.4	3.4 ± 0.3
	+	1.6 ± 0.5	1.0 ± 0.3	2.1 ± 0.3	2.8 ± 0.0
(C)		**Total Grain N Yield (mg $plant^{-1}$)**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	10 ± 0	17 ± 1	38 ± 5	43 ± 5
	+	12 ± 0	18 ± 1	47 ± 2	69 ± 6
Urea	-	17 ± 1	24 ± 1	39 ± 3	44 ± 6
	+	15 ± 1	21 ± 2	41 ± 8	62 ± 7
Ni	-	10 ± 0	17 ± 1	40 ± 4	47 ± 4
	+	11 ± 1	19 ± 2	47 ± 2	66 ± 7
Urea + Ni	-	19 ± 1	28 ± 3	45 ± 6	57 ± 8
	+	17 ± 1	25 ± 1	50 ± 4	80 ± 5

* Values are means and standard deviations of 4 independent replicates. For related statistics, refer to Table 3.5.

The straw dry weight increased by up to 150% with increasing soil N fertilization (Tables 3.5 and 3.9A). Urea-sprayed plants produced on average 12% more vegetative biomass than non-sprayed ones. This effect of urea was observed at all soil N levels except the high N level. According to ANOVA, Ni applications to the soil or foliage did not have any impact on the vegetative growth of durum wheat. However, Ni treatments enhanced the Ni concentration of the straw significantly (Tables 3.5 and 3.9B). On average, soil Ni fertilization provided an increase by 14% in the straw Ni concentration. Foliar Ni, on the other hand, seemed to increase the straw Ni concentration by a factor of 5, although surface contamination with Ni may have contributed to this effect (see discussion). In general, higher N supply via soil or foliar applications markedly reduced the straw Ni concentration, particularly in Ni-sprayed plants. All sources of variation except foliar Ni application and its interactions with the other treatments affected the straw N concentration significantly as shown in Tables 3.5. Generally, the straw N concentration tended to increase with increasing soil N (Table 3.9C). The most dramatic response of the straw N to soil N, however, was observed at the high level where it was almost doubled when compared to lower N levels. The straw N concentration was also elevated in urea-sprayed plants. Notably, the magnitude of this effect of foliar urea application was much higher at lower than at higher soil N levels. The apparently significant negative effect of soil Ni on the straw N concentration was limited to higher soil N levels and urea-sprayed plants.

Analysis of variance revealed that soil N, soil Ni and foliar Ni applications had significant effects on the harvest index (Table 3.5). Despite the fact that the high-N plants exhibited the lowest average harvest index, not only the lowest (33%) but also the highest (51%) harvest index value in Table 3.10A was observed in this group of plants. The reason behind was the significant interaction of soil N level with soil and foliar applications of Ni. Both types of Ni treatments markedly increased the harvest index, only in case the N supply was ample. With increasing N supply via soil or foliar applications, the NUE decreased significantly (Tables 3.5 and 3.10B). In contrast, the NUE responded positively to both soil and foliar Ni treatments. Soil Ni was in this respect ineffective at lower soil N levels, whereas it provided average increases of 25% and 45% at medium and high N levels, respectively. Another important interaction was observed between foliar urea and foliar Ni applications. Foliar Ni enhanced the NUE significantly only in urea-sprayed plants.

Table 3.9: (A) Straw dry weight, **(B)** straw Ni concentration and **(C)** straw N concentration of durum wheat (*Triticum durum* cv. Balcali2000) plants as affected by soil and foliar applications of Ni (2 mg Ni kg^{-1} soil; 0.01% w/v $NiCl_2.6H_2O$). The plants were supplied with very low (50 mg N kg^{-1} soil), low (100 mg N kg^{-1} soil), medium (300 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N, and half of them were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

(A)		Straw Dry Weight (g $plant^{-1}$)			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	*0.72 ± 0.02	0.89 ± 0.03	1.53 ± 0.12	2.04 ± 0.33
	+	0.75 ± 0.04	0.96 ± 0.04	1.56 ± 0.11	1.81 ± 0.09
Urea	-	0.85 ± 0.03	1.07 ± 0.05	1.79 ± 0.08	2.05 ± 0.18
	+	0.84 ± 0.04	1.00 ± 0.11	1.70 ± 0.13	2.07 ± 0.14
Ni	-	0.68 ± 0.04	0.94 ± 0.03	1.59 ± 0.06	1.94 ± 0.12
	+	0.76 ± 0.05	1.02 ± 0.05	1.50 ± 0.09	1.94 ± 0.25
Urea + Ni	-	0.90 ± 0.06	1.21 ± 0.11	1.81 ± 0.19	2.16 ± 0.24
	+	0.91 ± 0.12	1.21 ± 0.06	1.73 ± 0.05	1.85 ± 0.19
(B)		**Straw Ni Concentration (mg kg^{-1})**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	0.9 ± 0.3	1.1 ± 0.6	1.6 ± 0.4	1.7 ± 0.2
	+	1.1 ± 0.7	1.5 ± 0.8	3.2 ± 0.4	1.7 ± 0.6
Urea	-	1.0 ± 0.2	0.7 ± 0.2	2.0 ± 1.0	0.9 ± 0.0
	+	1.3 ± 0.5	1.5 ± 0.2	1.9 ± 0.4	0.9 ± 0.1
Ni	-	13.9 ± 1.6	11.2 ± 1.4	6.4 ± 0.8	3.2 ± 0.9
	+	14.5 ± 1.4	11.1 ± 2.1	8.6 ± 3.1	4.0 ± 0.3
Urea + Ni	-	6.0 ± 1.4	6.0 ± 0.7	4.3 ± 0.8	3.0 ± 0.3
	+	6.6 ± 1.6	5.4 ± 0.8	5.0 ± 1.7	4.6 ± 2.0
(C)		**Straw N Concentration (%)**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	0.33 ± 0.03	0.34 ± 0.02	0.59 ± 0.08	1.15 ± 0.03
	+	0.31 ± 0.03	0.35 ± 0.04	0.55 ± 0.02	1.06 ± 0.16
Urea	-	0.74 ± 0.10	0.86 ± 0.11	0.94 ± 0.09	1.58 ± 0.17
	+	0.84 ± 0.16	0.83 ± 0.07	0.83 ± 0.07	1.16 ± 0.13
Ni	-	0.35 ± 0.05	0.36 ± 0.03	0.52 ± 0.05	1.23 ± 0.10
	+	0.38 ± 0.05	0.39 ± 0.05	0.52 ± 0.06	1.11 ± 0.12
Urea + Ni	-	0.72 ± 0.11	1.00 ± 0.08	0.88 ± 0.06	1.57 ± 0.10
	+	0.80 ± 0.04	0.79 ± 0.06	0.79 ± 0.07	1.08 ± 0.17

* Values are means and standard deviations of 4 independent replicates. For related statistics, refer to Table 3.5.

Table 3.10: Effect of soil (2 mg Ni kg^{-1} soil) and foliar (0.01% w/v $NiCl_2.6H_2O$) Ni applications on the **(A)** harvest index and **(B)** nitrogen use efficiency of durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions. The plants were supplied with very low (50 mg N kg^{-1} soil), low (100 mg N kg^{-1} soil), medium (300 mg N kg^{-1} soil) or high (600 mg N kg^{-1} soil) N, and half of them were sprayed twice with 1% (w/v) urea at booting and inflorescence emergence.

(A)		Harvest Index (%)			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	*42 ± 1	45 ± 1	39 ± 4	33 ± 4
	+	41 ± 2	44 ± 2	46 ± 2	45 ± 4
Urea	-	42 ± 2	43 ± 2	36 ± 2	33 ± 2
	+	42 ± 1	44 ± 1	42 ± 4	40 ± 4
Ni	-	42 ± 2	45 ± 1	44 ± 1	35 ± 3
	+	41 ± 1	44 ± 0	48 ± 2	44 ± 4
Urea + Ni	-	41 ± 4	45 ± 1	39 ± 5	37 ± 4
	+	40 ± 3	46 ± 0	47 ± 2	51 ± 1
(B)		**Nitrogen Use Efficiency**			
Foliar App.	Soil Ni App.	Soil N			
		Very Low	Low	Medium	High
None	-	48 ± 2	41 ± 3	22 ± 2	12 ± 2
	+	47 ± 2	42 ± 4	30 ± 2	19 ± 2
Urea	-	24 ± 2	26 ± 2	18 ± 1	11 ± 1
	+	24 ± 1	24 ± 2	22 ± 3	15 ± 2
Ni	-	45 ± 1	41 ± 1	26 ± 1	13 ± 1
	+	49 ± 4	43 ± 2	30 ± 2	18 ± 2
Urea + Ni	-	25 ± 4	31 ± 2	21 ± 3	14 ± 2
	+	23 ± 2	31 ± 2	26 ± 2	20 ± 1

* Values are means and standard deviations of 4 independent replicates. For related statistics, refer to Table 3.5.

3.4. Discussion

Stunting, reduced tillering and uniform chlorosis typically starting in older leaves are well known symptoms of N deficiency in cereals (Marschner 2012). As shown in Fig. 3.1, durum wheat plants grown with low N supply were chlorotic at booting, however only in the absence of soil Ni application, whereas those grown with high N supply appeared healthy and vigorous irrespective of the Ni treatment. The observation that the leaf color was the only visible difference between Ni-treated and -untreated plants at the low N condition while the vegetative biomass and tiller production were unaffected by Ni suggests that Ni just delayed the N deficiency-induced senescence but did not alleviate N deficiency otherwise. Several reports in the literature documented that Ni could retard the senescence symptoms when applied to detached or attached leaves of cereals as well as cut flowers (Bushnell 1966; Mishra and Kar 1973; Jamali and Rahemi 2011). The results of this study demonstrate that root-absorbed Ni can also be effective in delaying senescence in intact plants. In conformity with the high phloem mobility of N (Marschner 2012), the most prominent reduction in chlorophyll concentration was observed in old leaves of low-N plants (Fig. 3.2).

Though not common, significant yield responses to Ni applications were reported for several crops not only in soil or sand culture studies conducted under greenhouse conditions (Atta-Aly 1999; Gad *et al.* 2007) but also under field conditions (Roach and Barclay 1946). This study revealed dramatic yield responses to Ni treatment in durum wheat, and these responses were apparently dependent on the N supply level. In both experiments, yield enhancements by soil and/or foliar Ni treatments were observed only in plants supplied with sufficiently high levels of N via soil and/or foliar applications (Fig. 3.3A; Table 3.6). Surprisingly, the positive effect of Ni on the visual appearance of low-N plants at booting was not reflected in the grain yield of these plants (Figs 3.1 and 3.3A). Probably, the yield depressive effects of low N treatment overshadowed the beneficial effects of Ni. Measuring the MS and tiller grain yields separately led to the remarkable finding that most of the yield increases provided by Ni applications could be accounted for by increases in tiller yields (Table 3.6). As higher N supply encourages tiller production (Marschner 2012), this observation can also explain why yield responses to Ni treatments were more pronounced in plants grown under high

N conditions. It is well known that not all tiller produce grains in wheat and many actually abort before anthesis (Acevedo *et al.* 2002). Here, Ni applications increased the ratio of productive tillers, which is known to be strongly influenced by genetic and environmental factors. It appears that higher levels of N supply somehow increased the Ni requirement of wheat in this study. There are many reports in the literature showing that higher N levels can increase the requirement of plants for other essential minerals and also induce or aggravate their deficiencies (Chaudry and Loneragan 1970; Willet *et al.* 1985; Van Den Driessche and Ponsford 1995; Marschner 2012).

If the soil N had been supplied in the form of urea, it would have been conceivable that the yield-enhancing effect of soil Ni treatments was related to the efficient use of urea fertilizer, considering the role of Ni as the cofactor of urease (Polacco *et al.* 2013). Accordingly, positive growth responses to soil Ni applications were reported in wheat grown with urea as the sole N source in the soil (Singh *et al.* 1990). But here, the major N source in the soil was nitrate, indicating that the observed effects of soil Ni can not be attributed to its role in the assimilation of external urea. Nevertheless, the additional improvements of grain yield by foliar applications of Ni may be related to the key role of Ni in the urea metabolism since such improvements were only observed in urea-sprayed plants (Table 3.6). It is also noteworthy that the plants in this study were apparently disease-free and yield improvements provided by Ni applications can therefore not be linked to the reported beneficial effects of Ni on disease resistance (Graham *et al.* 1985; Polacco *et al.* 2013).

Although it is difficult to report critical Ni deficiency concentrations for crops, solution culture studies revealed that 100 µg Ni per kg dry weight was sufficient not only to maximize the grain viability and shoot growth of mineral N-supplied cereals (Brown *et al.* 1987a, b) but also to achieve full urease activity and the maximum growth rate in urea-fed plants (Gerendas *et al.* 1999). The Ni concentrations reported here for both the grains and straw of durum wheat are well above this critical level under all conditions, ruling out Ni deficiency *per se* (Tables 3.2, 3.7 and 3.9). Soil Ni application slightly increased the grain Ni concentration at higher N levels in the absence of foliar Ni application, but its effect was statistically not significant. It is an interesting coincidence that the yield responses to soil Ni application were also observed only at higher N levels; however, the observed differences in Ni concentrations were probably too small to explain such yield effects (Fig. 3.3; Tables 3.2, 3.6 and 3.7). The effects of

soil Ni application on the shoot Ni content in the first experiment and the grain Ni yield in the second experiment were much more pronounced than its effects on the Ni concentrations, indicating that the extra Ni in the shoots of plants grown on Ni-applied soil was diluted as a result of yield increases.

Foliar Ni application resulted in dramatic increases in Ni concentrations of not only straw but also grain samples (Tables 3.7 and 3.9). Of course, apoplastic Ni as well as Ni fixed on leaf surfaces and could not be washed away may have contributed to the straw Ni concentrations of Ni-sprayed plants; but since Ni was sprayed before anthesis, when the spikes were still buried in the culm, the increases in the grain Ni concentrations provide a clear evidence for the absorption of foliar-applied Ni and its re-translocation to sink tissues via the phloem. This is in agreement with the results reported in Chapters 1, 2 and 4 as well as the findings of Page and Feller (2005), who demonstrated the high phloem mobility of Ni in wheat. It is also known that wheat tends to store high amounts of Ni in its root system (Coinchelin *et al.* 2012). In accordance, a small solution culture study showed that 96% of the total Ni in 30-day-old wheat plants was retained in the roots, and the relatively small amount of Ni in the shoot was preferentially allocated to developing leaves (Table 3.11). Therefore, it is conceivable that the soil-applied Ni had a greater impact on the Ni concentrations of the roots and shoot sinks during critical stages of development than on the grain and straw Ni concentrations of mature plants.

Table 3.11: The distribution of Ni in 30-day-old durum wheat (*Triticum durum* cv. Balcali2000) plants hydroponically grown with 0.2 μM Ni as $NiCl_2.6H_2O$.

Plant Part	Ni Conc. (mg kg^{-1})	Ni Content (μg $plant^{-1}$)
Developing Leaves	*1.5 ± 0.1	0.1 ± 0.0
Remaining Shoot	0.6 ± 0.0	1.1 ± 0.2
Root	41.7 ± 3.8	30.8 ± 4.2

* Values are means and standard deviations of 4 pot replicates, each containing 5 plants.

In this study, the observed effects of Ni may be linked to its role as an ethylene biosynthesis inhibitor (Pennazio and Roggero 1992; Polacco *et al.* 2013). The delay of senescence in low-N plants as a result of Ni application may also be attributable to the inhibition of ethylene production by Ni (Figs. 3.1 and 3.2). It is well documented that ethylene releasing chemicals accelerate the senescence whereas ethylene inhibitors retard it (Gepstein and Kenneth 1981; Beltrano *et al.* 1994). Moreover, the generative

development of plants is highly sensitive to ethylene (Klassen and Bugbee 2002; Hays *et al.* 2007). Overproduction of ethylene, as observed under various stress conditions, and applications of ethylene releasing chemicals can induce male sterility as well as kernel abortion and thus lower grain yield significantly in wheat and rice (Rowell and Miller 1971; Campbell *et al.* 2001; Hays *et al.* 2007). Even in the absence of any stress treatment, ethylene inhibitors were reported to promote male gametophyte survival and improve grain filling in rice (Naik and Mohapatra 1999, 2000). Although this study did not involve any stress application, the tillers could not realize their yield potential (Table 3.6). Unanticipated stress factors such as high planting density may have contributed to the sterility of tillers by inducing ethylene production, and Ni may have helped by inhibiting the ethylene production.

The chloride salt of Ni was used in this study for both soil and foliar applications of Ni. Chloride (Cl^-) is an essential micronutrient for all higher plants, and though not very common, its deficiency can result in significant yield losses (White and Broadley 2001; Marschner 2012). In cereals, including winter wheat, durum wheat and barley, Cl^- deficiency was shown to be the cause of a typical physiological leaf spot syndrome (Engel *et al.* 1997, 2001; Christensen and Hayes 2009). Under field conditions, cereals suffering from this syndrome produced lower grain yield and responded significantly to Cl^- fertilizers (Fixen *et al.* 1986; Engel *et al.* 1997; Freeman *et al.* 2006). The soil used in this study is not known to be deficient in Cl^-, but since no Cl^- salts other than that of Ni were used, an involvement of Cl^- in the observed yield responses would be conceivable. However, neither the typical leaf spot symptoms associated with Cl^- deficiency in wheat nor reduced vegetative biomass production due to Cl^- deficiency (Engel *et al.* 1997, 2001) was observed here in the absence of $NiCl_2.6H_2O$ application at any N level (Figs. 3.1 and 3.3; Table 3.9). It is therefore highly unlikely that Cl^- was a critical variable in this study.

Antagonistic interactions between Ni and other divalent micronutrient cations including Fe and Zn were reported in various studies (Wood 2008; Nishida *et al.* 2012). Here, the grain Fe and Zn concentrations were not affected by the soil (Table 3.3) or foliar (*data not shown*) application of Ni. So, Ni application is not a threat to the mineral nutritional value of wheat grain for human consumption. Parallel results were also reported in Chapter 1, where increasing levels of Ni application did not reduce the seed Fe and Zn concentrations in hydroponically grown soybean. The well-documented

positive impact of increasing N supply on the grain concentrations of Zn and Fe of wheat (Kutman *et al.* 2011) was also observed in this study (Table 3.3).

In general, positive yield responses to increasing N applications were accompanied by enhancements in grain N concentrations (Tables 3.4 and 3.8). The yield responses to Ni applications were so dramatic at higher N levels that the grain N concentrations were slightly reduced in many cases due to dilution although the total grain N yield was significantly enhanced (Tables 3.6 and 3.8). At the high N level and particularly in urea-sprayed plants, the N concentration of the straw was also lowered by soil-applied Ni, which can be explained by improved N remobilization from vegetative tissues to developing grains as a result of increased yield potential and thus higher sink activity (Tables 3.6 and 3.9A).

In the absence of soil Ni application, the vegetative biomass production was more responsive to extra N than grain yield, which was reflected in reduced HI values at higher N levels (Table 3.10A). Apparently, the application of Ni enhanced the yield by improving not only the total shoot biomass (straw biomass + grain yield) but also the dry matter allocation to grains (Tables 3.6, 3.9A and 3.10A). A higher grain yield response to additional N applications in Ni-treated plants implies by definition a higher NUE (Table 3.10B). In various studies, positive growth responses to Ni applications were observed only in urea-fed plants but not in mineral N-supplied ones and explained by improved uptake and/or utilization efficiency of urea (Chapter 2; Singh *et al.* 1990; Gerendas and Sattelmacher 1997; Gerendas *et al.* 1998b; Tan *et al.* 2000). However, in this study, the positive effects of Ni applications on the NUE are probably not attributable to the roles of Ni in urea metabolism since they were not dependent on urea fertilization (Table 3.10B). Of course, the recycling of endogenously produced urea also requires urease activity and thus Ni (Eskew *et al.* 1983; Walker *et al.* 1985), but as discussed above, the Ni concentrations measured in all treatment groups were sufficiently high (Gerendas *et al.* 1999) and therefore unlikely to impair the urease activity. Nitrogen itself was the yield limiting factor at lower N levels, whereas Ni availability limited the grain yield and thus indirectly the NUE under ample N supply.

3.5. Conclusion

Although the essentiality of Ni as a plant micronutrient is well documented, most studies reporting growth and yield improvements in response to Ni applications were based on urea as the sole N source and Ni-deprived solution culture conditions. The effects were explained in these studies by improved urea NUE due to the direct involvement of Ni in urea metabolism. However in this soil study, not only urea-sprayed plants but also plants supplied with nitrate as the only N source showed significant yield responses to Ni applications, when the N supply was ample. Nickel applications improve the yield of particularly tillers, the production of which is encouraged by higher N levels. The marked beneficial effects of Ni applications on wheat productivity, which can not be simply explained by the correction of Ni deficiency or better use of urea fertilizer, suggest a more complex developmental response, possibly involving phytohormonal effects based on the role of Ni as an ethylene inhibitor. Further studies are required to investigate the potential of Ni in wheat production and NUE under field conditions and elucidate the exact mechanism behind the observed beneficial effects.

CHAPTER 4

FOLIAR NICKEL APPLICATION ALLEVIATES DETRIMENTAL EFFECTS OF GLYPHOSATE DRIFT ON YIELD AND SEED QUALITY OF WHEAT

4.1. Introduction

In all the previous chapters, the effects of Ni nutrition were related to the form and/or level of N supply. Depending on the N nutrition, the Ni availability was able to improve the growth, yield and NUE of soybean and wheat. This final chapter deals with a completely different beneficial effect of Ni nutrition on the growth and yield of wheat and documents how Ni application can be used as a protective tool against glyphosate drift injury.

Glyphosate, which is the most commonly used herbicide in the world, exerts its main herbicidal activity by specifically inhibiting the 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) in the shikimate pathway and thus blocking the biosynthesis of aromatic amino acids, auxin, lignin and other phenolic compounds (Hernandez *et al*. 1999; Dill 2005; Duke and Powles 2008). The inhibition of this critical pathway leads to impairments in protein and photosynthetic carbon metabolism (Geiger *et al*. 1986; Geiger *et al*. 1999; Baylis 2000). Foliar-applied glyphosate moves to actively growing shoot and root tips with high sink activities and causes shikimate accumulation in these young tissues (Hetherington *et al*. 1999; Feng *et al*. 2003; Ozturk *et al*. 2008; Cakmak *et al*. 2009).

Applications of glyphosate as a pre-plant burn-down or to fields of glyphosate-resistant (GR) transgenic crops are frequently associated with glyphosate drift injuries

in neighboring fields (Roider *et al.* 2007). Off-target movement of glyphosate is often the result of improper application techniques and high wind speeds. Glyphosate drift to susceptible crops can cause toxicity symptoms, developmental disorders and significant yield losses. In wheat, 10% of the labeled usage rate of glyphosate was shown to cause yield losses by over 90%, depending on climatic conditions and treatment stage (Deeds *et al*. 2006). Another study documented yield losses by up to 70% when wheat was sprayed with practically relevant drift doses of glyphosate at the first node stage (Roider *et al*. 2007).

Besides yield loss, the typical growth anomalies associated with sublethal glyphosate injury in grasses are reduced stem elongation (stunting) and increased tillering (Coupland and Caseley 1975; Baur *et al*. 1977; Al-Khatib *et al*. 2003; Roider *et al*. 2007). Dicots also exhibit abnormal growth symptoms in response to sublethal glyphosate, including increased axillary branching (Baur 1979; Lee 1984; Maxwell *et al*. 1987) and epinasty (Baur 1979; Smid and Hiller 1981; Baylis 2000). Reportedly, the disruption of phytohormone balance by glyphosate contributes to sublethal glyphosate injury (Baylis 2000). Induction of tillering in grasses and axillary branching in dicots by glyphosate indicates a temporary loss of apical dominance, which was associated with glyphosate-induced inhibition of polar auxin transport from the actively growing apex (Baur 1979; Maxwell *et al*. 1987; Baylis 2000). According to Baur (1979), glyphosate may do so indirectly by inducing the production of ethylene, known to disrupt auxin transport (Beyer and Morgan 1969; Suttle 1988). Glyphosate-induced ethylene production was documented for common bean (*Phaseolus vulgaris*) (Abu-Irmaileh *et al*. 1979) and white birch (*Betula papyrifera*) (Stasiak *et al*. 1992).

Another aspect of glyphosate drift to non-target plants is the effects of glyphosate on mineral nutrition. Due to its affinity to divalent cations (Motekaitis and Martell 1985; Duke *et al.* 2012), glyphosate can reduce the tissue concentrations and impair the uptake and translocation of essential nutrients, particularly Ca, Mg, Fe and Mn, as documented in various species including soybean (Duke *et al*. 1983, 1985; Cakmak *et al*. 2009), sunflower (Eker *et al*. 2006) and turfgrass (Su *et al*. 2009). Furthermore, glyphosate was reported to indirectly reduce the Fe uptake by impairing the root ferric reductase activity (Ozturk *et al*. 2008; Bellaloui *et al*. 2009). In a recent report, it was suggested that impairment of root growth by glyphosate may also contribute to reduced mineral uptake in non-target plants (Duke *et al*. 2012). The direct

interaction between glyphosate and divalent metals has important implications not only for plant nutrition but also for the herbicidal activity of glyphosate, which may be reduced due to complex formation in spray solutions (Thelen *et al.* 1995; Bernards *et al.* 2005; Chahal *et al.* 2012).

Due to the relatively high *in vitro* affinity of Ni^{2+} to glyphosate (Motekaitis and Martell 1985) and its role as an ethylene biosynthesis inhibitor *in planta* (Lau and Yang 1976; McGarvey and Christoffersen 1992; Itamura *et al.* 1997), Ni may interact with glyphosate in crops directly at a chemical and/or indirectly at a functional level. To our knowledge, this is the first report on the effects of Ni on glyphosate drift injury in a non-target crop. This study was conducted to investigate the possibility of using soil or foliar Ni applications for alleviating glyphosate drift damage to durum wheat (*Triticum durum*). Glyphosate drift was simulated under greenhouse conditions by applying different levels of sublethal glyphosate to wheat plants at different developmental stages. Visual injury symptoms, various growth parameters, shikimic acid accumulation, grain yield and seed germination were investigated to demonstrate the effects of Ni on glyphosate damage.

4.2. Materials and Methods

This chapter reports and discusses the results of 3 soil experiments and a germination test, all conducted with durum wheat (*Triticum durum* cv. Balcali2000). The soil culture and greenhouse conditions were described in "General Materials and Methods. All soil experiments had completely randomized designs, and each treatment group consisted of 4 pot replicates, each containing 6 individual plants. As a pre-plant fertilizer, 300 mg kg^{-1} N as $Ca(NO_3)_2.4H_2O$ was applied to each pot, in addition to other nutrients described in "General Materials and Methods". The plants which were grown until grain maturation were fertilized with an additional 100 mg N per kg soil at anthesis.

For simulating glyphosate drift, Roundup® STAR (Monsanto) containing 441 g/L N-(phosphonomethyl) glycine (glyphosate) potassium salt was used in this study. The recommended application dose of this commercial herbicide is 300 ml per 1000 m^2 in

30 L water. Throughout the chapter, n% glyphosate refers to n% of the recommended herbicidal glyphosate application dose.

4.2.1. First Experiment

In the first experiment, 1% and 1.5% of the recommended dose were selected for simulating glyphosate drift, corresponding to 0.21 and 0.32 mM glyphosate, respectively. There were 5 Ni treatment groups in this experiment: One group was left untreated as control, another one was fertilized with 2 mg Ni per kg soil at the beginning of the experiment, and the remaining 3 groups were sprayed with Ni at different concentrations when the plants were 33 days old and at tillering stage (Zadoks Stage (ZS): 21-24). Nickel sprays contained 0.002% (referred to as low), 0.01% (referred to as medium) or 0.02% (referred to as high) (w/v) $NiCl_2.6H_2O$ (corresponding to 0.08, 0.42, 0.84 mM Ni, respectively) and 0.01% (w/v) Tween-20 as surfactant. The remaining pots were sprayed with the same amount of dH_2O containing only 0.01% (w/v) Tween-20. Two days later, one third of the pots were treated with 1% and one third with 1.5% glyphosate, while the remaining one third were sprayed with just water as control. When control plants were at booting stage (ZS: 45-47), 50 days after sowing (DAS), main stem heights (up to the joint of the youngest leaf blade) were measured. Samples taken from the 2nd youngest fully expanded leaves, which were the youngest common non-necrotic leaves in all glyphosate treatment groups, were used for shikimate analysis according to the method described by Ozturk *et al.*[6] Plant shoots were harvested, washed 3 times with dH_2O and dried at 70°C for 2 days. The dry samples were ground, digested and analyzed for Ni concentration as described in "General Materials and Methods".

4.2.2. Second Experiment

The second soil experiment was designed as a fully factorial experiment where wheat plants were grown until grain maturation. At the beginning, half of the plants were fertilized with 2 mg Ni per kg soil. When the main stems were swelling (ZS: 41-45), 43 days after sowing, half of the pots were sprayed with 0.01% (w/v) $NiCl_2.6H_2O$ and 0.01% (w/v) Tween-20 and the rest with only 0.01% (w/v) Tween-20. Two days

later, half of the pots were treated with 1% glyphosate, while the remaining were sprayed with just water. When the plants completely senesced, the straw and spikes were harvested separately. The samples were dried, ground, digested and analyzed for Ni as described in "General Materials and Methods".

4.2.3. Third Experiment

The effects of the timing of glyphosate treatment were studied in the third soil experiment. Plants were treated with glyphosate either at tillering (ZS: 21-24; 29 days after sowing) or booting (ZS: 47-49; 50 days after sowing) stage. Foliar Ni applications were carried out two days before glyphosate treatments. For foliar Ni application, plants were sprayed with 0.01% (w/v) $NiCl_2.6H_2O$ solution containing 0.01% (w/v) Tween-20 or only Tween-20. Glyphosate treatments were conducted by spraying plants with 0.5% or 1% of the recommended dose or just dH_2O. Half of the earlier treated pots were harvested when control plants reached were at the stem elongation (ZS: 37-39; 43 days after sowing) whereas the other half as well as the later treated pots were grown until grain maturation. The main stem heights (up to the joint of the youngest leaf blade for vegetative stage plants and up to the beginning of the spike for mature plants) were measured just before harvest in all treatment groups. Whole shoots of vegetative stage plants were harvested and washed with dH_2O. In the case of mature plants, the straw and spikes were harvested separately. All samples were dried at 70°C for 2 days. The dry samples were ground, digested and analyzed for Ni concentration as described in "General Materials and Methods".

4.2.4. Germination Test

A germination test was conducted using the seeds produced by the plants subjected to glyphosate at booting in the third soil experiment. From each seed batch (produced by plants grown in one pot), 50 seeds were selected randomly. Since the soil experiment had 4 replicates, the germination test was also a 4-replicate experiment where each glyphosate x Ni group was represented by 200 seeds in total. Seeds were sown in perlite moistened with 2 mM $CaSO_4.2H_2O$ and germinated in the greenhouse

for 8 days. The germination percentage and shoot (coleoptile + primary leaf) length of the seedlings were determined.

4.3. Results

In the first experiment, where the impact of soil and foliar applications of Ni on actively growing wheat plants subject to sublethal glyphosate concentrations were studied, the shoot dry weight and main stem height were significantly affected by Ni and glyphosate treatments as well as their interaction (Table 4.1A, B). When the plants were harvested 15 days after glyphosate treatment, 1% and 1.5% of the recommended lethal glyphosate dose reduced the shoot biomass on average by 30% and 40%, respectively (Fig. 4.1; Table 4.1A). Low foliar and soil Ni applications had no significant effect on the shoot dry weight, whereas medium and high rates of foliar Ni reduced the loss in shoot biomass of wheat plants treated with 1% glyphosate. The main stem height, which was halved by 1% glyphosate in the absence of any Ni application, was almost completely restored to control levels by medium and high foliar Ni doses (Fig. 4.1; Table 4.1B). In the case of 1.5% glyphosate, the main stem height was even more drastically reduced, and none of the Ni treatments provided any benefit.

At the time of harvest, the shikimate concentration measurements revealed no shikimate accumulation in the young leaves of wheat plants treated with 1% glyphosate (Table 4.1C), probably because the time between glyphosate application and leaf sampling was too long (14 days), and the analysis could not be carried out in the youngest leaves and shoot tips which died upon 1.5% glyphosate treatment (see discussion). However, the application of 1.5% glyphosate caused a statistically significant 30% increase in shikimate levels, which was not prevented by any Ni treatment. Glyphosate and soil Ni treatments did not affect the shoot Ni concentration, whereas increasing levels of foliar Ni application resulted in marked increases in shoot Ni concentration (Table 4.1D).

Table 4.1: Effects of low (0.002% $NiCl_2.6H_2O$), medium (0.01% $NiCl_2.6H_2O$) and high (0.02% $NiCl_2.6H_2O$) foliar Ni (33 DAS), soil (2 mg kg^{-1}) Ni and glyphosate (35 DAS) applications on shoot dry weight **(A)**, main stem height **(B)**, shikimate concentration **(C)** and shoot Ni concentration **(D)** of 50-day-old durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions

(A)	**Shoot DW (g plant^{-1})**		
Ni Application	**Glyphosate Dose (% of rec.)**		
	0	**1.0**	**1.5**
No Ni	2.26 ± 0.08	1.45 ± 0.18	1.21 ± 0.09
Low Foliar Ni	2.07 ± 0.14	1.28 ± 0.07	1.19 ± 0.18
Medium Foliar Ni	2.10 ± 0.10	1.64 ± 0.04	1.13 ± 0.10
High Foliar Ni	2.16 ± 0.08	1.82 ± 0.17	1.24 ± 0.13
Soil Ni	2.21 ± 0.09	1.51 ± 0.20	1.45 ± 0.11

$HSD_{0.05}$ (Ni; Gly; NixGly) = (0.15; 0.10; 0.32)

(B)	**Main Stem Height (cm)**		
Ni Application	**Glyphosate Dose (% of rec.)**		
	0	**1.0**	**1.5**
No Ni	35.1 ± 1.6	17.0 ± 1.3	13.1 ± 1.0
Low Foliar Ni	33.4 ± 2.1	16.9 ± 3.3	13.6 ± 1.1
Medium Foliar Ni	33.6 ± 2.6	30.1 ± 4.1	13.5 ± 1.1
High Foliar Ni	34.5 ± 2.6	29.4 ± 2.9	14.1 ± 1.6
Soil Ni	34.5 ± 2.5	20.1 ± 3.2	12.8 ± 1.0

$HSD_{0.05}$ (Ni; Gly; NixGly) = (2.4; 1.6; 5.3)

(C)	**Shikimate Conc. (µmol g^{-1} FW)**		
Ni Application	**Glyphosate Dose (% of rec.)**		
	0	**1.0**	**1.5**
No Ni	1.69 ± 0.07	1.64 ± 0.32	2.14 ± 0.49
Low Foliar Ni	1.43 ± 0.22	1.48 ± 0.23	2.00 ± 0.20
Medium Foliar Ni	1.47 ± 0.30	1.39 ± 0.06	1.89 ± 0.42
High Foliar Ni	1.57 ± 0.14	1.47 ± 0.52	2.13 ± 0.46
Soil Ni	1.50 ± 0.48	1.55 ± 0.08	1.99 ± 0.27

$HSD_{0.05}$ (Ni; Gly; NixGly) = (*n.s.*; 0.25; *n.s.*)

(D)	**Shoot Ni Conc. (mgkg^{-1})**		
Ni Application	**Glyphosate Dose (% of rec.)**		
	0	**1.0**	**1.5**
No Ni	2.9 ± 0.4	2.1 ± 0.3	1.7 ± 0.1
Low Foliar Ni	3.3 ± 0.4	3.8 ± 0.2	4.0 ± 0.5
Medium Foliar Ni	9.4 ± 1.1	11.5 ± 1.3	10.1 ± 0.6
High Foliar Ni	19.3 ± 2.0	20.6 ± 2.9	24.8 ± 3.6
Soil Ni	2.8 ± 0.3	2.6 ± 0.1	2.1 ± 0.4

$HSD_{0.05}$ (Ni; Gly; NixGly) = (1.6; *n.s.*; 3.5)

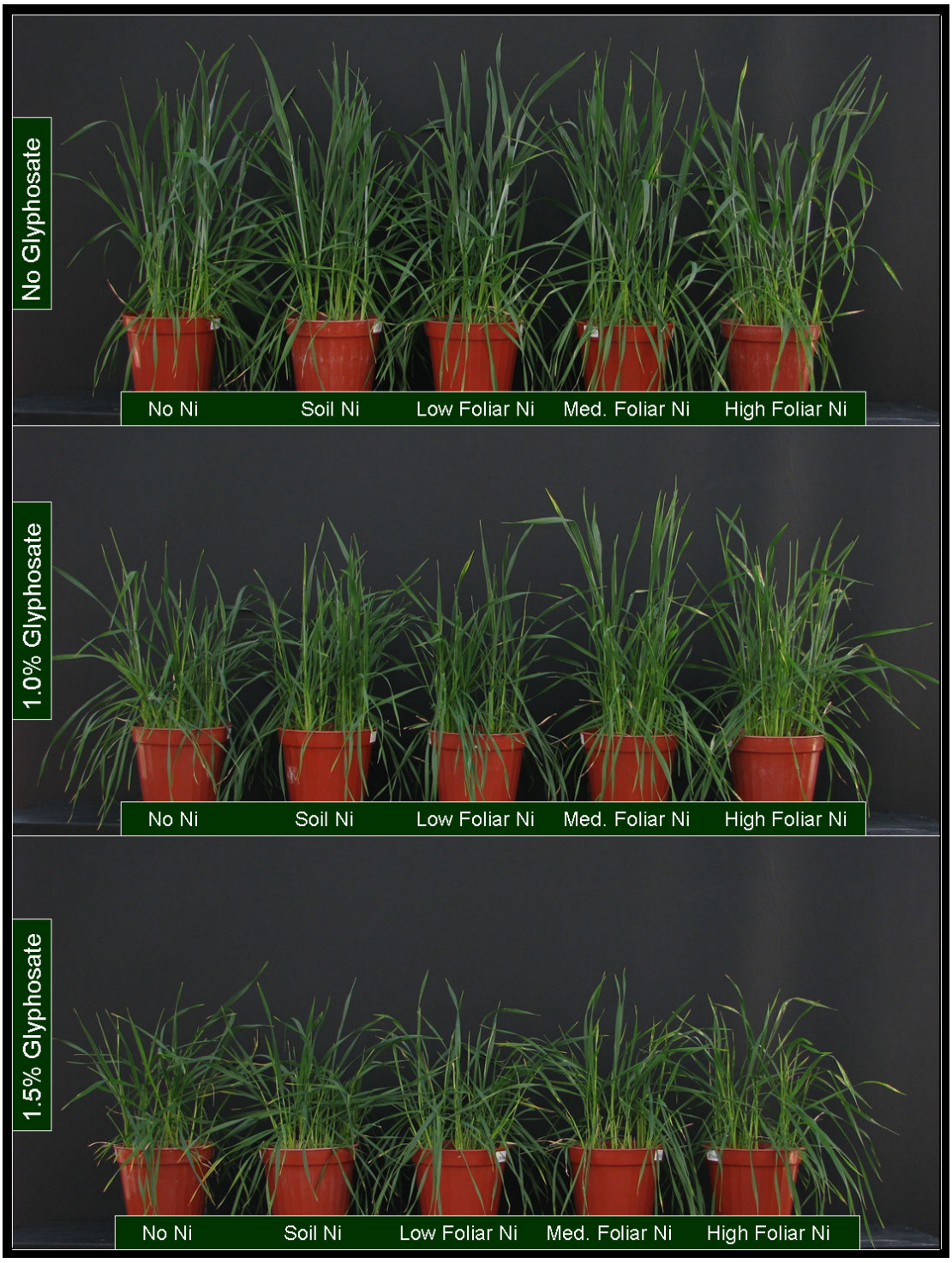

Figure 4.1: Effects of low (0.002% $NiCl_2.6H_2O$), medium (0.01% $NiCl_2.6H_2O$) and high (0.02% $NiCl_2.6H_2O$) foliar Ni (33 DAS), soil (2 mg kg^{-1}) Ni and glyphosate (35 DAS) applications on 50-day-old durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions

Table 4.2: Effects of soil Ni, foliar Ni (43 DAS) and glyphosate (45 DAS) treatments on grain yield, straw dry weight, grain Ni and straw Ni concentration of durum wheat (Triticum durum cv. Balcali2000) plants grown under greenhouse conditions

Soil Ni (A) (mg kg^{-1})	Foliar Ni (B) (% $NiCl_2.6H_2O$)	Gly. Dose (C) (% of std.)	Grain Yield (g $plant^{-1}$)	Straw DW (g $plant^{-1}$)	Grain Ni Conc. (mg kg^{-1})	Straw Ni Conc. (mg kg^{-1})
0	0	0	3.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.4	0.8 ± 0.1
		1.0	0.5 ± 0.3	2.4 ± 0.2	5.8 ± 1.7	0.9 ± 0.1
	0.01	0	3.2 ± 0.3	2.4 ± 0.2	8.3 ± 1.8	5.2 ± 1.2
		1.0	2.4 ± 0.2	2.1 ± 0.1	6.8 ± 1.3	5.1 ± 0.9
2	0	0	3.2 ± 0.1	2.1 ± 0.1	2.9 ± 0.3	1.0 ± 0.1
		1.0	0.7 ± 0.1	1.9 ± 0.1	4.9 ± 0.4	1.1 ± 0.3
	0.01	0	3.1 ± 0.2	2.2 ± 0.1	8.2 ± 1.6	5.3 ± 0.1
		1.0	2.4 ± 0.3	2.2 ± 0.1	8.9 ± 1.4	6.6 ± 0.8

Grain Yield: $HSD_{0.05}$ (A; B; C; AxB; AxC; BxC; AxBxC) = (*n.s.*; 0.2; 0.2; 0.3; *n.s.*; 0.3; *n.s.*)
Straw Dry Weight: $HSD_{0.05}$ (A; B; C; AxB; AxC; BxC; AxBxC) = (0.1; *n.s.*; 0.1; 0.2; *n.s.*; *n.s.*; *n.s.*)
Grain Ni Concentration: $HSD_{0.05}$ (A; B; C; AxB; AxC; BxC; AxBxC) = (*n.s.*; 0.9; 0.9; *n.s.*; *n.s.*; 1.7; *n.s.*)
Straw Ni Concentration: $HSD_{0.05}$ (A; B; C; AxB; AxC; BxC; AxBxC) = (*n.s.*; 0.5; *n.s.*; *n.s.*; *n.s.*; *n.s.*; *n.s.*)

The effects of soil and foliar Ni applications on glyphosate-induced grain yield loss in wheat were investigated in the next experiment. Based on the results of the previous experiment, 1% glyphosate and the medium foliar Ni rate (0.01% $NiCl_2.6H_20$) were selected as effective application levels for this study. Dramatic yield losses were observed in the absence of foliar Ni treatment when the plants were sprayed with glyphosate at booting (Table 4.2). Irrespective of soil Ni application, foliar Ni treatment quadrupled the grain yield of wheat plants subjected to glyphosate by preventing nearly 75% of the damage caused by glyphosate. Soil application of Ni did not have any significant effect on grain yield under these experimental conditions. In contrast to grain yield, straw dry weight did not exhibit marked responses to glyphosate or Ni applications. Glyphosate and soil Ni treatments tended to slightly decrease straw dry weight whereas foliar Ni did not affect straw dry weight at all. Grain Ni concentration increased markedly in response to foliar Ni treatment, but it did not respond to soil Ni application. In the absence of foliar Ni, glyphosate-treated plants produced grains with higher Ni concentrations. Straw Ni concentration showed a 4-fold increase upon foliar Ni application, irrespective of glyphosate and soil Ni treatments.

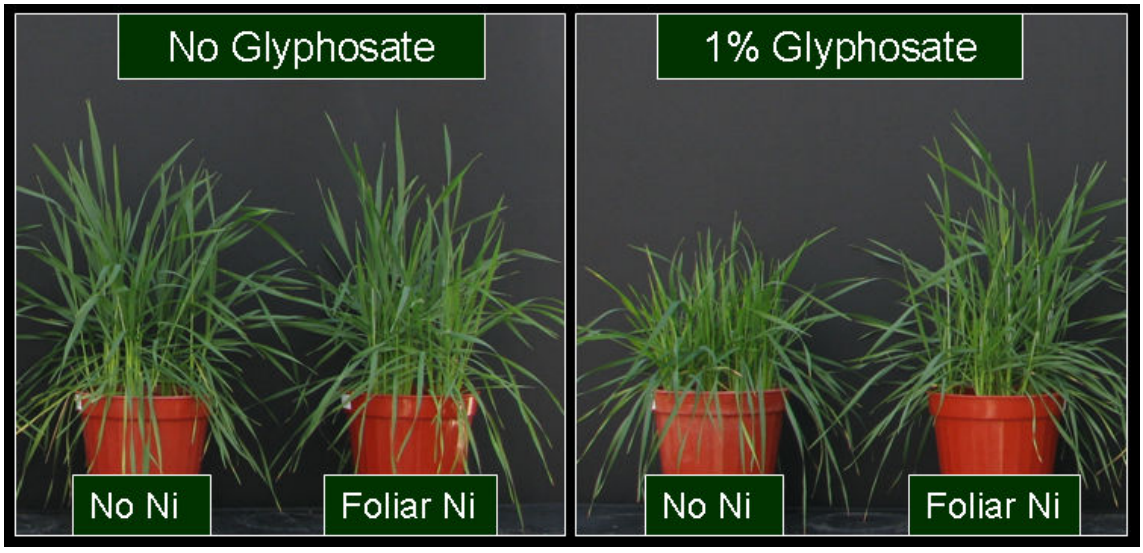

Figure 4.2: Effects of foliar Ni (0.01% $NiCl_2.6H_2O$; 27 DAS) and glyphosate (1% of rec.; 29 DAS) treatments on 43-day-old durum wheat (*Triticum durum* cv. Balcali2000) plants grown under greenhouse conditions

The next experiment was conducted in order to study the interactive effects of foliar Ni and sublethal glyphosate at different application stages on vegetative growth and grain yield of wheat. When foliar treatments were carried out at tillering and plants were harvested two weeks later at stem elongation, dwarfing and excessive tillering were observed in plants treated with 1% glyphosate but not sprayed with Ni (Fig. 4.2). Neither shoot dry weight nor main stem height was significantly affected by 0.5% glyphosate (Table 4.3). In the absence of Ni, 1% glyphosate decreased shoot dry weight by 25%. This reduction of shoot biomass by glyphosate was significantly but only partially (by 40%) prevented by foliar Ni. In agreement with the results of the first experiment, an even more pronounced protective effect of foliar Ni was observed in the context of main stem height (Figs. 4.1 and 4.2; Table 4.1 and 4.3). When the plants were not sprayed with Ni, 1% glyphosate reduced main stem height by 40% (Fig. 4.2; Table 4.3). Foliar Ni application completely protected the plants from the effects of glyphosate on stem elongation. Shoot Ni concentrations of Ni-sprayed plants were on average 70% higher than those of non-treated plants (Table 4.3).

Table 4.3: Shoot dry weight, main stem height and shoot Ni concentration of 43-day-old durum wheat (*Triticum durum* cv. Balcali2000) plants treated with foliar Ni (27 DAS) and glyphosate (29 DAS) at tillering under greenhouse conditions

Foliar Ni (% $NiCl_2.H_2O$)	Gly. Dose (% of rec.)	Shoot DW (g $plant^{-1}$)	Main Stem Height (cm)	Shoot Ni Conc. (mg kg^{-1})
0	0	1.27 ± 0.07	26 ± 4	4.5 ± 0.8
	0.5	1.20 ± 0.03	25 ± 3	5.4 ± 0.4
	1.0	0.95 ± 0.09	15 ± 3	4.1 ± 0.9
0.01	0	1.32 ± 0.09	23 ± 3	7.8 ± 0.6
	0.5	1.30 ± 0.04	25 ± 2	7.8 ± 1.2
	1.0	1.08 ± 0.07	23 ± 4	7.9 ± 0.8

Shoot DW: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (0.09; 0.06; *n.s.*)
Main Stem Height: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (3; *n.s.*; 6)
Shoot Ni Conc.: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (*n.s.*; 0.7; *n.s.*)

Table 4.4: Grain yield, grain number, straw dry weight, main stem height and grain Ni concentration of durum wheat (*Triticum durum* cv. Balcali2000) plants treated with foliar Ni and glyphosate at tillering or booting under greenhouse conditions

Treatment Stage	Foliar Ni (% $NiCl_2.6H_2O$)	Gly. Dose (% of rec.)	Grain Yield (g plant^{-1})	Grain No (per plant)	Grain Ni Conc. (mg kg^{-1})	Straw DW (g plant^{-1})	Main Stem Height (cm)
Tillering	0	0	4.8 ± 0.3	111 ± 8	2.5 ± 0.3	3.8 ± 0.1	73 ± 4
		0.5	4.6 ± 0.3	108 ± 5	2.3 ± 0.2	3.6 ± 0.3	71 ± 3
		1.0	3.5 ± 0.2	90 ± 18	2.6 ± 0.5	3.8 ± 0.2	62 ± 2
	0.01	0	4.7 ± 0.2	117 ± 6	2.8 ± 0.2	4.0 ± 0.2	72 ± 3
		0.5	4.5 ± 0.2	112 ± 11	2.6 ± 0.4	3.8 ± 0.2	73 ± 2
		1.0	4.3 ± 0.2	108 ± 8	2.9 ± 0.3	3.9 ± 0.1	71 ± 3
Booting	0	0	4.7 ± 0.3	110 ± 7	2.9 ± 0.7	3.9 ± 0.1	73 ± 3
		0.5	3.8 ± 0.5	75 ± 16	2.9 ± 0.4	3.9 ± 0.4	66 ± 3
		1.0	2.3 ± 0.4	51 ± 11	4.0 ± 0.6	4.1 ± 0.4	54 ± 5
	0.01	0	4.6 ± 0.4	106 ± 15	5.1 ± 0.7	3.9 ± 0.4	71 ± 2
		0.5	4.1 ± 0.3	109 ± 6	5.1 ± 0.6	4.2 ± 0.2	69 ± 1
		1.0	3.7 ± 0.7	81 ± 19	4.8 ± 0.8	4.1 ± 0.5	63 ± 5

Tillering:

Grain Yield: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (0.4; 0.2; 0.7)
Grain No: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (15; 10; *n.s.*)
Grain Ni Conc: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (*n.s.*; 0.3; *n.s.*)
Straw DW: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (*n.s.*; *n.s.*; *n.s.*)
Main Stem Height: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (4; 2; 7)

Booting:

Grain Yield: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (0.6; 0.4; 1.1)
Grain No: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (17; 12; 29)
Grain Ni Conc: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (*n.s.*; 0.6; *n.s.*)
Straw DW: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (*n.s.*; *n.s.*; *n.s.*)
Main Stem Height: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (4; 2; 6)

Application of 0.5% glyphosate to wheat plants at tillering did not cause a considerable yield loss, whereas the same glyphosate dose reduced grain yield significantly when applied at booting (Table 4.4). The yield loss due to 0.5% glyphosate at booting was about 20% in plants not treated with foliar Ni, but only 10% in Ni-sprayed plants. However, 1% glyphosate reduced grain yield not only when applied at booting but also at tillering. At booting, it halved the grain yield in the absence of foliar Ni, but the loss was limited to 20% in the presence of foliar Ni. The protective effect of foliar Ni was also observed at tillering, where foliar Ni totally prevented a yield loss of 25% due to 1% glyphosate. Number of grains produced per plant exhibited similar trends to grain yield in response to glyphosate and Ni treatments. At booting, foliar Ni effectively counteracted glyphosate, which caused marked grain number reductions in a dose-dependent manner. To a lesser extent, the negative effect of glyphosate on grain number was also observed when 1% glyphosate was applied at tillering, and it was almost completely eliminated by foliar Ni. Foliar Ni application enhanced grain Ni concentrations significantly at both treatment stages, but particularly when applied at booting.

In contrast to yield and grain number, straw dry weight was unaffected by glyphosate, foliar Ni or their interaction (Table 4.4). Nevertheless, stem elongation was impaired by glyphosate. The final main stem height was significantly lowered by only 1% glyphosate in the case of tillering application but by both 0.5% and 1% glyphosate in the case of booting application (Fig. 4.3; Table 4.4). Foliar Ni treatment was partially or completely successful in preventing plants from the dwarfing effect of glyphosate. The plants treated with 1% glyphosate at tillering but not sprayed with Ni were not only dwarfed, but also bore greater numbers of tillers during the generative development (Fig. 4.3), as it was also the case during the vegetative stage (Fig. 4.2).

Figure 4.3: Durum wheat (*Triticum durum* cv. Balcali2000) plants treated with foliar Ni (0.01% $NiCl_2.6H_2O$) and glyphosate (1% of rec.) at tillering or booting under greenhouse conditions

Plants subjected to glyphosate at booting produced deformed grains (Fig. 4.4A). Both the number of wrinkled seeds and the severity of deformation increased with increasing glyphosate concentration. This form disorder was not observed in seeds produced by Ni-sprayed plants. In order to investigate if this visual phenomenon was also linked to a physiological impairment, these seeds were germinated (Fig. 4.4B). The germination test revealed that germination was adversely affected by glyphosate and significantly improved by foliar Ni application to parental plants (Fig. 4.4B; Table 4.5). In addition to lower germination percentages, seeds of 1% glyphosate-treated plants exhibited impaired shoot growth. In the absence of foliar Ni application to parental plants, the mean shoot length of these seedlings was 30% lower than that of controls (Table 4.5). Foliar Ni treatment of the previous generation almost fully prevented this growth disorder.

Table 4.5: Germination percentage and shoot (coleoptile + primary leaf) length of 8-day-old durum wheat (*Triticum durum* cv. Balcali2000) seedlings grown in perlite from seeds produced by plants treated with foliar Ni and glyphosate at booting

Foliar Ni (%)	Gly. Dose (% of rec.)	Germination Percentage (%)	Shoot Length (cm)
0	0	86 ± 5	8.8 ± 1.0
	0.5	79 ± 7	8.6 ± 0.3
	1.0	69 ± 7	6.1 ± 1.1
0.01	0	89 ± 10	8.7 ± 0.6
	0.5	87 ± 7	8.7 ± 0.6
	1.0	81 ± 8	8.2 ± 0.3

Germination Percentage: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (10; 7; *n.s.*)
Shoot Length: $HSD_{0.05}$ (Gly; F.Ni; GlyxF.Ni) = (1.1; 0.7; 1.9)

Figure 4.4: (A) Seeds produced by durum wheat (*Triticum durum* cv. Balcali2000) plants treated with foliar Ni (0.01% $NiCl_2.6H_2O$) and glyphosate (0.5% or 1% of rec.) at booting **(B)** 8-day-old durum wheat seedlings grown in perlite from seeds shown in (A)

4.4. Discussion

Reduced vegetative biomass production in young plants subjected to sublethal glyphosate rates was documented in the literature for several crops (Eker *et al*. 2006; Cakmak *et al*. 2009) and can probably be explained by reduced photosynthetic carbon fixation (Geiger *et al*. 1986; Baylis 2000). Conformably, young wheat plants treated with drift doses of glyphosate in this study produced lower biomass than control plants (Figs. 4.1 and 4.2; Tables 4.1A and 4.3). Also in agreement with previous glyphosate drift simulation studies on cereals (Al-Khatib *et al*. 2003; Ellis *et al*. 2003; Roider *et al*. 2007), the stem elongation of wheat plants was inhibited by sublethal concentrations of glyphosate (Figs. 4.1, 4.2 and 4.3; Tables 4.1B, 4.3 and 4.4). Foliar applications of Ni at sufficiently high concentrations effectively counteracted these adverse effects of glyphosate on biomass production and stem elongation. As shown in Table 4.1, the medium foliar Ni dose, corresponding to 0.42 mM Ni, was able to improve the biomass and, even more so, the height of plants sprayed with 1% of the recommended herbicidal dose of glyphosate, corresponding to 0.21 mM glyphosate. This relative concentration of foliar Ni to glyphosate is, in theory, more than sufficient to make an interaction between Ni and glyphosate in a 1:1 ratio possible. As discussed below, such an interaction could take part in the alleviation of the toxic effects of glyphosate by prior foliar Ni treatment. However, once the glyphosate level was increased from 1% (0.21 mM) to just 1.5% (0.32 mM), neither the medium (0.42 mM) nor the high (0.84 mM) foliar Ni dose provided any benefit at all (Fig 4.1; Table 4.1). These intriguing results suggest that the protective role of Ni against glyphosate drift damage may involve more complex mechanisms than a direct interaction.

Accumulation of shikimic acid due to inhibition of EPSPS is well documented in glyphosate-treated sensitive plants, particularly in young tissues (Hetherington *et al*. 1999; Feng *et al*. 2003; Cakmak *et al*. 2009). In this study, despite its devastating effects on plant growth parameters, 1% glyphosate had not resulted in shikimate accumulation two weeks after application, whereas 1.5% glyphosate had (Table 4.1C). The youngest leaves at the time of glyphosate application turned completely necrotic in two weeks in the 1.5% glyphosate treatment group, and they could therefore not be analyzed for shikimate accumulation at the harvest time. Possibly, if they had been analyzed instead of the second youngest leaves at an earlier stage, significant shikimate accumulation

could have been detected also in the 1% glyphosate group, and also dramatically higher shikimate concentrations could have been measured in the 1.5% glyphosate group.

The economic burden of glyphosate drift to non-target crops is mainly due to losses in yield. Some glyphosate-sensitive crops like soybean and cotton may partially or fully recover from glyphosate drift injury at early developmental stages, (Al-Khatib and Peterson 1999; Ellis and Griffin 2002; Ellis *et al*. 2002), whereas the grain yield of cereal crops such as wheat, corn and rice is much more sensitive to sublethal glyphosate (Ellis *et al*. 2003; Deeds *et al*. 2006; Roider *et al*. 2007; Reddy *et al*. 2010). When compared to vegetative parts, generative tissues of plants are known to be more sensitive to glyphosate injury as they can accumulate much higher levels of glyphosate by acting as terminal sinks and are probably even more dependent on the products of the shikimic acid pathway (Becerril *et al*. 1989; Cakmak *et al*. 2009; Pline *et al*. 2002). Another reason for the higher sensitivity of generative tissues to glyphosate might be the relatively low concentration of some divalent nutrient cations with limited phloem mobility such as Ca, Mn and Fe (Cakmak *et al*. 2009). Possibly, reduced *in planta* complexation of glyphosate with these metals might potentiate glyphosate damage in reproductive organs.

According to Deeds *et al*. (2006) and Roider *et al*. (2007), wheat yield is most sensitive to glyphosate when the generative organ primordia are developing. In practice, glyphosate is commonly applied as a pre-plant burn-down to summer crop fields in the early spring when wheat plants in nearby fields may be particularly susceptible to glyphosate drift injury. Depending on the glyphosate rate and the application stage, yield losses between 20-85% were observed in this study, and these losses could be to a significant extent prevented by foliar Ni treatment (Tables 4.2 and 4.4). It is noteworthy that the glyphosate rate (1% of the recommended concentration) responsible for dramatic yield losses in this greenhouse experiment was close to the lowest rate used in glyphosate drift simulation studies conducted under field conditions where drift doses as high as 12.5% were tested (Ellis *et al*. 2003; Roider *et al*. 2007; Reddy *et al.* 2010). Although glyphosate can be slowly degraded to less toxic compounds in at least some species like soybean, dilution due to continuing biomass production is the major way of gradual detoxification of sublethal glyphosate *in planta* (Maxwell *et al*. 1987; Duke and Powles 2008). Here, wheat subjected to simulated glyphosate drift at booting suffered greater yield reductions than wheat treated with the same glyphosate rate at tillering

(Table 4.4), possibly because earlier applied glyphosate was diluted when plants reached the most glyphosate sensitive stage of their reproductive development.

The marked correlation between the grain yield and the number of grains produced per plant indicates that glyphosate reduces the yield by disrupting the grain formation rather than the grain filling (Fig 4.5A). Notably, the effect of glyphosate on the grain yield correlates also quite well with its effect on the final plant height (Fig. 4.5B); in agreement with Deeds *et al*. (2006) reporting that visual glyphosate injury is a reliable indicator for yield loss in wheat. However, glyphosate treatments had no effect on the straw dry weight (Tables 4.2 and 4.4). These findings conform to the literature indicating higher susceptibility of generative organs to glyphosate injury than vegetative biomass production (Cakmak *et al*. 2009; Pline *et al*. 2002).

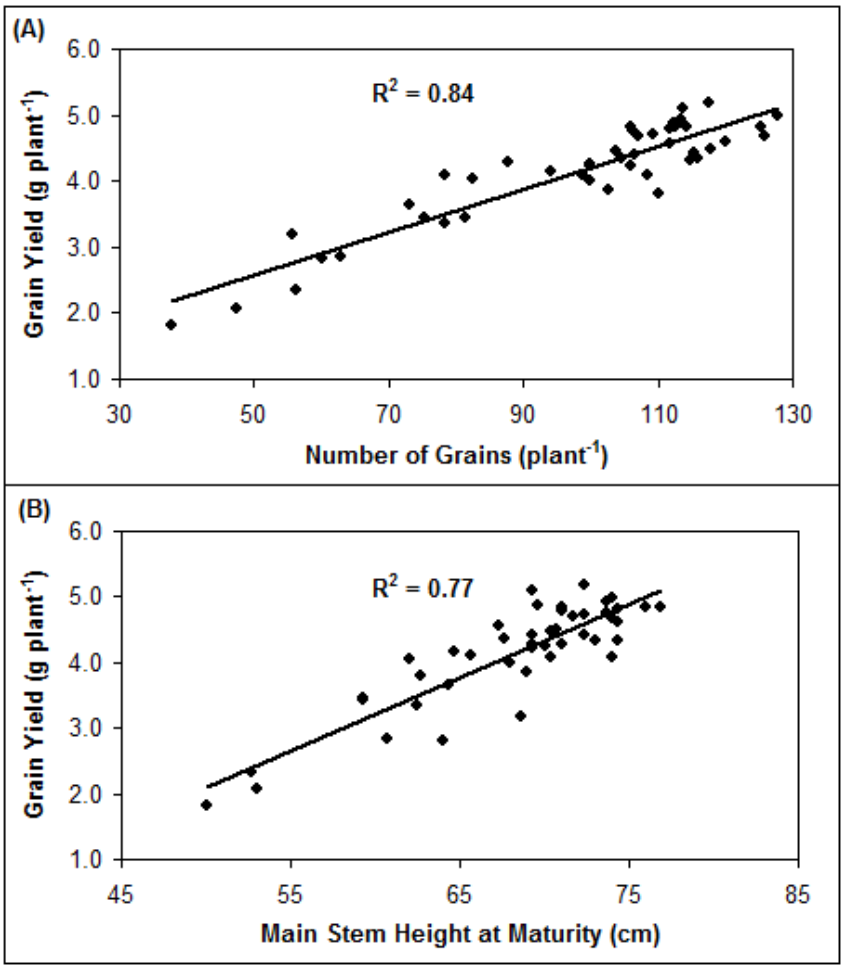

Figure 4.5: Correlation between **(A)** grain yield and number of grains produced per plant and **(B)** grain yield and main stem height at maturity for durum wheat (*Triticum durum* cv. Balcali2000) plants grown for the glyphosate drift simulation study under greenhouse conditions (Data points are taken from Table 3.)

The negative effects of glyphosate on shoot dry weight of young plants, stem elongation and grain yield of wheat were alleviated or eliminated by foliar Ni applications at sufficiently high rates (Tables 4.1, 4.2, 4.3 and 4.4), but not by soil Ni fertilization (Tables 4.1 and 4.2). This situation can be explained by the shoot and grain Ni concentrations, which did not respond to soil Ni application under the present experimental conditions but significantly increased with foliar Ni. The calcareous nature of the soil (described above) might have restricted the bioavailability of soil-applied Ni. Moreover, this finding may be explained by limited shoot translocation of soil Ni. Nickel is known to be mobile and translocated to growing parts of wheat (Page and Feller 2005), but physiologically excess amounts of Ni taken up by the roots are sequestered in the root system and not translocated to the shoot of wheat, which is known as a Ni excluder (Coinchelin *et al*. 2012) In the case of foliar Ni application, leaf apoplastic Ni could account for part of the shoot and straw Ni concentrations reported in Tables 4.1, 4.2 and 4.4, although the samples were washed thoroughly after harvest. However, the grain Ni concentration results provide evidence for the uptake and re-translocation of foliar-applied Ni. Remarkably, neither foliar Ni nor glyphosate applications caused any reduction in the grain concentrations of Ca, Mg, Zn and Fe, which are essential for human health (data not shown).

Despite the facts that Ni is an essential micronutrient for all higher plants (Brown *et al*. 1987a; Marschner 2012; Polacco *et al*. 2013) and its deficiency can be observed even under field conditions (Wood *et al*. 2004), the positive effects of foliar Ni applications in the present study can not be explained by the correction of Ni deficiency; because under given conditions, Ni applications did not provide any benefit in the absence of glyphosate treatment, and glyphosate treatments did not lower the Ni concentrations in any plant part analyzed (Tables 4.1, 4.2, 4.3 and 4.4). It is well documented that sublethal doses of glyphosate can interfere specifically with the uptake and/or translocation of Ca, Mg, Fe and Mn in non-target plants (Duke *et al*. 1983, 1985; Eker *et al*. 2006; Cakmak *et al*. 2009; Su *et al*. 2009). The possibility of an *in planta* interaction between glyphosate and Ni was investigated by Zobiole *et al*. (2010), who reported reduced leaf Ni concentrations in GR soybean upon glyphosate application and suggested that restricted Ni availability to symbiotic bacteria could be responsible for impaired N_2 fixation in glyphosate-treated soybean. However, according to the results

presented here, drift doses of glyphosate do not affect Ni levels in wheat (Tables 4.1, 4.2, 4.3 and 4.4).

It is a well documented phenomenon that tank-mixing of divalent nutrients such as Mn, Zn, Ca and Fe with glyphosate or even hard water can reduce the phytotoxicity of glyphosate, most probably because of the formation of glyphosate-metal complexes. This complex formation may affect the cuticular penetration (Thelen *et al*. 1995; Bailey *et al*. 2002; Chahal *et al*. 2012) and/or cellular uptake of glyphosate by diffusion or active transport mechanisms possibly involving phosphate transporters (Hetherington *et al*. 1998). Losses in glyphosate efficacy due to foliar nutrient applications can be avoided by applying them separately, preferably later than glyphosate (Duke *et al*. 2012). This tank-mix effect can also be eliminated in most cases where foliar nutrients are applied prior to glyphosate (Bernard *et al*. 2005). Since in the present study, the purpose of foliar Ni applications was not just Ni fertilization but the protection of non-target plants from possible glyphosate drift damage, Ni was applied separately to wheat plants a few days before glyphosate and not tank-mixed with it. Nevertheless, Ni ions remaining on the cuticle and in the apoplast may have interacted with glyphosate, interfered with its uptake and inactivated it.

The protective effect of Ni against glyphosate drift in wheat may also be based on the inhibitory role of Ni in ethylene biosynthesis (Lau and Yang 1976; Pennazio and Roggero 1992; Polacco *et al*. 2013). It was documented that plants subjected to sublethal glyphosate can produce higher levels of ethylene (Abu-Irmaileh *et al*. 1979; Stasiak *et al*. 1992), which is well known as a stress hormone (Taiz and Zeiger 2006). Application of ethephon, which is converted into ethylene in plants, to grasses at vegetative growth causes anomalies remarkably similar to glyphosate symptoms, including excessive tillering and height reduction (Poovaiah and Leopold 1973; Moes and Stobbe 1991; Foster *et al*. 1992). Both glyphosate and ethylene can disrupt apical dominance by inhibiting polar auxin transport (Baur 1979; Suttle 1988; Baylis 2000). The observation of epinasty in glyphosate-treated dicots further suggests the involvement of ethylene in symptoms of glyphosate injury, since epinasty is a well known ethylene response (Baur 1979; Smid and Hiller 1981; Baylis 2000). Elevated levels of ethylene in the ambient air as well as ethephon applications can reduce wheat yield by inducing male sterility (Rowell and Miller 1971; Campbell *et al*. 2001; Klassen and Bugbee 2002), which may also be the cause of disrupted grain setting in

glyphosate-affected wheat (Fig 4.5A; Table 4.4). However, further studies are required to clarify if glyphosate injury is linked to ethylene and also if inhibition of ethylene synthesis by Ni is behind its protective role against glyphosate.

The results presented herein show that glyphosate drift affects not only the wheat yield (Tables 2 and 4) but also the physical quality (Fig. 4.4A) and germination capacity of wheat grain (Fig. 4.4B; Table 4.5), in contrast to the results by Deeds *et al*. (2006) who claimed that glyphosate did not impair the germination of harvestable wheat grains. Apparently, foliar Ni applications can prevent the detrimental effects of glyphosate on the germination capacity of harvestable seeds as well as the seedling vigor (Fig. 4.4B; Table 4.5), which might have severe implications on the yield of the next generation.

4.5. Conclusions

The most commonly used herbicide; glyphosate is still gaining popularity with the increasing adoption of glyphosate-resistant crops and no-tillage cropping systems. Glyphosate drift to non-target crops in nearby fields is a growing practical problem and can cause serious economic losses, mainly due to its detrimental effects on yield. Wheat, which is a very important staple crop, is highly susceptible to glyphosate injury, particularly at early stages of generative development. The results presented in this study indicate that glyphosate rates as low as 1% or even 0.5% of the recommended herbicidal rate can disrupt seed set and thus significantly reduce wheat yield under controlled conditions. Foliar Ni applications at sufficiently high concentrations can apparently enhance the resistance of wheat to glyphosate drift damage. Not only yield loss but also the adverse effects of glyphosate on plant growth and seed quality can be partially or totally prevented by foliar Ni treatment. Direct binding of Ni to glyphosate and/or the role of Ni as an ethylene inhibitor may be behind the reported protective effects of Ni; but the exact mechanism remains to be elucidated. Foliar Ni application appears to have a great potential as a means to eliminate glyphosate drift injury to wheat and possibly other non-target crops and should be optimized under field conditions.

(C) GENERAL DISCUSSION AND CONCLUSIONS

Nickel was the last mineral nutrient to be accepted as essential for all higher plants (Marschner 2012). Since it is required in very low amounts, it is classified as an ultra-micronutrient and has generally been neglected as a plant nutrient (Asher 1991). Soilless culture systems such as hydroponics were preferred in most studies on Ni nutrition of plants as most soils contain more than sufficient Ni to meet the demand of plants (Brown *et al.* 1987a; Gerendas *et al.* 1999). However, clear evidence for Ni deficiency under field conditions was documented in pecan orchards (Wood *et al.* 2004). Although commercial Ni fertilizers are available, they are still not commonly used in crop production. Mounting evidence suggests that Ni deficiencies may be relevant in practice, especially in the production of crops with relatively high Ni requirements such as ureide-transporting nuts and legumes (Bai *et al.* 2006). Nickel deficiency can also be induced or aggravated by excessive liming practices, use of high-purity fertilizers and increasing applications of potentially competing minerals, such as Cu, Mn and Zn, for fertilization or fungicidal purposes, which can decrease the bioavailability of Ni (Brown 2006). Moreover, due to the critical role of Ni in urea metabolism, extensive use of urea fertilizers can increase the Ni requirement of crops (Polacco *et al.* 2013). It is also noteworthy that hidden, *i.e.* non-symptomatic deficiencies of essential nutrients are common in agriculture and can result in significant yield losses (Marschner 2012). Hidden deficiencies of Ni that go unnoticed may also be common.

Since urea assimilation is the only proven metabolic process which Ni is directly involved in, almost all Ni deficiency studies conducted in hydroponics focused on reduced urease activity and the accumulation and toxicity of either internally produced or externally supplied urea (Eskew *et al.* 1983, 1984; Walker *et al.* 1985; Gerendas and Sattelmacher 1997). In the literature, impairment of urea assimilation due to Ni deficiency was associated with reductions in vegetative growth and leaf chlorophyll levels as well as disruptions in amino acid and organic acid metabolisms in urea-fed

plants (Brown *et al.* 1990; Gerendas *et al.* 1999; Bai *et al.* 2006). The experiments described in Chapters 1 and 2 were designed to gain a deeper insight into the role of Ni in the urea metabolism of soybean and investigate the potential of seed Ni reserves in this respect. In Chapter 1, urea was applied to the foliage of soybean plants fed with marginal levels of NO_3^- via the nutrient solution whereas in Chapter 2, either urea or NO_3^- was supplied via the nutrient solution as the sole N source. Foliar urea applied to Ni-deprived plants caused toxicity symptoms, including marginal necrosis and whole leaf chlorosis followed by leaf abscission, and could not be efficiently utilized (Chapter 1). In plants supplied with urea as the only N source in nutrient solution, the consequences of Ni starvation were significantly impaired growth, physiological N deficiency, limited N uptake and reduced NUE (Chapter 2). Using Ni-rich seeds were almost as effective as Ni supplied via the nutrient solution in alleviating the problems associated with urea nutrition in both chapters (Chapters 1 and 2). These findings indicate that the seed can be a physiologically relevant Ni reservoir, at least for early vegetative growth.

Improved Ni nutrition also enhanced N remobilization from source tissues, even in the absence of foliar urea (Chapter 1), which is most probably related to improved recycling of metabolic urea. Seed Ni may also be critical for the efficient utilization of seed N reserves during germination where substantial amounts of urea are produced by Arg catabolism (Witte 2011). Moreover, it is well documented that most of the seed urease activity is accounted for by the activity of embryo specific urease which has a defensive rather than an assimilatory role (Follmer *et al.* 2004; Carlini and Polacco 2008). Therefore, Ni-poor seeds having severely depressed urease activities (Chapter 1) may also have an increased susceptibility to pathogens.

Critical Ni deficiency levels for crops were mentioned in only a few reports in the literature where 100 µg Ni per kg dry weight appeared to be sufficient for maximizing vegetative growth, grain viability and urease activity (Brown *et al.* 1987a, b; Gerendas *et al.* 1999). It was also documented that Ni is highly mobile in the phloem and easily translocated from source to sink tissues (Neumann and Chamel 1986; Page and Feller 2005). In agreement, youngest leaves of both soybean and wheat were remarkably richer in Ni than the remaining shoot tissues, and wheat grains had significantly higher Ni concentrations than wheat straw (Chapters 1, 2, 3 and 4). This may indicate a relatively high requirement of growing parts for Ni and/or be an inevitable consequence

of the high phloem mobility of Ni. Due to this biased distribution of Ni within the plant body, defining critical Ni deficiency levels on the whole shoot basis would be deceptive.

According to the results presented in Chapter 3, soil-grown wheat containing much higher Ni concentrations than the proposed critical levels can still significantly benefit from additional applications of Ni in a conditional manner. Higher N supply appears to increase the demand of wheat for Ni, which enhances the productivity of tiller culms and thus increases both the HI and NUE. The beneficial effects of foliar Ni applications were observed only in urea sprayed plants grown with higher N, whereas those of soil Ni application were not dependent on urea fertilization. These findings suggest that the Ni responses described here can not be explained just by the role of Ni in urea metabolism but probably involve more complex mechanisms such as alterations in phytohormone metabolism as discussed below.

A totally different and novel effect of Ni applications was documented in Chapter 4. The detrimental effects of sublethal glyphosate on the growth, development, grain yield and seed quality of durum wheat were ameliorated by prior foliar Ni applications. Based on the well documented interactions between glyphosate and divalent metals (Motekaitis and Martell 1985; Cakmak *et al.* 2009; Duke *et al.* 2012) and the role of Ni as an ethylene inhibitor (Lau and Yang 1976; Itamura *et al.* 1997), two possible mechanisms were discussed: Foliar-applied Ni can directly detoxify glyphosate by forming glyphosate-Ni complexes on the cuticle, in the apoplast or within plant cells, particularly in the growing parts where both glyphosate and Ni are known to accumulate. Alternatively, the adverse effects of glyphosate can be attributable to induced ethylene production (Abu-Irmaileh *et al.* 1979; Stasiak *et al.* 1992) and enrichment of plant tissues with Ni can protect wheat from glyphosate by acting as an ethylene biosynthesis inhibitor. If the latter mechanism is relevant *in planta*, it would suggest that extra Ni can be beneficial for plants under various stress conditions such as drought (Beltrano *et al.* 1997), heat (Hays *et al.* 2007), and flooding (English *et al.* 1995) which can all boost ethylene production. Although there was no stress application in the previous chapter (Chapter 3), unanticipated stress factors such as high planting density may be behind the observed beneficial effect of Ni. Due to the role of Ni as an ethylene biosynthesis inhibitor, Ni application can also be a promising tool for

increasing the shelf-life of fruits (Zheng *et al.* 2006) and vase-life of flowers (Jamali and Rahemi 2011).

Statistically significant yield losses due to Ni deficiency were observed in first generation soybean plants grown with NO_3^- (Chapter 1). For the first time in the literature, significant yield responses to Ni applications were reported in a model experiment in the absence of urea fertilization (Polacco *et al.* 2013). Depending on the conditions, grain yield of wheat was also improved by Ni applications as reported in Chapters 3 and 4. In all cases where seed yield responded to Ni fertilization (Chapters 1, 3 and 4), the yield increases were due to improved seed setting rather than seed filling. This finding shows that Ni nutrition has a critical role in the early reproductive development of plants.

Despite the evidence indicating that Ni is essential for also animals and humans, Ni deficiency has not been observed in humans (Anke *et al.* 1984; Nielsen 1984; Spears 1984). In animals, symptoms of Ni deficiency include depressed growth, anemia and disturbances in iron and carbohydrate metabolism. For humans, Ni toxicity is generally a more relevant concern, although high levels of Ni are required to cause toxicity, owing to the tight homeostatic control of Ni in the body. Consumption of plant foods is the most important route of exposure to Ni (Flyvholm *et al.* 1984). In this thesis, the Ni concentrations for soybean and wheat seeds (Chapters 1, 3 and 4) were generally within the normal range reported for Ni-rich foods. Moreover, experiments with both soybean and wheat revealed that Ni applications did not have any negative impact on the concentrations of important minerals essential for human health like Fe and Zn (Chapters 1 and 3).

The results presented in this thesis show that Ni deserves further attention as a plant micronutrient. Nickel-deficient soybean plants exhibit impaired growth, yield losses, toxicity symptoms upon foliar urea applications, reduced root uptake of urea, lower NUE and physiological N deficiency symptoms (Chapters 1 and 2). Use of seeds with high Ni concentrations can be an environmentally friendly, economical and effective alternative to Ni fertilization. In soil-grown wheat, Ni applications can also improve grain yield and NUE under ample N supply and provide protection against glyphosate drift injury (Chapters 3 and 4). Future studies should address the potential of Ni nutrition under field conditions, the applicability of these results to other crops and the exact mechanisms behind the reported positive effects of Ni in plant production.

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