Potential of imaging techniques for *in-situ* investigation of roots – Root growth response to different N-forms in soil

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Abstract

The investigation of root growth in the soil represents a challenge because the direct observation and analysis of roots *in-situ* is not possible without suitable tools. Classical methods usually lead to the destruction of the intact soil-root system. Typical analytical methods on the laboratory scale additionally require soil removal and clean root washing for later analysis. This means that it is not possible to observe the same plant over time and information on the spatial arrangement of the root architecture is lost. However, this information is important, as the spatial characteristics of the root system in the soil are directly related to the uptake of water and nutrients by the plant. This is connected to the aim of modern agriculture to increase nutrient use efficiency.

Imaging techniques have the potential to overcome these shortcomings. Originally developed for medical applications, neutron tomography, magnetic resonance imaging and especially industrial X-ray computed tomography (CT) are among the most important imaging techniques of our time. Each method has its specific advantages and disadvantages, as well as typical areas of application. For the investigation of root growth in soil, X-ray CT has shown to be particularly valuable, although there are limitations for this method as well.

Within the scope of this work, X-ray CT was the method of choice for root-soil imaging. The potential negative impact of X-rays on root growth of *Vicia faba* and *Hordeum vulgare* was investigated in detail. Thereafter, time resolved X-ray CT was used to analyse the reaction of root growth to heterogeneous supply of different nitrogen (N) forms (nitrate and ammonium) in the soil. The simultaneous analysis of the soil solution chemistry associated with urea-based N application and its turnover within the soil was a central part of this thesis. This methodological combination made it possible to study root growth dynamics in the direct context of soil solution dynamics.

The vast majority of past studies investigating the response of root growth to nitrate (NO_3^-) and ammonium (NH_4^+) supply have been obtained in nutrient solutions and other artificial growth media. The general conclusion derived from these studies is that NO_3^- leads to an increased elongation of the roots, while NH_4^+ causes an increased branching of the roots. Transfer of these results to soil based systems is critical and has been inconclusive so far because soil is a much more complex system (in terms of *e.g.* N-turnover, N-mobility and sorption, physical constraints on root expansion etc.) and methods that enable the study of chemical dynamics simultaneously to changes in root system architecture have been lacking. Here, the issue was

revisited using X-ray CT in combination with soil solution sampling for two plant species with contrasting root systems.

To enable these investigations, soil column experiments were performed under climate chamber conditions using a natural silty clay loam soil (subsoil of a haplic Luvisol). Plant growth experiments with *Vicia faba* (faba bean) and *Hordeum vulgare* (barley) were complemented by chemical analyses of soil solution and classical extraction methods for the determination of mineral N-forms in the soil. Root growth was recorded by X-ray CT or by classical destructive sampling and analysis with WinRHIZO after root washing.

Both, the root growth reaction to X-ray exposure and the root growth response to NO_3^- and NH_4^+ revealed distinct differences between the two plant species. Root growth of *Vicia faba* was significantly reduced by the chosen X-ray dose, while root growth of *Hordeum vulgare* was not affected by the same dose. Conversely, the root growth of *Hordeum vulgare* showed a marked response to both N-forms, while no significant differences were found for *Vicia faba*.

The methodological approach of combining soil chemical analysis with simultaneous root growth assessment has proven to be useful and valuable. This has enabled to link the root system architecture with the chemical conditions of the soil solution at different points in time. With this approach it is possible to detect changes in the chemical properties of the soil and simultaneously analyse how, where and when root growth reacts to these changes.

X-ray CT as an imaging technique is particularly suitable for such time-resolved studies, as the root growth of one and the same plant can be examined non-destructively over time. However, the experimental setup and CT settings must be carefully chosen to achieve promising results. Improvements are necessary in order to cover plant species with small root diameters like barley and other grasses in high quality.

Additional experiments using similar approaches would be useful in order to further expand the acquired knowledge and to verify the results presented in this thesis. This can contribute to a better understanding of the adaptation strategies of root growth to local nutrient availability in the soil and to improve nutrient use efficiency. This needs to be addressed for different plants, soils and fertilisers. With the necessary adaptations, the approach can also be used for other questions with similar spatial and temporal demands, such as the effect of pollutants on root growth or mucilage exudation.

Zusammenfassung

Die Untersuchung des Wurzelwachstums im Boden stellt eine Herausforderung dar, da die direkte Beobachtung und Analyse von Wurzeln *in situ* ohne geeignete Werkzeuge nicht möglich ist. Klassische Untersuchungsmethoden ziehen meist eine Zerstörung des intakten Boden-Wurzel-Systems nach sich. Auch die typischen Analysemethoden auf der Laborskala erfordern im Normalfall die Entfernung des Bodens und ein sauberes Auswaschen der Wurzeln für eine spätere Untersuchung. Dadurch ist eine Untersuchung derselben Pflanze im zeitlichen Verlauf nicht möglich und Informationen zur räumlichen Struktur der Wurzelarchitektur gehen verloren. Diese Informationen sind jedoch wichtig, da die räumlichen Eigenschaften des Wurzelsystems im Boden in direktem Zusammenhang mit der Erschließung des Bodens und damit der Aufnahme von Wasser und Nährstoffen durch die Pflanze stehen. Dies steht in Verbindung mit dem Ziel moderner Landwirtschaft, die Effizienz der Nährstoffnutzung zu erhöhen.

Bildgebende Verfahren haben das Potenzial, diese methodischen Defizite zu überwinden. Die für die medizinische Anwendung entwickelten Verfahren Neutronentomographie, Magnetresonanztomographie und insbesondere die industrielle Röntgen-Computertomographie (CT) zählen zu den wichtigsten bildgebenden Verfahren unserer Zeit. Jedes Verfahren hat seine spezifischen Vor- und Nachteile, sowie typische Anwendungsgebiete. Für die Untersuchung des Wurzelwachstums im Boden hat sich CT als besonders wertvoll erwiesen, jedoch existieren auch für diese Methode Einschränkungen.

Im Rahmen dieser Arbeit war CT die Methode der Wahl für die Bildgebung des Systems Wurzel-Boden. Die potentiell negativen Auswirkungen der Röntgenstrahlen auf das Wurzelwachstum von *Vicia faba* (Ackerbohne) und *Hordeum vulgare* (Geste) wurden im Detail untersucht. Anschließend wurde Röntgen-CT als Methode der Wurzelerfassung verwendet, um die Reaktion des Wurzelwachstums auf verschiedene, heterogen im Boden vorliegende Stickstoff (N)-Formen (Nitrat und Ammonium) zu analysieren. Die gleichzeitige Analyse der bodenchemischen Bedingungen im Zusammenhang mit der auf Harnstoff basierenden Stickstoff-Applikation und ihrer Umsetzung im Boden waren zentraler Bestandteil dieser Arbeit. Diese methodische Kombination ermöglichte es, die Dynamik des Wurzelwachstums im direkten Kontext der chemischen Dynamik der Bodenlösung zu untersuchen.

Die überwiegende Mehrheit der bisherigen Studien, welche die Reaktion des Wurzelwachstums auf die Verfügbarkeit von Nitrat (NO_3^-) und Ammonium (NH_4^+) untersucht haben, wurde in

Nährlösung und anderen künstlichen Wachstumsmedien durchgeführt. Die daraus abgeleitete generelle Schlussfolgerung lautet, dass NO_3^- zu einer verstärkten Elongation der Wurzeln führt, während NH_4^+ eine erhöhte Verzweigung der Wurzeln bewirkt. Die Übertragung dieser Ergebnisse auf bodenbasierte Systeme ist kritisch und nicht eindeutig, da der Boden ein wesentlich komplexeres System darstellt (hinsichtlich z.B. N-Umsatz, N-Mobilität und Sorption, physikalische Einschränkungen des Wurzelwachstums etc.). Außerdem gab es bisher nur wenige Ansätze, die chemische Dynamik im Boden gleichzeitig mit Veränderungen der Wurzelsystemarchitektur zu untersuchen. In der vorliegenden Arbeit wurde dieses Thema durch die Kombination aus bildgebender CT-Anwendung und der chemischen Untersuchung der Bodenlösung für zwei Pflanzenarten mit kontrastierenden Wurzelsystemen neu untersucht.

Um diese Untersuchungen zu ermöglichen, wurden Säulenexperimente unter Klimakammerbedingungen mit einem natürlichen schluffigen Lehmboden (Unterboden einer eutrophen Parabraunerde) durchgeführt. Pflanzenwachstumsexperimente mit *Vicia faba* und *Hordeum vulgare* wurden durch chemische Analysen der Bodenlösung und klassische Extraktionsverfahren zur Bestimmung mineralischer N-Formen im Boden kombiniert. Das Wurzelwachstum wurde mittels CT oder durch klassische destruktive Probenahme und Analyse mit WinRHIZO nach dem Auswaschen erfasst.

Sowohl die Wurzelwachstumsreaktion auf die Exposition mit Röntgenstrahlen als auch die Reaktion auf eine heterogene Versorgung mit NO_3^- und NH_4^+ zeigten deutliche Unterschiede zwischen den beiden Pflanzenarten. Das Wurzelwachstum von *Vicia faba* wurde durch die verwendete Röntgendosis signifikant reduziert, während das Wurzelwachstum von *Hordeum vulgare* durch die gleiche Dosis nicht beeinflusst wurde. Umgekehrt zeigte das Wurzelwachstum von *Hordeum vulgare* eine deutliche Reaktion auf beide N-Formen, während für *Vicia faba* keine signifikanten Unterschiede festgestellt wurden.

Der methodische Ansatz der Kombination von bodenchemischer Analyse mit gleichzeitiger Erfassung des Wurzelwachstums hat sich als nützlich und wertvoll erwiesen. Dadurch konnte die Architektur des Wurzelsystems mit den chemischen Bedingungen der Bodenlösung zu verschiedenen Zeitpunkten verknüpft werden. Mit diesem Ansatz ist es möglich, Veränderungen der chemischen Eigenschaften des Bodens zu erkennen und gleichzeitig zu analysieren, wie, wo und wann das Wurzelwachstum auf diese Veränderungen reagiert.

CT als bildgebendes Verfahren eignet sich besonders für solche zeitaufgelösten Studien, da das Wurzelwachstum ein und derselben Pflanze über die Zeit im Boden untersucht werden kann.

Allerdings müssen die experimentellen Randbedingungen und CT-Einstellungen sorgfältig ausgewählt werden, um aussagekräftige Ergebnisse zu erzielen. Technische Verbesserungen sind notwendig, um Pflanzenarten mit kleinem Wurzeldurchmesser (wie Gerste und andere Gräser) in hinreichender Qualität erfassen zu können.

Weitere Experimente mit ähnlicher Herangehensweise wären sinnvoll, um die gewonnenen Erkenntnisse weiter auszubauen und zum Beispiel für weitere Pflanzenarten, unterschiedliche Böden und andere Düngemittel zu überprüfen. Dies kann zu einem besseren Verständnis der Anpassungsstrategien des Wurzelwachstums an lokale Nährstoffverfügbarkeit im Boden und damit zur Verbesserung der Nährstoffnutzungseffizienz beitragen.

Mit den notwendigen Anpassungen kann der Ansatz auch für andere Fragestellungen mit ähnlichen räumlichen und zeitlichen Anforderungen genutzt werden, wie z.B. zur Untersuchung der Wirkung von Schadstoffen auf das Wurzelwachstum oder die Exsudation von Mucigel.

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List of Abbreviations

2D	two-dimensional
3D	three-dimensional
BfS	German Federal Office for Radiation Protection
CEC	cation exchange capacity
C_{org}	organic carbon
CT	computed tomography
cv.	*cultivarietas*
DAP	days after planting
DCD	dicyanodiamide
e.g.	*exempli gratia*
FAO	Food and Agriculture Organization of the United Nations
Fig.	figure
HMD	half mean distance
i.e.	*id est*
L.	botanical author citation of Carl Linnaeus
LR	lateral root
MRI	magnetic resonance imaging
NH_4^+	ammonium
NI	nitrification inhibitor
NO_3^-	nitrate
ns	not significant
NT	neutron tomography
NUE	nutrient use efficiency
Ø	diameter
p	level of significance
RDH	root distance histogram
ROI	region of interest
RSA	root system architecture

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Publications

The present doctoral thesis is directly based upon the following publications:

- **Blaser SRGA**, Schlüter S, Vetterlein D (2018): "How much is too much? Influence of X-ray dose on root growth of faba bean (*Vicia faba*) and barley (*Hordeum vulgare*). *PLoS ONE* 13(3):e0193669
- **Blaser SRGA**, Koebernick N, Schlüter S, Vetterlein D (2020): "The 3-D imaging of roots growing in soil". Book Chapter in "The Root Systems in Sustainable Agricultural Intensification", *John Wiley & Sons (accepted).*
- **Blaser SRGA**, Koebernick N, Spott O, Thiel E, Vetterlein D (2019): "When Drew meets soil - Dynamics of localised supply of nitrogen-species in soil and their relevance for root system morphology – A comparison between faba bean (*Vicia faba*) and barley (*Hordeum vulgare*)", *Scientific Reports (under revision).*

In addition, the present doctoral thesis is partly based on methods, ideas and applications from the following publications with co-authorship:

- Carminati A, Vetterlein D, Koebernick N, **Blaser S**, Weller U, Vogel H.J. (2012): Do roots mind the gap? Plant and Soil, 367, 651-661.
- Koebernick N, Schlüter S, **Blaser S**, Vetterlein D (2018): Root-soil contact dynamics of *Vicia faba* in sand. Plant and Soil, 431:417-431 doi:10.1007/s11104-018-3769-4.
- Schlüter S, **Blaser S**, Weber M, Schmidt V, Vetterlein D (2018): Quantification of root growth patterns from the soil perspective via root distance models. Frontiers in Plant Science, 9:1084. doi: 10.3389/fpls.2018.01084.
- Nwanko C, **Blaser S**, Vetterlein D, Neumann G, Herrmann L (2018): Seedball-induced changes of root growth and physicochemical properties – a case study with pearl millet. Journal of Plant Nutrition and Soil Science 181:768-776.
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- Gao W, **Blaser S**, Schlüter S, Shen J, Vetterlein D (2019): Effect of localised phosphorus application on root growth and soil nutrient dynamics in situ – comparison of maize (*Zea mays*) and faba bean (*Vicia faba*) at the seedling stage. Plant Soil doi:10.1007/s11104-019-04138-2

1 Aims, outline and objectives

1.1 Aim of the thesis

The aim of the present thesis is to address the topic of root growth development in soil and its visualisation in general, and specifically the response of root growth and root architecture to different forms of nitrogen (N) in soil.

The thesis comprises two major topics that are both related to the non-invasive visualisation and characterisation of root growth dynamics in soil: i) the influence of X-ray dose on root growth for two contrasting plant species (*Vicia faba* and *Hordeum vulgare*) and ii) the response of these two plant species to a localised application of urea-based N fertilisers.

The study of root growth dynamics in soil is a challenge, especially due to the opaque nature of soil. New imaging techniques can help to obtain information about the true geometric spatial details of root system architecture *in-situ*. In addition, emerging root growth dynamics can be observed over time by repeated recording. All techniques which may be considered to overcome this challenge like magnetic resonance imaging (MRI), neutron tomography (NT) and X-ray computed tomography (CT) have strengths but also limitations. Moreover, every method of investigation has to be tested for potential adverse effects on root growth itself.

X-ray computed tomography has evolved to a widely used imaging method to visualise and analyse dynamics of root system development in soil. Large steps of technical development have been made, if early studies from the 1990's (Watanabe *et al.*, 1992; Tollner *et al.*, 1994; Heeraman *et al.*, 1997) are compared to recent studies (Flavel *et al.*, 2017; Blaser *et al.*, 2018; Schlüter *et al.*, 2018; Gao *et al.*, 2019b). The potential influence of the X-ray radiation itself on root development was neglected in most cases so far. Only a few studies included a control treatment to observe a potential impact (Flavel *et al.*, 2012), and even fewer studies are available that systematically addressed the issue (Zappala *et al.*, 2013). Due to the laborious nature of experimental investigations including X-ray CT as the method of choice and the resulting limitation in regards of replicate samples and number of treatments, a control treatment without application of X-rays was omitted in most studies. To overcome this issue, specific experiments were designed within this work to study the influence of a moderate X-ray dose that is typically applied in root growth experiments, varying the total X-ray dose that is received per plant by application of two different scanning frequencies, namely every second and every fourth day,

in comparison to a non-scanned control treatment. This setup is carried out for two plant species with contrasting root systems.

Root growth response to different N forms was mainly studied in artificial systems like pure quartz sand percolated with nutrient solution in the well-known work from Drew and colleagues in the 1970's (Drew *et al.*, 1973; Drew, 1975; Drew and Saker, 1975; Drew and Saker, 1978). Other systems that had often been applied for these studies are agar plates (*e.g.* Gruber *et al.* (2013), Lima *et al.* (2010), Remans *et al.* (2006), Zhang *et al.* (1999), Zhang and Forde (1998)), or nutrient solution (*e.g.* Bloom *et al.* (2006), Granato and Raper (1989), Ogawa *et al.* (2014), Schortemeyer *et al.* (1993), Tian *et al.* (2008)). These systems were usually operated under constant conditions (in terms of nutrient concentration and pH of the growth media), which can only be transferred to soil conditions to a very limited extent. Studies that have used soil (*e.g.* Anghinoni and Barber (1988), Bloom *et al.* (1993), Pan *et al.* (2016), Van Vuuren *et al.* (1996), Xu *et al.* (2014), Zhang and Barber (1993)), varied markedly in their methodological approaches and results. Furthermore, unfortunately most of these studies have omitted either the analysis of N-dynamics in soil, soil solution or root growth and usually focussed on low N-concentrations.

In this thesis the topic is brought to a more natural condition, using real soil material and commercial urea fertilisers with and without nitrification inhibitor. Monitoring of the nitrogen turnover in the soil in combination with monitoring of plant growth response to the soil conditions related to the local N-supply concomitantly over time is the second major topic of this thesis. Urea turnover in soil is well known in general, but the reaction velocity is different for every soil. Soil chemistry and plant growth development are monitored together under climate chamber conditions to enhance understanding of both dynamics.

1.2 Outline of the thesis

The thesis is structured in seven Chapters. After this first short introductory chapter, the theoretical background and state-of-the-art is given in Chapter 2. "The 3-D imaging of roots growing in soil", represents Chapter 3 (book chapter). Chapter 4 is entitled "How much is too much? Influence of X-ray dose on root growth of faba bean (*Vicia faba*) and barley (*Hordeum vulgare*)" (published paper). Chapter 5 is called "When Drew meets soil - Dynamics of localised supply of nitrogen-species in soil and their relevance for root system morphology – A comparison between faba bean (*Vicia faba*) and barley (*Hordeum vulgare*)" (paper under

revision). Afterwards, a combined discussion of the previous chapters is given, followed by the final Chapter 7, giving conclusions and an outlook on the respective topics.

This present thesis is denoted as a monography. However, it is directly based on a book chapter and two papers (one is published, one is under revision), representing the central backbone of this thesis. Many experiments and pre-experiments were carried out to test several setups and conditions. These early studies are not considered explicitly in this thesis but they were needed to work towards a proper setup and to reduce methodical errors as far as possible. This has enabled an intensive examination of the following objectives and marked progress in many of the following questions.

1.3 Objectives of the thesis

The objectives discussed in this thesis will be:

1. Plant roots – definition, growth and analysis
    a. Why are roots important to us?
    b. How do roots explore the soil?
    c. What possibilities do we have to visualise and analyse roots and their growth behaviour?
2. Influence of X-ray radiation on root growth and plant development
    a. Does X-ray radiation have an influence on root growth and plant development?
    b. Is it different for two specific, typical scanning frequencies?
    c. Are root and shoot affected similarly?
    d. Do two plant species with different root system architecture react differently?
3. Urea turnover in a natural soil substrate, N-dynamics, chemical conditions in soil solution and root growth response to different N-forms
    a. How is the turnover of urea with and without NI specifically characterised for the soil used in the experiments in this thesis under controlled laboratory conditions?
    b. Can we draw conclusions for the influence of plant nutrient uptake by monitoring urea turnover and chemical composition of soil solution in locally fertilised soil patches?
    c. How do different plant species with varying root system architecture respond to the given N species dynamics in soil solution?

2 Theoretical background and state-of-the-art

2.1 Roots – definition, growth and analysis

Vascular plants (also known as higher plants) are an essential part of the Kingdom of PLANTAE (Haeckel, 1866; Engler, 1907). These also comprise gymnosperms and angiosperms, the latter including *Vicia faba* and *Hordeum vulgare*, which have a central role in the present thesis.

Angiosperms (also known as flowering plants) are composed of leaves, stem (both together are considered as the shoot) and roots. While the above ground organs are essentially responsible for photosynthesis, the roots have three main tasks: anchorage in the soil, water uptake and nutrient uptake (Gregory, 2008). Additionally, roots also participate in secondary functions like phytohormone synthesis and photoassimilate storage (Osmont *et al.*, 2007).

Root system architecture

In order to fulfil these tasks, roots explore the soil and thereby form a certain spatial structure, the root system architecture (RSA). RSA is defined as the three-dimensional structure of the root system, including the primary root, branch roots and root hairs (Osmont *et al.*, 2007). The term "branch roots" can be misleading and is less common in recent literature, although the term "branch" reflects the morphologic appearance of the root system well.

While the primary root is formed during embryogenesis, lateral roots (LR) are formed postembryonically from existing roots. Mainly originating from the pericycle, the process of LR formation can take place at roots of different orders and subsequently create the next level of root order. Apart from these root types, there are adventitious roots originating from the shoot, aerial roots, crown, storage or cluster roots, leading to a wide variety of final root shapes and root system architectures.

The morphological diversity of root systems is remarkable across plant species and hence, a simple classification scheme is not applicable (Fitter, 1987). However, in most cases a distinction between two major root system morphologies is sufficient for angiosperms: First, taproot systems (also termed allorhizic) and second, homorhizic systems. *Vicia faba* and *Hordeum vulgare*, the two main species which have been selected for the experiments presented in this thesis, are characteristic representatives for those two major root system morphologies.

Allorhizic systems are typical for dicotyledon plants and apart from *Vicia faba* (faba bean), *Solanum lycopersicum* (tomato) and *Arabidposis* (wall-cress) are typical representatives. The allorhizic system is dominated by the primary root (termed tap root in this case), from which the first order LRs emerge (Esau, 1965). These LR can then, in turn, also form LRs which then are considered second order laterals. If they branch further, the resulting roots are called third order lateral roots and so on.

In contrast, the root system of monocotyl plants like *Hordeum vulgare* (barley) or *Triticum aestivum* (wheat) is characterised by the growth of adventitious roots in addition to the primary roots, which are often termed seminal roots. Some monocots like *Zea mays* (corn) or *Oryza* (rice) can form shoot-borne roots, termed brace or crown roots (Hochholdinger *et al.*, 2004). As LR can be formed from all of these root types, the morphologic appearance is referred to as "bushy" (Osmont *et al.*, 2007; Lima *et al.*, 2010; Mounier *et al.*, 2014). This can result in a particularly intensive exploration of the soil volume, whereas the root zones from tap root systems in the soil tend to overlap less.

Root tropisms

These very contrasting RSA for dicots and monocots emphasise the genetic factors responsible for the spatial arrangement of the roots. In addition, various biotic and abiotic exogenous factors are responsible for the developmental plasticity of root system architecture (Osmont *et al.*, 2007). "Morphological plasticity" (de Kroons and Hutchings, 1995; Fransen *et al.*, 1998; Johnson and Biondini, 2001; Bingham and Bengough, 2003), or "the plastic plant" (Hodge, 2004; Hodge, 2006) are terms related to that phenomenon. The growth response to certain stimuli is called "tropism" (Esmon *et al.*, 2004; Gilroy, 2008; Gregory, 2008). There are many forms of tropisms; among the most important are gravitropism to gravity, thigmotropism to touch, phototropism to light and hydrotropism to water. Most of those are known for a long time and some trace back to Charles Darwin (Darwin, 1897), but more detailed studies about these tropisms were conducted later (Onderdonk and Ketcheson, 1973; Fitter, 1987; Kaspar and Bland, 1992; Boonsirichai *et al.*, 2002; Correll and Kiss, 2002; Eapen *et al.*, 2005).

An explicitly defined "tropism" towards nutrients, or specifically nitrogen, does not exist, but the idea that root growth responds to the availability and concentration of nutrients is generally recognised. For instance, this was addressed by Schnepf *et al.* (2018) with the term "chemotropism". The works of Drew and colleagues in the 1970s (Drew *et al.*, 1973; Drew,

1975; Drew and Saker, 1975; Drew and Saker, 1978) are particularly known for this, but there have also been studies on this topic before, such as by Wiersum (1958) or Hackett (1972).

Root visualisation and analysis

To analyse any type of tropism or root growth response in general, an appropriate setup has to be chosen. Experimental conditions, visualisation and subsequent analysis are needed. It has always been a challenge to capture root growth, especially when growing in soil due to the opaque nature of the natural growth habitat. This is nicely reflected in the title "Roots – the Hidden Half" by Waisel *et al.* (1996).

In 1960 Lore Kutschera published the first version of the well-known "Wurzelatlas" (root atlas) with detailed drawings of excavated root systems (Kutschera, 1960). Examples for *Vicia faba* and *Hordeum vulgare* are given in Figure 3.1 (page 17). Until today (the seventh volume of this atlas series was released in 2009 (Kutschera *et al.*, 2009) a lot of well-deserved attention is paid to these drawings, especially as root plasticity is also set out in a splendid way as many root systems of the same plant are shown for different soils or climatic conditions. These results are memorable but of course they lack quantitative information about root length for different root types and orders and especially regarding the three dimensional arrangement of root systems in the soil volume.

In part, this knowledge gap could be closed by separating the roots from the soil by washing them out and subsequently measuring their length. At the beginning this was simply done with a tape measure or ruler, before the line intersect method was released by Tennant (1975). The next development step was the introduction of the software WinRHIZO (Regent Instruments Inc.) in the 1990s (Arsenault *et al.*, 1995). This method made it possible to scan washed-out root systems and then examine their length and diameter automatically with a high degree of detail. This software is still widely used today and is also applied in the experiments carried out for this thesis. The advantages are the easy handling and the robust, comparable results. But of course, an undisturbed, three-dimensional analysis of the root growth in the growth medium is not possible with this method.

Other methods were applied in the field or in the laboratory scale, *e.g.* profile wall methods, resin embedding techniques, rhizotrons, in-growth bags, transparent artificial growth media, rhizoboxes or other inclined boxes with the underlying idea that the roots grow along a transparent surface so that they can be detected and analysed there (Neumann *et al.*, 2009; Iyer-Pascuzzi *et al.*, 2010; Trachsel *et al.*, 2011; Downie *et al.*, 2012; Smit *et al.*, 2013). Although

many of these methods have the advantage of being cost-effective, all of these listed methods also have decisive disadvantages. Either no recording over time is possible, an insufficient proportion of roots is detected, or a conclusion about the three-dimensional characteristics of the root system is hardly possible.

Modern imaging methods may overcome these problems, though having been developed mainly for medical applications. These are, in particular, neutron tomography (NT), magnetic resonance imaging (MRI) and X-ray computed tomography (CT). Metzner *et al.* (2015), comparing the latter two methods concluded that CT was advantageous because of the higher spatial resolution, but MRI would be better situated for larger pots due to the high contrast between root and soil. But since MRI is sensitive to ferro-magnetic particles in the soil, and the acquisition costs are higher, it is not surprising that CT is used more frequently in practice, as in the experiments of this thesis.

A detailed description of the 3-D imaging of roots growing in soil, as well as a comparison of the three major methods of soil root imaging can be found in Chapter 3.

2.2 X-ray computed tomography – basics and application for root research

X-ray computed tomography (CT) was invented in the 1970's in the medical sector, long time after Wilhelm Conrad Röntgen discovered the X-radiation in 1895. Already in the 1980's other scientific research areas recognised the enormous potential of this technique (Petrovic *et al.*, 1982; Hainsworth and Aylmore, 1983; Grevers *et al.*, 1989). Although the technical details and the computing power behind the processors have increased tremendously since then, the primary principle remains the same, independent of its usage as medical scanner or an industrial scanner that is mainly used in the scientific respect and in the studies within this thesis.

First, a tungsten filament is heated up in a vacuum due to a bypassing current. By this, electrons are released that are accelerated afterwards, and hit the positively charged anode, also called the target (tungsten material in most cases). In the course of this collision, electrons are slowed down rapidly and several processes take place. These are outer shell interaction (electromagnetic radiation), inner shell interaction (characteristic radiation) and nucleus field interaction (Bremsstrahlung). X-ray photons are released when a higher shell electron moves to an empty space within the k- or l-shell (characteristic radiation) and by inelastic interactions with the nuclei of atoms, when the electron path is deflected by the electric field of the nucleus and energy is transferred to an emitted photon (Bremsstrahlung). The majority (about 80%) of the photons in the X-ray beam are emitted via Bremsstrahlung, which gives rise to the

continuous spectrum of photon energies that are released, while characteristic X-rays are specific for the anode material and appear as sharp peaks in the spectrum (Cember and Johnson, 2009; Dance *et al.*, 2014).

The X-ray beam then passes through the sample, which is rotating inside industrial scanners. During the passing process, the beam is transmitted, absorbed and scattered. This leads to an attenuation of the X-rays, described by Lambert-Beer's law:

$$I = I_0 \exp(-\mu D)$$

This simplified version defines the attenuated intensity I to be dependent on the incident intensity I_0, the sample thickness D, and a linear attenuation coefficient μ. For multiphase media like soils, respective phase-dependent coefficients and other factors like density, porosity and water saturation need to be considered (Wildenschild *et al.*, 2002).

The attenuated X-ray beam is projected on a scintillation detector after passing through the sample. Each projection consists of a data matrix of pixels that receive the respective attenuation values, a function of the electron density of the sample material (Mahesh, 2002). A large number of these projections are taken during one scan, usually 1000-3000 per scan. In the course of a process called reconstruction, these single projections are combined together to achieve the 3D volume dataset. The most prominent algorithm for that process is the filtered back projection, based on the Radon-transformation from Johann Radon in 1917.

Also root scientists started to profit from the non-invasive nature of this imaging method. Watanabe *et al.* (1992) seem to have been the first to use X-ray CT as a method for root visualisation and analysis. Other early studies were carried out by Tollner *et al.* (1994), Grose *et al.* (1996) and Heeraman *et al.* (1997). Image resolution was rather coarse (in the range of mm per voxel) back in the time, limiting the degree of details that could be detected and distinguished. Further progress was made by Gregory *et al.* (2003), Lontoc-Roy *et al.* (2006), Kaestner *et al.* (2006), and Flavel *et al.* (2012). Nowadays, much finer image resolutions can be achieved, down to the nanometre scale. Nevertheless, the trade-off between sample size and image resolution still exists. With increasing sample size, image resolution is getting coarser. This means that sample size in most experiments is rather small, limited to a sample diameter of some centimetres usually. This holds especially true when plants with fine roots, *i.e.* small diameters are used, like for cereals. As a consequence, duration of plant growth experiments is limited to several days or a few weeks. Hence, the exploration of roots through the soil is restricted and is not the same as in the field. For more information on the general aspects and

the development in the X-ray technology and image processing, suggested reviews are from Wildenschild *et al.* (2002), Mooney *et al.* (2012), Helliwell *et al.* (2013), and Schlüter *et al.* (2016).

The potential influence of the X-ray radiation on living root tissue was neglected or not investigated in most previous studies. Because this may present a major limitation to the usefulness of X-ray CT to examine root growth development over time, this topic is an important part of this thesis.

It is well known that X-rays as ionising radiation can have negative effects on living tissue. In extreme cases, X-rays are even used explicitly to sterilize materials. Specific threshold values have been formulated in the medical field and in occupational health and safety. According to the German Federal Office for Radiation Protection (BfS), the threshold value (maximum permissible dose) of the annual radiation exposure for persons of the general population (*e.g.* resulting from the release of radioactive substances from nuclear facilities) is 1 mSv per year. The typical dose range for a whole body computed tomography of an adult is 10-20 mSv. A dose of 20 mSv also corresponds to the limit value (maximum permissible dose) of the annual radiation exposure for occupationally exposed persons in Germany.

These values are given in Sievert (Sv), which is the unit of measurement for different weighted doses of ionising radiation. This is based on the absorbed dose in Gray (Gy) and is modified by weighting factors depending on the type of radiation and, among other things, on the tissue. For industrial applications Sievert is often equated directly with Gray.

Comparable thresholds or maximum permitted doses have not yet been defined for plants. Zappala *et al.* (2013) derived a limiting value of about 30 Gy from the literature and their own experiment with rice (*O. sativa* spp. Azucena).

Whether the radiation, that typically occurs in an industrial CT scanner, has an influence on plant development and root growth, and whether two contrasting plant species behave similarly is described in Chapter 4.

2.3 Nitrogen – essential macronutrient and driver for root system development?

Nitrogen (N) is a macronutrient and therefore particularly important for plant nutrition. N is a primary component of biomolecules like amino acids and proteins and therefore essential for life (Xu *et al.*, 2012). Moreover, it is also a main constituent of chlorophyll, the green pigments that are essential for photosynthesis. A lack of nitrogen has devastating effects on crop

development, including reduced growth and poor yield formation and yield quality. Therefore, it is particularly important in the agricultural context to guarantee a sufficient supply of N to the plants.

Essentially, plants take up nitrogen through their roots. The two mineral forms nitrate (NO_3^-) and ammonium (NH_4^+) are of decisive importance. There are also indications of the uptake of organic N-forms, such as glutamate (Walch-Liu *et al.*, 2006; Forde and Lea, 2007) and urea (Liu *et al.*, 2003; Arkoun *et al.*, 2012), although these are assigned a subordinate role in comparison to NO_3^- and NH_4^+. Both mineral N-forms are present in the soil, whereby under aerobic conditions the nitrate ion is present in a much higher concentration than ammonium, which is more common in flooded wetland or acidic soils (Xu *et al.*, 2012; Blume *et al.*, 2015).

The nitrogen cycle describes the essential components and processes in which nitrogen occurs and in which the various N-forms are converted. Additionally to the natural processes that are classically included in the N cycle, agricultural input in form of fertilisation need to be considered. Bacteria play a crucial role in the cycle, as they are responsible for many of the processes, including nitrogen-fixing from atmospheric N, ammonification, nitrification and denitrification. Both, fungi and bacteria are considered as decomposers that act via mineralisation and transfer organic compounds back into mineral forms, in particular NH_4^+. The uptake of NO_3^- and NH_4^+ by plants is referred to as assimilation.

In addition to its function as a nutrient, both mineral N-forms have a signalling effect for root growth (Crawford, 1995; Forde, 2002; Miller *et al.*, 2008; Li *et al.*, 2013). This is also a result of an adaptation strategy of the plants to the dynamic and heterogeneous conditions present in the soil. The concentrations of nitrate and ammonium in soil and soil solution can vary enormously, both temporally and spatially (Blume *et al.*, 2015). To cope with this situation, root systems have developed a remarkable plasticity (de Kroons and Hutchings, 1995; Fransen *et al.*, 1998; Bingham and Bengough, 2003; Hodge, 2004; Hodge, 2006; Gruber *et al.*, 2013).

This plasticity results from both physiological and morphological responses. Physiological responses usually occur before morphological responses and lead to an increased uptake rate per root unit (Drew and Saker, 1978; Burns, 1991; Caldwell, 1994; Robinson, 1994; Robinson *et al.*, 1994; Van Vuuren *et al.*, 1996). Hodge (2004) deduced that the increased nutrient uptake as the physiological response can act as a signal and thus, stimulate subsequent root growth as the morphologic response. This thesis only covers the morphological root growth response.

There are several publications on the topic of root growth response to local patches of nutrients. This is especially known for Phosphorus (P). P might be a very relevant example, due to the low mobility of P in the soil and the majority of P is taken up by the plant in the direct vicinity – less than 1 mm from the root surface (Nye and Tinker, 1977). The well-known work on the reaction of root growth to various nutrients – P, but and also to NO_3^- and NH_4^+ - was done by Drew and his colleagues in the 1970s (Drew *et al.*, 1973; Drew, 1975; Drew and Saker, 1975; Drew and Saker, 1978). Hence the clear reference to Drew's work in Chapter 5 of this thesis: "When Drew meets soil - Dynamics of localised supply of nitrogen-species in soil and their relevance for root system morphology – A comparison between faba bean (*Vicia faba*) and barley (*Hordeum vulgare*)". However, Drew, like many other scientists, did not necessarily examine the topic from the perspective of the soil, but from that of the plant. Mainly from Drew, but also from other similar studies the general conclusion is drawn that "localised zones of high N concentration promote rather than suppress lateral root growth", as cited by Osmont *et al.* (2007). The generalisation of this reaction is debatable, because it conceals the sometimes very artificial conditions in such studies, as well as the diversity of soils and the different plasticity of various plant species. To remain with the example of Drew (1975): Very low N concentrations (0-0.1 mM N) above and below a layer with higher concentration were used in these studies, forcing a N deficiency in these areas. The reaction of root growth within the layer with a higher concentration (1 mM N) was therefore particularly clear and a very distinct alteration of root morphology was found in that layer. High NO_3^- led to an increased root elongation, while high NH_4^+ resulted in a highly branched root system architecture (Fig 2.1).

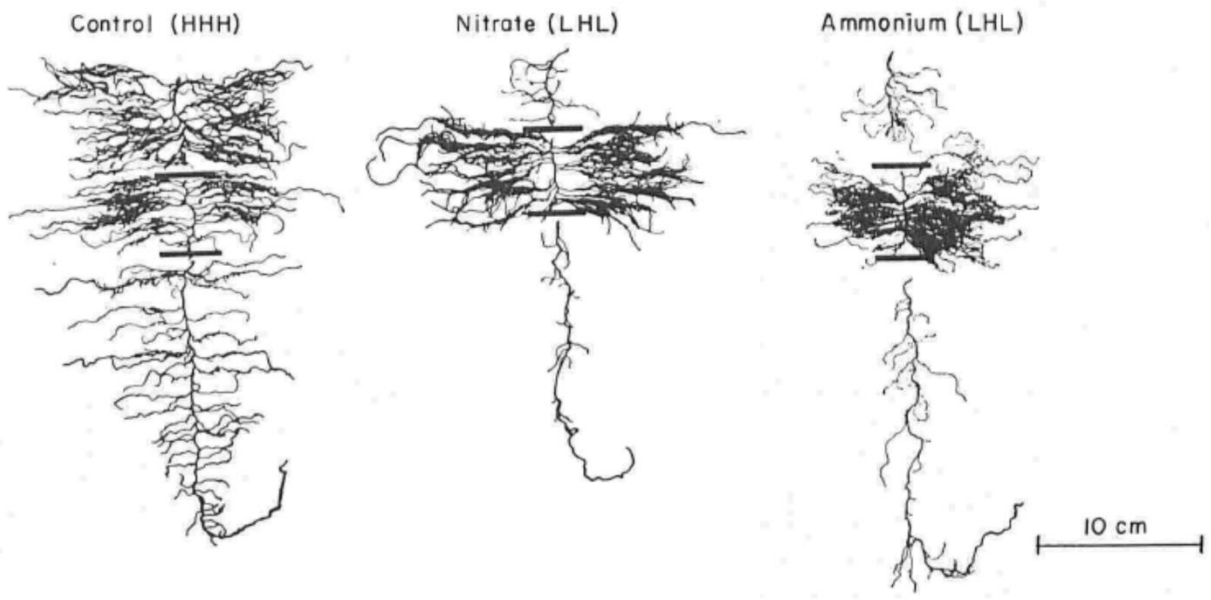

Figure 2.1: Effect of a localised supply of nitrate and ammonium on barley root morphology. Control plants (HHH) received a complete nutrient solution to all parts of the root system. The other treatments received nutrient solution including the respective N-form only in the middle zone, while top and bottom being supplied with a solution deficient in the specified nutrient. Modified from Drew (1975).

In Drew (1975), barley was the only plant species analysed and quartz sand was used as growth media. Due to its physical and chemical inertia, quartz sand cannot be compared to most real soils. Drew's barley plants were definitely at the upper end of responsiveness, albeit under contrived laboratory conditions.

For such observations as in Drew (1975) but also other studies on gene expression (Raghuram and Sopory, 1999; Wang *et al.*, 2000; Araki and Hasegawa, 2006; Miller *et al.*, 2007) or transcription factors and affinity transporters (Zhang and Forde, 1998; Remans *et al.*, 2006; Lima *et al.*, 2010), the use of other growth media, in particular nutrient solution, agar or quartz sand that is rinsed with nutrient solution, are very suitable. Under these conditions it is possible to work very accurately, all boundary conditions can be controlled and defined. These conditions are characterised in particular by constant chemical properties. Dynamic interactions and competition (*e.g.* with microorganisms) can be excluded, no specific adsorption on soil particles or organic matter takes place, so that the nutrients applied are mostly freely available and surround the root system.

As soon as soil is used as a growth medium, most of these boundary parameters cannot be controlled as precisely anymore. But the comparability with the conditions experienced by the

plants in agricultural practice increases. Due to the soils heterogeneity, nutrients are available to plants in patches (Fitter *et al.*, 1994). In natural soils this heterogeneity is mainly determined by the distribution of soil organic matter and the microbial turnover (Van Vuuren *et al.*, 1996; Hodge *et al.*, 1998). By the use of granular fertilisers, this natural patchiness is further increased (Robinson *et al.*, 1999). The aim here was to investigate the reaction of root growth to the two N-forms under conditions closer to agriculture by using a natural soil and the application of urea fertiliser granules instead of laboratory chemicals.

However, this also means that sometimes less pronounced results are to be expected, due to natural soil being a very complex system. A soil is characterised by a physical structure consisting of a soil matrix with particles or aggregates of different sizes and a network of pores, differing in size and connectivity. In contrast to the situation in nutrient solution or on agar, the root system is not always evenly in contact with a constant concentration of nutrients, instead water content and nutrient concentration within the soil volume can differ significantly in space and time.

Further differences to artificial growth media are the adsorption capacity of the organic matter and the clay minerals, the dynamic turnover of the N-forms by microorganisms, as well as the background concentration of N which is present in almost every soil, whereby extremely steep concentration gradients occur only rarely.

Urea is of interest and great relevance because it is the most common N fertiliser on a global scale (Glibert *et al.*, 2006; Li *et al.*, 2015). In addition, urea is produced with various inhibitors which can inhibit or strongly delay both the initial hydrolysis to ammonium by the enzyme Urease, and the further nitrification to nitrate by microorganisms. These products can be purchased as commercial and certified fertilisers and are often used in agricultural practice. The use of the inhibitors leads in particular to the minimization of N-losses (Kirschke *et al.*, 2019). Both, gaseous losses in form of NO_x and leaching in ground and surface waters have a negative impact on the environment and also represent an economic burden for farmers. In addition, N losses that cannot contribute to yield formation reduce nitrogen use efficiency (NUE). The improvement of the NUE is one of the main goals of modern agriculture in order to minimise and optimise fertiliser application (Dobermann and Cassman, 2004; Giller *et al.*, 2004; Good *et al.*, 2004; Xu *et al.*, 2012).

The fact that urea in soil is rapidly converted to NH_4^+ and subsequently to NO_3^- allows detailed investigation of this turnover and the reaction of root growth and plant development to these

conditions. The use of a nitrification inhibitor influences both, temporal and concentration dynamics of the two N-forms in the soil and the soil solution.

Whether the root growth of the two species faba bean and barley react in a similar way to that of Drew and other studies is addressed in Chapter 5.

3 The 3-D imaging of roots growing in soil

3.1 Why do we need 3D imaging of roots in soil?

Roots are the hidden half

In 1996 a book with the title "Roots – the Hidden Half" was published by Waisel *et al.* (1996). This title wonderfully reflects the fact that roots are much more difficult to access than the above-ground organs of the plant. While the shoot and leaves are directly visible and accessible, much more preparation and advanced techniques are needed to explore and study roots in opaque soil.

Not all roots are hidden, as some plants are able to form above-ground roots, such as aerial roots in epiphytes, mangroves and some trees such as figs. Nevertheless, the vast majority of the roots are located in soil. This natural habitat is opaque, not transparent to the human eye and, hence, more ingenuity is needed to observe the hidden half, which is the focus of this Chapter.

Long term interest in root growth and definition of root system architecture

The interest of humans in their environment and especially in the realm of plants is probably as old as mankind itself - not least because plants have always been part of the food chain. With the beginning of agriculture, soil management and plant nutrition have clearly gained in importance and are experiencing a renaissance through today's efforts to ensure the nutrition of a growing world population with less fertilisers and a better and sustainable soil management.

In addition to anchoring the plant in the soil, the central task of the root system is to take up water and nutrients in order to maintain the plant's vital functions and enable a healthy and lush growth. In order to fulfil these tasks, roots explore the soil in a specific spatial arrangement, which has been termed the root system architecture (RSA). RSA is defined as the three-dimensional structure of the root system, including the primary root, branch roots and root hairs (Osmont *et al.*, 2007). The term "branch roots" can be misleading and is less common in recent literature, although the term "branch" reflects the morphologic appearance of the root system very well. Instead, the term lateral root is preferred.

The primary root is formed during embryogenesis, and lateral roots are formed post-embryonically from existing roots. Mainly originating from the pericycle, the process of lateral root formation can take place at roots of different orders and subsequently create the next level of root order. Apart from these root types, there are adventitious roots (originating from the

shoot), aerial roots, crown, storage or cluster roots, leading to a wide variety of possible root network morphologies and root system architectures.

Root growth does not depend on genetic program only

The development of root growth and the spatial characteristics of the root architecture depend on many environmental and ontogenetic factors, *i.e.* the individual origin and development of an organism within its lifetime (Gould, 1977). However, the genetic programme of the plant can be described as having the overarching influence.

The morphological diversity of root systems is remarkable across plant species and hence a simple classification scheme is not applicable (Fitter, 1987). However, in most cases a distinction between two major root system morphologies can be made for angiosperms: with taproot (also termed allorhizic) and and without (homorhizic systems). Two characteristic representatives for those major types are *Vicia faba* and *Hordeum vulgare*, respectively (see Fig. 1).

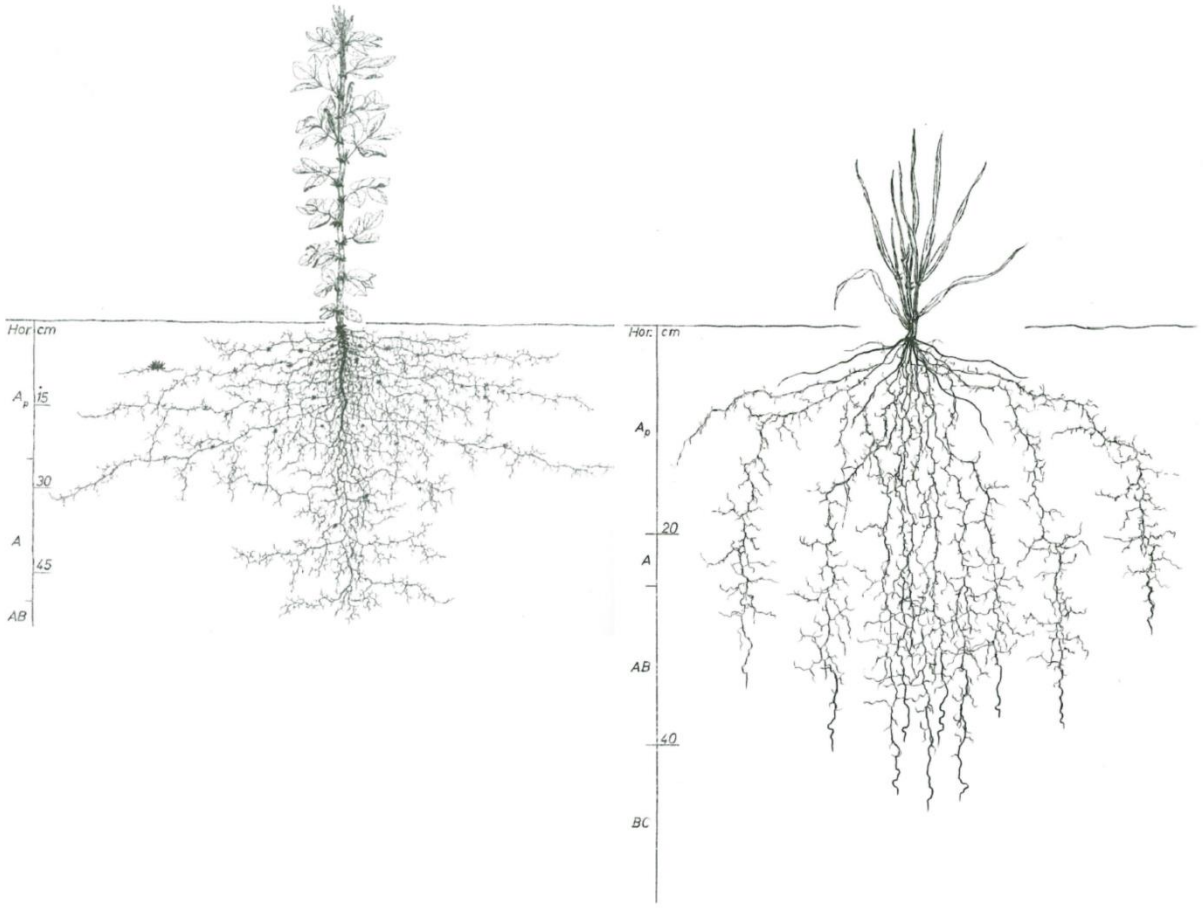

Figure 3.1: Root system architecture drawings of faba bean (*Vicia faba*) on the left and barley (*Hordeum vulgare*) on the right. From Kutschera *et al.* (2009). Please note the different depth axis scales.

Allorhizic systems are typical for dicotyledonous plants. The allorhizic system is dominated by the primary root (termed taproot), from which the first order lateral roots emerge (Esau, 1965). These laterals can then, in turn, also form lateral roots which then are considered the second order laterals. If they branch further, the resulting roots are called the third order lateral roots and so on.

In contrast, the root system of monocotyledonous plants such as *Hordeum vulgare* (barley) or *Triticum aestivum* (wheat) is characterised by the growth of adventitious roots in addition to the primary roots (often termed seminal roots). Some monocots such as *Zea mays* (maize) or *Oryza sativa* (rice) can form shoot-borne roots, termed brace or crown roots (Hochholdinger *et al.*, 2004). As lateral roots can be formed from all of these root types, the morphologic appearance is referred to as "bushy" (Osmont *et al.*, 2007; Lima *et al.*, 2010; Mounier *et al.*, 2014). This can result in a particularly intensive exploration of the soil volume, typically more intensive than by the taproot systems.

Other biotic and abiotic stimuli influence root growth

The contrast in RSA of dicots and monocots emphasises the genetic control of the spatial arrangement of the root system. In addition, various biotic and abiotic exogenous factors result in a remarkable developmental plasticity of root system architecture (Osmont *et al.*, 2007; Morris *et al.*, 2017). Related terms to that phenomenon are "morphological plasticity" (de Kroons and Hutchings, 1995; Fransen *et al.*, 1998; Johnson and Biondini, 2001; Bingham and Bengough, 2003), or "the plastic plant" (Hodge, 2004; Hodge, 2006).

The growth response to certain stimuli is called "tropism" (Esmon *et al.*, 2004; Gilroy, 2008; Gregory, 2008). There are many tropisms; among the most important are gravitropism to gravity, thigmotropism to touch, phototropism to light and hydrotropism to water. Most of those are known for a long time and trace back to Charles Darwin (Darwin, 1897), but more detailed studies about these tropisms have been conducted much later (Onderdonk and Ketcheson, 1973; Fitter, 1987; Kaspar and Bland, 1992; Boonsirichai *et al.*, 2002; Correll and Kiss, 2002; Eapen *et al.*, 2005).

An explicitly defined 'tropism' towards nutrients does not exist, but the idea that root growth responds to the availability and concentration of nutrients is generally recognised. This reaction can also be understood as being part of 'chemotropism' (Newcombe and Rhodes, 1904), and this has been used and applied *e.g.* in Leitner *et al.* (2010) (a simulation study for phosphate uptake by maize roots). The publications of Drew and colleagues in the 1970s (Drew *et al.*, 1973; Drew, 1975; Drew and Saker, 1975; Drew and Saker, 1978) are particularly well-known, investigating plant response to a localised supply of nutrients. Earlier studies date back to Wiersum (1958) or Hackett (1972).

Root visualisation and analysis

To analyse any type of tropism or root growth response in general, an appropriate setup with controlled, experimental conditions is needed, as well as visualisation and subsequent analysis. It has always been a challenge to capture root growth, especially when growing in soil due to the opaqueness of this natural growth habitat.

In 1960 Lore Kutschera published the first version of the well-known "Wurzelatlas" (root atlas) with detailed drawings of excavated root systems (Kutschera, 1960). Examples of *Vicia faba* and *Hordeum vulgare* are given in Figure 1. Until today - the seventh volume of this atlas series was released in 2009 - a lot of deserved attention has been paid to these drawings, especially as

root plasticity is illustrated superbly with many root systems of the same plant are shown for different soils or climatic conditions (Kutschera *et al.*, 2009). While intriguing, these drawings largely lack quantitative information about root length for different root types and orders and especially regarding the three-dimensional arrangement of the RSA in the soil volume.

This knowledge gap regarding quantification of root length can be closed partially via separating the roots from soil by washing them out and subsequently measuring their length. At the beginning this was done simply by a tape measure or a ruler, before the line intersect method was developed by Tennant (1975). The next large step was the introduction of high-resolution document scanners combined with image analysis software such as WinRHIZO (Regent Instruments Inc., Québec, Canada) in the 1990s (Arsenault *et al.*, 1995). This method made it possible to scan washed-out roots and then examine their length and diameter automatically with a high-degree of detail on a computer. This software is still widely used today. The advantages are the easy handling and the robust, comparable results. However, the three-dimensional analysis of the undisturbed arrangement of roots in soil is not possible with this method. Some open-source alternatives to WinRHIZO are now available, such as RootNav (Pound *et al.*, 2013), Root System Analyser (Leitner *et al.*, 2014) or DART (Le Bot *et al.*, 2010).

Other methods were applied in the field or in the laboratory, *e.g.* soil profile wall methods, resin embedding techniques, in-growth bags, transparent artificial growth media, rhizoboxes or other inclined boxes with the underlying idea that the roots grow along a transparent surface of a box so that they can be detected and analysed (Neumann *et al.*, 2009; Iyer-Pascuzzi *et al.*, 2010; Trachsel *et al.*, 2011; Downie *et al.*, 2012; Smit *et al.*, 2013). Although many of these methods have the advantage of being cost-effective, all of these methods also have grave disadvantages. Only for some methods recording over time is possible, in some a large proportion of roots is not detectable, so that a conclusion about the three-dimensional characteristics of the root system is hardly possible and in others the experimental environments are very different from natural conditions, resulting in artefacts.

3.2 Overview of non-invasive imaging techniques – NT, MRI and X-ray CT

To overcome the problems mentioned above, non-invasive imaging methods are now increasingly used, most of which have initially been developed for medical applications. The three techniques mainly used for the three-dimensional imaging of roots are neutron tomography (NT), magnetic resonance imaging (MRI) and particularly industrial X-ray computed tomography (CT). These techniques are summarised in Table 1.

Table 3-1: Comparison among the NT, MRI and industrial X-ray CT methods for the root system architecture characterisation. For the ranked attributes: * = low, ** = medium and * = high.**

Method	Neutron tomography (NT)	Magnetic resonance imaging (MRI)	Industrial X-ray computed tomography (CT)
Pioneering work	Willatt *et al.* (1978)	Brown *et al.* (1986)	Watanabe *et al.* (1992)
Type of radiation used	Neutrons ((indirect) ionising radiation)	Electromagnetic waves (not ionising)	X-rays (ionising radiation)
Measurement principles	Strongest neutron attenuation by hydrogen while most other materials are easily penetrated	Nuclear magnetic resonance effect (NMR). Atomic nuclei are excited and their excitation response is monitored in terms of the emitted radiation in the time domain.	Attenuation and scattering of the X-ray beam are dependent on electron densities as a function of atomic number and bulk density
Accessibility	*	**	***
Advantages	Sensitive to hydrogen and hence good detection of water	Good contrast between soil and roots; water content can be measured as well	Quick, only few restrictions on the root growth media used, good accessibility (apart from synchrotron-CT)
Disadvantages	Evokes radioactivity in the sample and therefore less scope for	Sensitive to ferro-magnetic particles, longer duration,	Potential influence of radiation on root growth, artefacts, poor

	studies over time, low accessibility and decreasing penetration of the sample with increasing water content	artefacts, mediocre accessibility	contrast between roots and water-filled pores
Image resolution	**	*	***
Sample size possibilities	*	***	**
Typical application	Water distribution in soil and roots	Root growth over time	Root growth over time, simultaneous information on soil structure
Recommended reviews	• Oswald *et al.* (2008) • Strobl *et al.* (2009)	• Borisjuk *et al.* (2012) • Stingaciu *et al.* (2013) • van Dusschoten *et al.* (2016)	• Taina *et al.* (2008) • Mooney *et al.* (2012) • Helliwell *et al.* (2013) • Wildenschild and Sheppard (2013)
Comparative Reviews	*e.g.* Li *et al.* (2014b), Downie *et al.* (2015), Pohlmeier *et al.* (2018)		

All of these methods have the fundamental disadvantage that they cannot be used directly in the field – at least not in the near future. Therefore, it is necessary to prepare appropriate samples in order to carry out the measurements.

Neutron tomography (NT) relies on the physical principle of neutron absorbance by the sample material. Because hydrogen is attenuating exceptionally strongly, water can be detected and visualised particularly well with NT. A main disadvantage is the low availability of the technology, and usually only thin slabs can be used for 2D radiography studies (*e.g.* 1-cm

thickness in Zarebanadkouki *et al.* (2016)) and relatively small columns for tomography studies (*e.g.* 27-mm diameter in Moradi *et al.* (2011)). The initial root research using NT goes back to the late 1970s (Willatt *et al.*, 1978; Willatt and Struss, 1979; Couchat *et al.*, 1980). The recent studies used NT to *e.g.* capture root systems and water distributions (Rudolph-Mohr *et al.*, 2017) and analyse the 3D water flow influenced by roots (Totzke *et al.*, 2017). An example of neutron radiography application is shown in Figure 2.

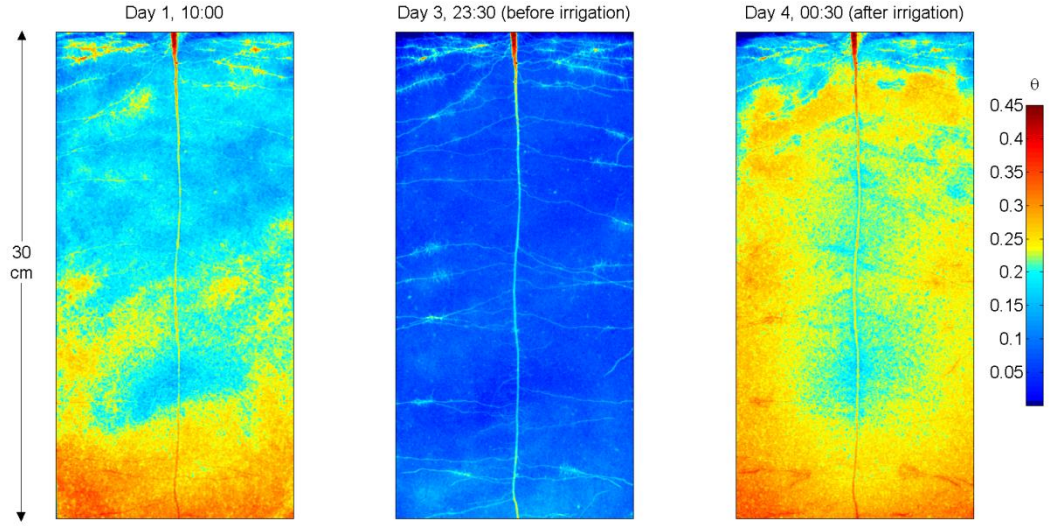

Figure 3.2: Neutron radiography during soil drying and after irrigation. The colour map is proportional to the water content (θ). Note the higher water content near the upper lupin (*Lupinus albus*) roots at day 1. After irrigation (day 4), the rhizosphere of the upper roots, of the tap root, and that of the proximal parts of the lower roots remained markedly drier compared to the rest of the sample. In contrast, a region with high water content is visible around the root tips in the lower parts of the sample. From Carminati (2013).

Magnetic resonance imaging (MRI) is based on the nuclear magnetic resonance effect. Within an external magnetic field, the spin system of the H atom in water is excited by radio-frequency. The response signal, coming from the relaxation after the excitation, is recorded and contains the information needed for reconstruction and subsequent analysis. Similar to NT, MRI is particularly suitable for analyses of the water distribution in the sample. The accessibility of MRI is better than that of NT, but there are limitations with MRI regarding the soil material used (Hall *et al.*, 1997). The basic work in the context of root research with MRI goes back to the late 1980s (Brown *et al.*, 1986; MacFall *et al.*, 1990; Macfall *et al.*, 1991). The recent studies have used MRI to study changes in soil water content due to plant water uptake (Pohlmeier *et*

al., 2008; Pohlmeier *et al.*, 2010) and to conduct plant root system analysis (Schulz *et al.*, 2012; Schulz *et al.*, 2013; van Dusschoten *et al.*, 2016).

The polychromatic X-ray CT (using industrial or medical scanners) has emerged in recent years as the technique most commonly used for *in-situ* root analysis in soil due to relatively easy access to scanners, the high spatial resolution and the relatively few restrictions in the choice of soil to grow plants. Apart from industrial or medical CT scanners, synchrotron radiation is now increasingly used for root-soil imaging. The high brilliance of 3rd generation synchrotron sources enables extremely high-resolution images to be taken with a short-exposure time (Wildenschild and Sheppard, 2013). Because the beam in this case can be monochromatic, the special reconstruction techniques such as phase contrast imaging can be employed to increase the contrast in biological tissues (Chen *et al.*, 2016; Kastner *et al.*, 2017). However, due to the necessary technology (especially a particle accelerator), the accessibility of such devices is considerably lower than with normal industrial X-ray CT and is roughly comparable to the accessibility of NT.

A clear disadvantage of X-ray CT compared to other imaging methods is the (relatively) poor contrast in the images. In particular, the distinction of a root from a water-filled pore is usually not possible by the grey-scale value alone.

In comparing MRI and X-ray CT, Metzner *et al.* (2015) concluded that CT was advantageous because of the higher spatial resolution, but MRI would be better suited for larger pots due to the high contrast between roots and soil. Figure 3 gives the comparison between MRI and CT.

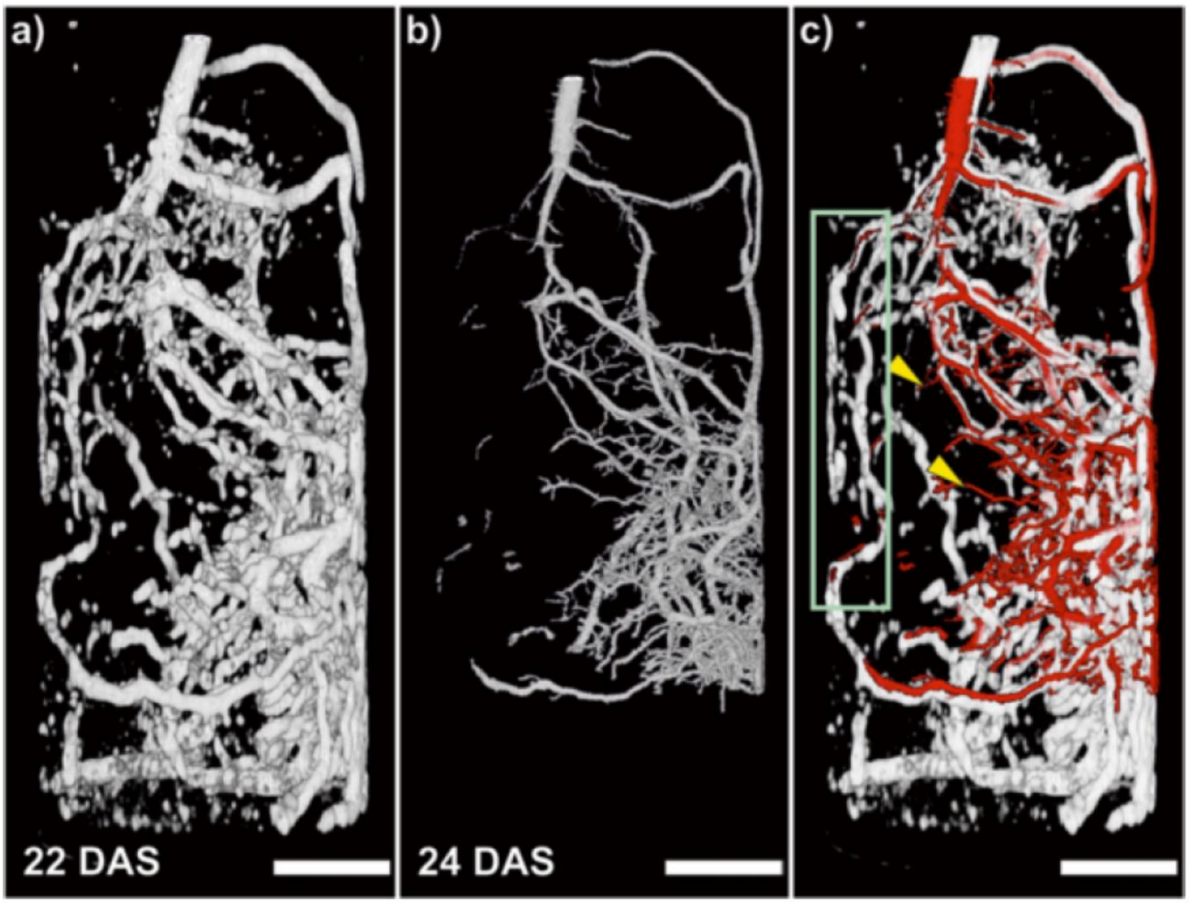

Figure 3.3: MRI and CT images of a bean (*Phaseolus vulgaris*) root system in a soil-filled pot. The same plant grown in a soil-filled pot (an inner diameter of 56 mm and a height of 200 mm) was imaged sequentially by MRI and CT. (a) MRI image measured with a voxel size 375 x 375 x 1000 µm. b) The CT image shows the root system measured with a voxel size of 68 x 68 x 68 µm. Roots in the lowest part of the pot could not be segmented and are therefore not shown. C) CT-MRI co-registration: the CT image is in red and the MRI image in grey. Arrowheads highlight roots visible in CT but not in MRI. Please note that the CT image was taken 2 days after the MRI image so that it is possible that some of the roots have only grown during this period. Box highlights area where few roots are visible in CT. Scale bar: 10 mm. From Metzner *et al.* (2015).

The trade-off between sample size and spatial resolution is an intrinsic problem of most imaging techniques. The larger the sample, the lower the spatial resolution of the images. Therefore, larger samples are often stitched from multiple scans. This is particularly useful for samples in column shape because several images can be taken on top of each other and then assembled for analysis (Flavel *et al.*, 2012; Koebernick *et al.*, 2014; Blaser *et al.*, 2018). However, even then, fine structures, especially higher-order lateral roots, cannot always be captured due to their small size. Therefore, either samples with a small diameter are selected (Flavel *et al.*, 2012; Tracy *et al.*, 2012b; Haling *et al.*, 2013), or the samples are not recorded in their entirety, but

only a certain section (Carminati *et al.*, 2009; Koebernick *et al.*, 2018). The latter is also called the region of interest imaging.

The selection of the sample size and the type of image acquisition is therefore linked to the question of what is to be detected - is it more about the details, focussing on fine roots or is it important to image the root system as a whole?

3.3 Image analysis

After the measurement it is necessary for all methods to process the 3D images in order to extract meaningful information. First, the images are usually filtered to reduce the image noise while preserving the features of interest. Depending on the signal-to-noise ratio and the target quantity to be analysed, different filters are used.

In the next step, roots are distinguished from the soil. This process is also referred to as "segmentation", depicted in Figure 4. Segmentation is difficult due to overlapping grey-scale values of soil and roots. Segmentation results can be improved by shape information, *i.e.* the fact that roots form a continuous network (Flavel *et al.*, 2012; Tracy *et al.*, 2012b; Koebernick *et al.*, 2014; Blaser *et al.*, 2018) and that they have an elongated tubular shape (Schulz *et al.*, 2013). The current publications with focus on root segmentation are by Gao *et al.* (2019b), Flavel *et al.* (2017) and Mairhofer *et al.* (2012). A more general overview of processing images from X-ray microtomographies is given by Schlüter *et al.* (2014).

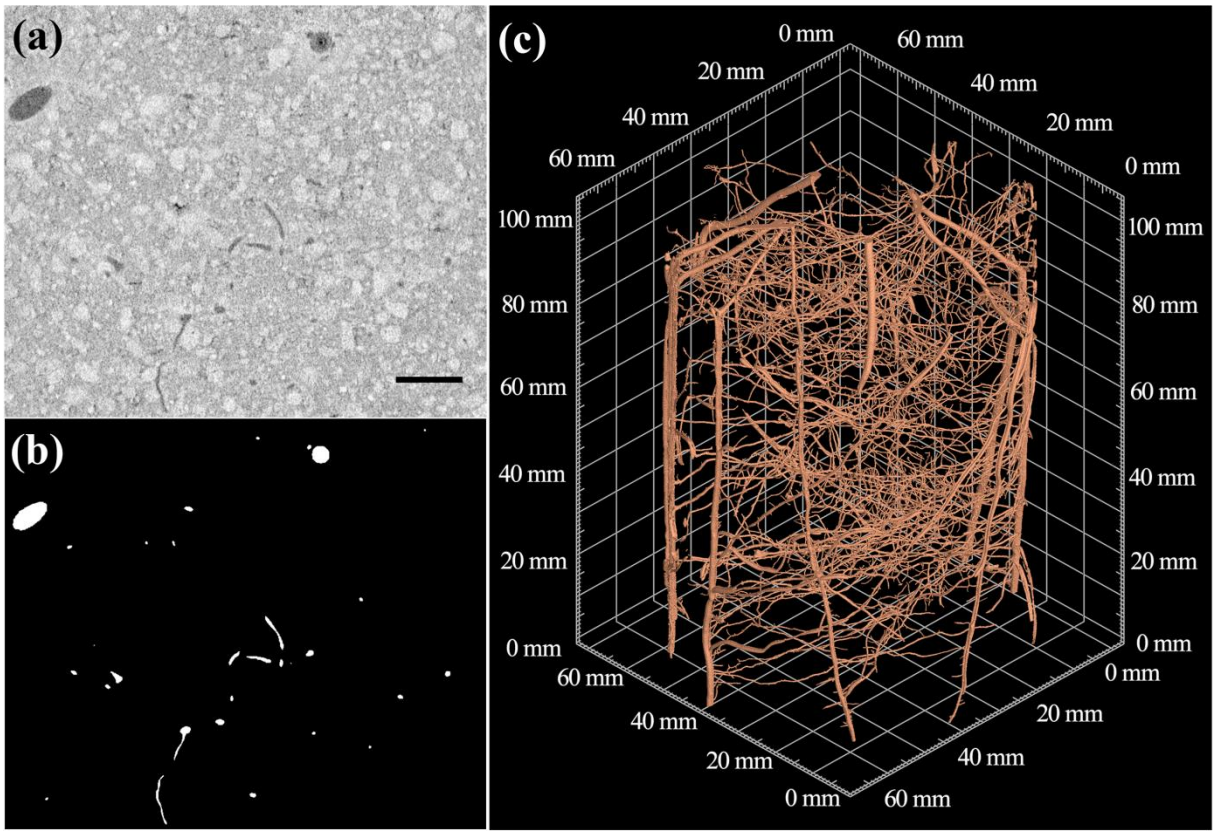

Figure 3.4: Root segmentation of maize (*Zea mays*) with the algorithm Rootine 1.0 (Gao *et al.*, 2019b). (a) 2D projection of the original raw CT image, (b) Segmented roots from (a); (c) 3D root network rendered in VG Studio Max 2.1. The scale bar in (a) represents a length of 5 mm.

3.4 Typical applications for 3D imaging of roots growing in soil

Apart from very general or qualitative studies (Garbout *et al.*, 2012; Oswald *et al.*, 2015; Totzke *et al.*, 2017; Nwankwo *et al.*, 2018), typical applications and main topics of the 3D and 4D information acquired by imaging techniques are root length and architecture, root age, root:soil contact, root hairs, soil-root-distances, soil compaction by roots, and creation of data bases for modelling.

Root length or root length density distributions in space are basic measures, which in part can also be obtained from destructive sampling. In addition to the root length, more spatial information and/or the differentiation according to root orders is included in the analysis of the root system architecture, as well as information on root demography and transport distances. The central topic is the spatial exploration of the soil volume by the growing, developing root system. Plant species, genotypes or soils can be compared. Often, however, a much more complex question is in the background, such as the reaction of root growth to locally-placed fertilisers (especially P and N), soil structure, water distribution, physical differences (such as

the availability of biopores, or areas in the soil with higher or lower bulk density), and the growth response to plant diseases such as *e.g.* apple replant disease.

Root length and root system architecture

A multitude of methods to derive quantitative information from root images have been developed over the years. Beginning with relatively simple approaches that have shown the suitability of CT for root research (Watanabe *et al.*, 1992; Tollner *et al.*, 1994), the analysis of root length and root system architecture has continued to evolve. The further milestone publications on root length analysis using CT were Gregory *et al.* (2003), Moran *et al.* (2000), Pierret *et al.* (1999) and Heeraman *et al.* (1997), with an increased level of detail and a higher proportion of detected roots. From there on, the technical development was rapid and recent studies are covering a wide range of topics such as root-soil contact (Schmidt *et al.*, 2012; Koebernick *et al.*, 2018), root shrinkage under drought stress (Carminati *et al.*, 2009; Carminati, 2013), root decay (Haling *et al.*, 2013), response to local fertiliser (Flavel *et al.*, 2014; Ahmed *et al.*, 2016b; Gao *et al.*, 2019a), influence of soil structure (Moran *et al.*, 2000), soil compaction (Tracy *et al.*, 2012a; Tracy *et al.*, 2012b), biopores (Pagenkemper *et al.*, 2013; Daly *et al.*, 2015), or pathogens (Sturrock *et al.*, 2015), showing root length and root system architecture in increasing details, even though not everything is technically feasible.

Root age

An essential strength of non-invasive imaging techniques is the possibility to record the same sample several times during growth and thus dynamically record the development of the roots (Figure 5). From this, the growth rate can be derived, a parameter that is very labour-intensive and difficult to determine without the use of non-invasive imaging. CT and MRI are better suited for repeated use due to the indirect ionising radiation when neutrons are used and the resulting danger of radioactivity being evoked in the sample. Nevertheless, caution is advised for the other methods as well, as repeated measurements result in a cumulative radiation dose. There is currently no evidence that this would be an issue for MRI. For X-ray CT there is mixed evidence for the influence of accumulated radiation on root growth, depending on different factors, especially the plant species (Johnson, 1936; Zappala *et al.*, 2013; Blaser *et al.*, 2018).

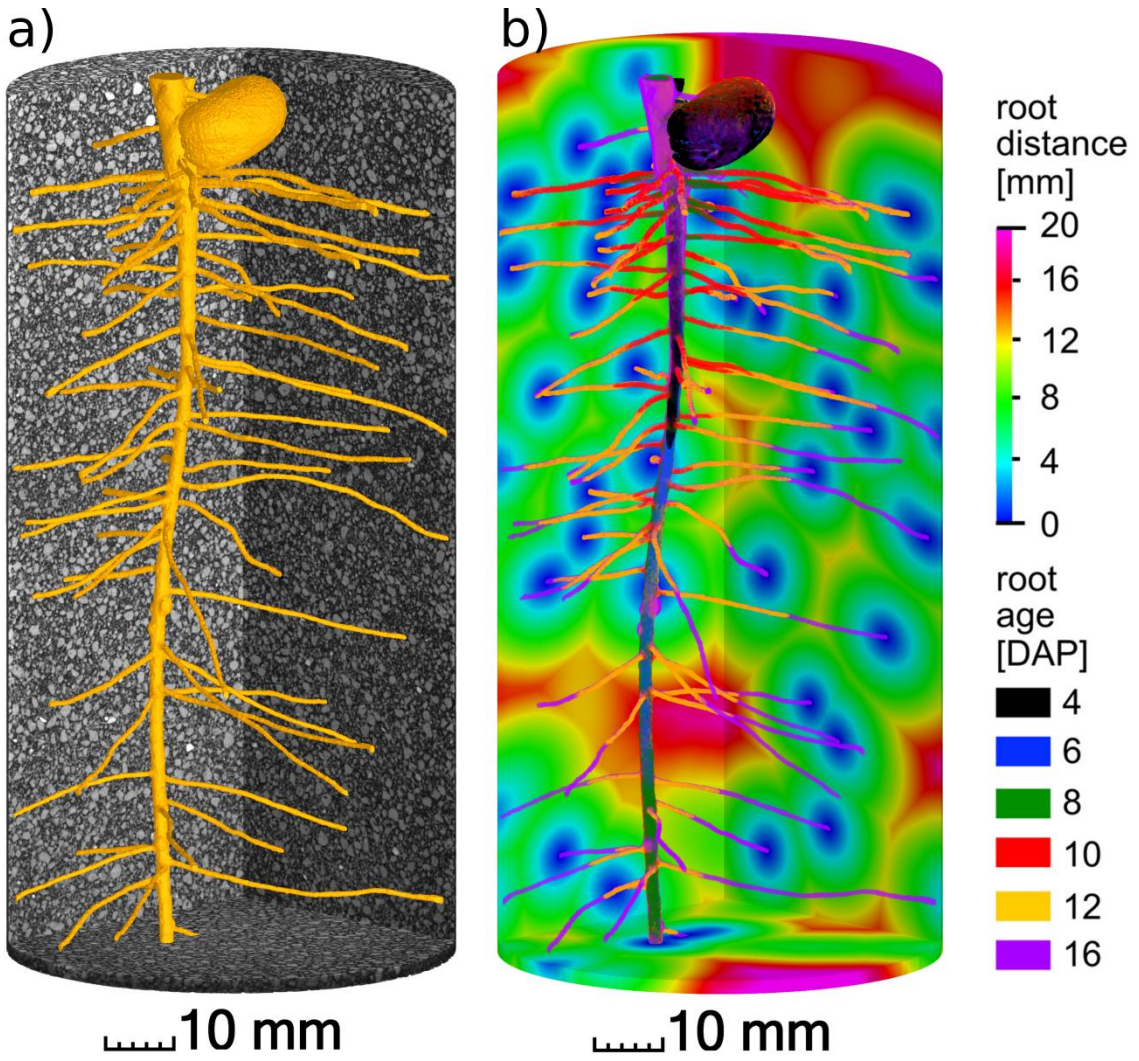

Figure 3.5: Root system of faba bean (*Vica faba*) segmented from the surrounding soil, acquired by repeated application of X-ray CT. a) Root system of faba bean 16 days after planting (DAP), embedded in the soil matrix, virtually cropped for visualisation; b) Combined colour-coded representation (same as in Schlüter *et al.* (2018)) of root age and soil-root distances 16 days after planting for the sample in (a).

Repeated measuring reveals which parts of the root system developed when (Carminati, 2013; Koebernick *et al.*, 2014; Blaser *et al.*, 2018). This allows basic statements to be made on the development of the root system and the roots of different order themselves, but also in such a way that different tasks and properties can be assigned to the respective root segments. This applies, for example, to the question where roots take up water (Zarebanadkouki *et al.*, 2013; Koebernick *et al.*, 2015) and influence of mucilage on the rhizosphere properties and root water uptake (Ahmed *et al.*, 2014; Ahmed *et al.*, 2016a). Some of these topics were also discussed in Vetterlein and Doussan (2016).

Root:soil contact

An optimal contact between root and soil is essential for the plant to ensure both anchorage in the soil and sufficient supply of water and nutrients. A complete root-soil contact may reduce gas diffusion from the atmosphere into the soil; in contrast, a low root:soil contact can be limiting for uptake of water and solutes by confining the hydraulic pathway (Veen *et al.*, 1992). When the soil dries or when roots grow into a macropore with a larger diameter than that of the root (Tinker, 1976), the contact may be poor. Shrinkage of the root tissue itself may also be involved in the gap formation (Huck *et al.*, 1970; Cole and Alston, 1974; North and Nobel, 1997b; North and Nobel, 1997a).

The phenomenon of air gap formation was observed decades ago, and theoretical considerations were formulated in the 1950s (Philip, 1957; Bernstein *et al.*, 1959). Visualisation and quantification was practically impossible without non-invasive imaging techniques such as X-ray CT. With the help of these techniques, it was possible to visualise and measure the gaps (Carminati *et al.*, 2009), as well as to evaluate the root:soil contact, by quantifying the contact area (Schmidt *et al.*, 2012; Koebernick *et al.*, 2018). In Figure 6 an illustrative example of the development of such an air gap and the effect of the water content is given. By simultaneously measuring the stomatal conductance and the transpiration, the interactions between the root:soil contact and plant water stress could be analysed. It was concluded that root shrinkage is not the initial cause of water stress, but rather the consequence (Carminati *et al.*, 2013; Koebernick *et al.*, 2018). Moreover, it was shown that plant species can differ in the extent of shrinkage of different root orders (Carminati *et al.*, 2013). A recent study was carried out to quantify the seed-soil contact of sugar beet using X-ray CT, showing about double the seed-soil contact for pelleted and coated seeds in comparison to naked (untreated) seeds, which is likely to enhance germination (Blunk *et al.*, 2017).

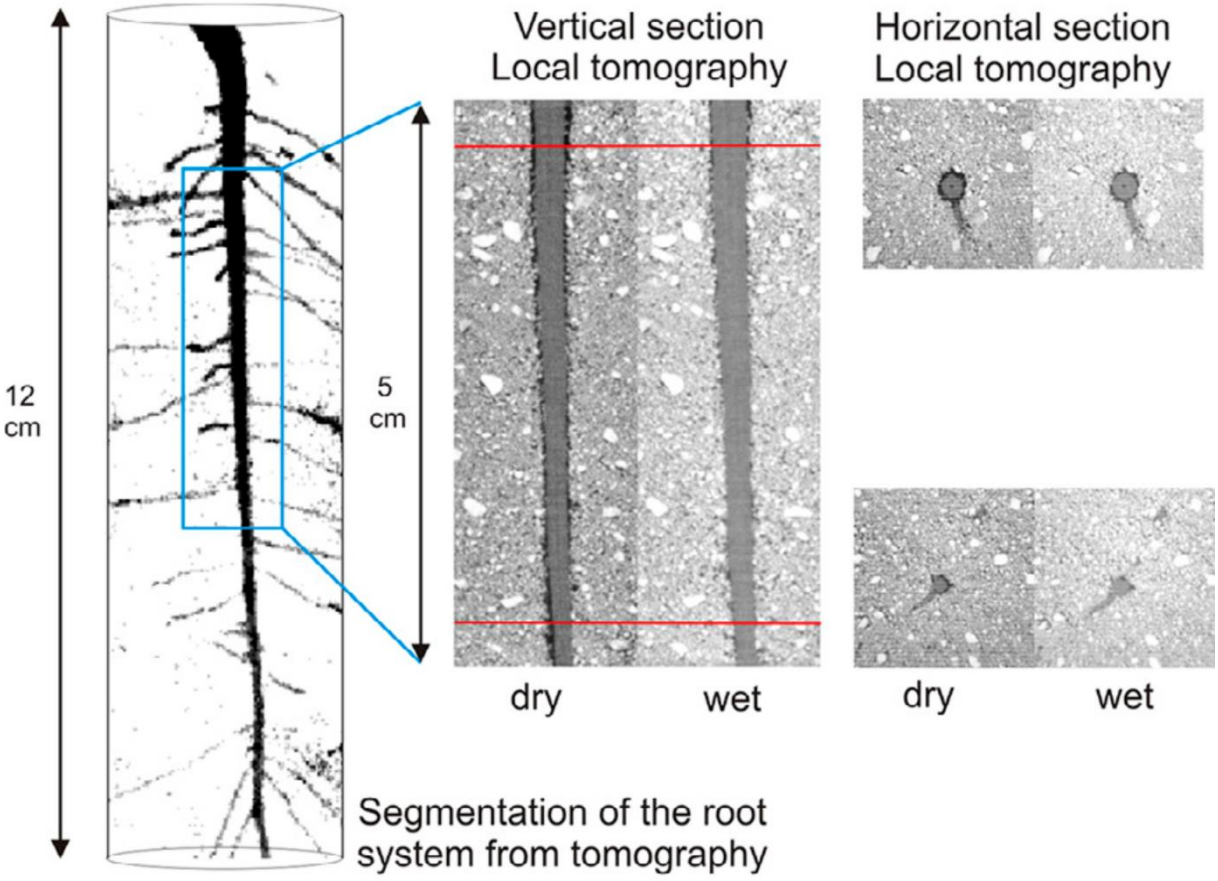

Figure 3.6: X-ray tomography of roots in soil: (left) three-dimensional root architecture of white lupin (Lupinus albus) growing in sandy soil; (middle) a two-dimensional vertical section of the local tomography under dry (left) and wet (right) conditions; (right) horizontal sections of the local tomography at two different depths (red lines in the centre image correspond to the depths), showing air gaps between roots and soil that are visible under dry conditions (black), while under wet conditions the gaps are partially closed. From Carminati *et al.* (2009).

Root hairs

Root hairs are tubular outgrowths from the epidermis of plant roots. They occur in the zone of maturation, are lateral extensions of a single cell and are rather small (a diameter between 5 and 20 µm and a length of 0.1-1.5 mm) (Gregory, 2008). Their role or function has been under discussion for a long time. There are indications that they are involved in water and nutrient uptake (especially phosphorus) due to an increase in the effective root surface area (Bates and Lynch, 1996; Gahoonia *et al.*, 1997; Carminati *et al.*, 2017). Moreover, they stimulate synergies with microorganisms, help anchor plants in the soil, and are involved in rhizosheath formation (Haling *et al.*, 2014).

The small size of root hairs, their relatively short lifetime of about 2-3 weeks, and the penetration into small pores and inter-aggregate spaces make them difficult to investigate. In the recent studies it was possible to visualise root hairs of *Triticum aestivum* and *Hordeum vulgare* using synchrotron radiation to study their role in P uptake (Keyes *et al.*, 2013; Keyes *et al.*, 2017a); and the rhizosphere structure formation (Koebernick *et al.*, 2017). A very small sample size in combination with a powerful technique and image processing are needed to achieve the level of detail needed for analysis of root hairs (Figure 7). Given the comparable scale, it is also conceivable that mycorrhizal associations with plant roots can be studied by visualising extraradical hyphae.

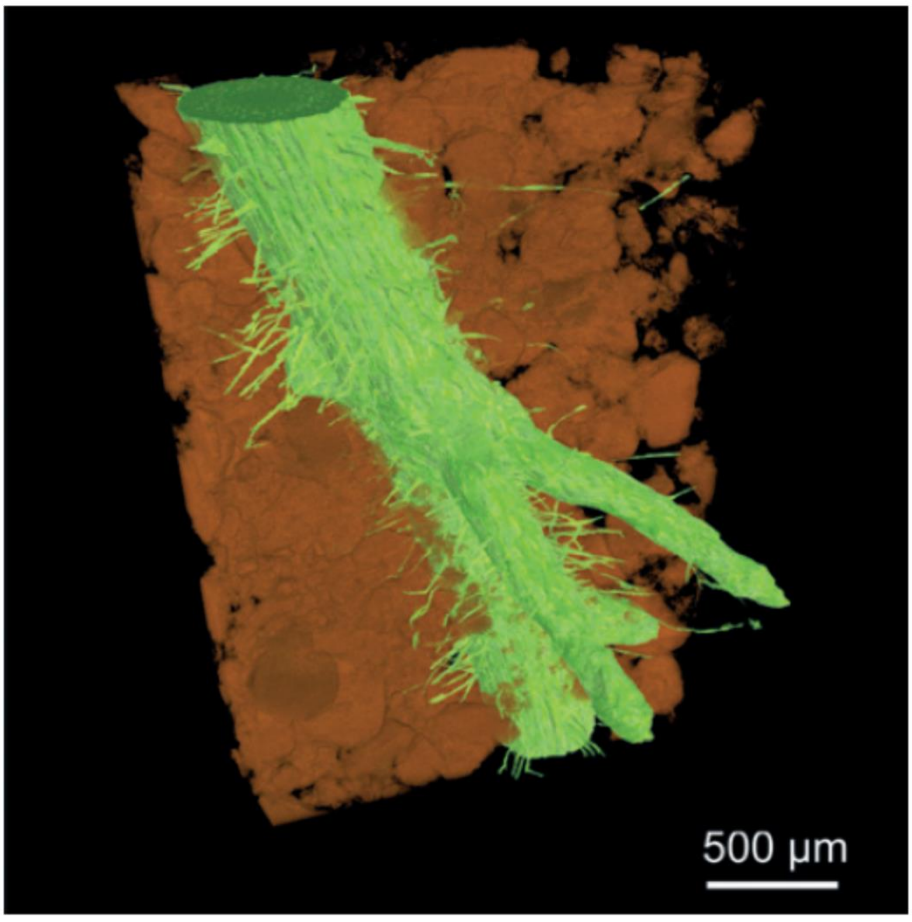

Figure 3.7: Digitally rendered 3D volume from a high resolution Synchrotron Radiation X-ray Tomographic Microscopy (SRXTM) at a resolution of approximately 1 µm. The soil phases are partially cut away to reveal a section of a seminal root of *Triticum aestivum*, including lateral roots and root hairs. From Keyes *et al.* (2013).

Soil root distances as a new perspective for soil exploration by roots

From the root perspective, RSA is typically quantified in terms of root length density and branching patterns. In contrast, from the soil perspective, root distances are a concept to consider regarding RSA (see Figure 5). The Euclidean distance from any location in the soil to the nearest root can be expressed either as distance distribution or distance maps (Koebernick *et al.*, 2014). This is a more direct estimate on how efficiently a plant explores the soil than root length, as these distances are a proxy for diffusion lengths of nutrients and water. The root distance distribution of *Vicia faba* was shown to develop (Figure 5) in predictable ways, which could be reproduced with a simple, empirical model (Schlüter *et al.*, 2018). This can be the basis for the sophisticated, dynamic models of root water and nutrient uptake.

Soil compaction by roots

When roots grow through the soil they often use already existing cavities and biopores (Passioura, 1991) or in general they preferentially follow a path of low penetration resistance or impedance (Sands *et al.*, 1979). Root mucilage serves as a gel-like lubricant that reduces the penetration resistance of the soil temporarily and in a small region around the root tip (McCully, 1999; Czarnes *et al.*, 2000). In the further course of root growth, there is also a shift and relocation of soil particles associated with changes in the spatial extension of pores and the pore network (Jones *et al.*, 2004).

Using optical microscopy and an artificial system, Vollsnes *et al.* (2010) were able to visualise particle displacement in 2D due to root growth of maize in a thin layer of sand between two glass microscope slides. By using X-ray CT, Keyes *et al.* managed to track root growth and associated rigid-body movement of soil particles in 3D (Figure 8) (Keyes *et al.*, 2016; Keyes *et al.*, 2017b).

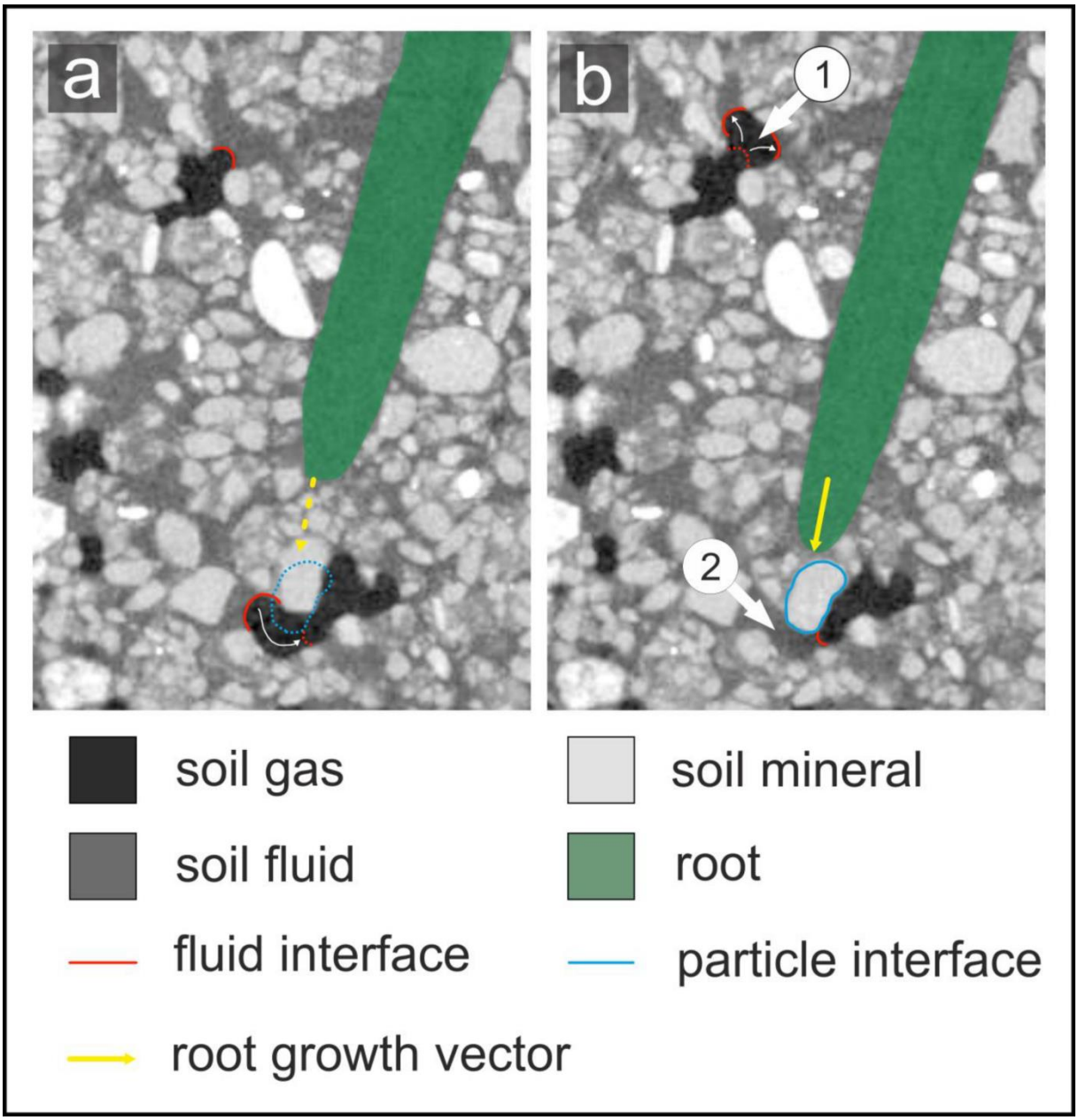

Figure 3.8: Deformation behaviour is shown qualitatively for a 2D section of X-ray CT data over a single growth step. During the 1-h interval between (a) and (b), the root tip extended along vector approximated by the yellow arrow, and the fluid/gas interfaces at locations (1) and (2) moved. At location (1), this interface movement (*i.e.* Haines jump) occurred without obvious soil matrix deformation in the pore region. At location (2), the interface movement coincided with obvious rigid-body movement of a soil grain driven by the extension of the root tip, resulting in a macro-pore volume change. From Keyes *et al.* (2016).

Aravena *et al.* (2011) showed that root-induced particle movement can result in a compression of large inter-aggregate pores. By numerical modelling based on the actual CT images, they showed that the root-induced deformation of the aggregated soil around a root increased unsaturated water flow in direction of the root.

Recent evidence suggests that soil compaction in the rhizosphere might not be a general trend, but that roots may also increase the porosity near the root surface by enhancing the aggregation of soil particles (Helliwell *et al.*, 2017; Koebernick *et al.*, 2017; Koebernick *et al.*, 2018).

Modelling

3D modelling of root uptake of water or nutrients from soil requires information on root architecture. Mostly, this information is generated using root system architecture models because direct measurements have not been available (Dunbabin *et al.*, 2013). Koebernick *et al.* (2015) were the first to replace modelled root architecture by time-resolved measured root architecture derived from X-ray CT data. In addition, they used measured soil matric potential dynamics as a reference for the different scenarios addressed by modelling.

For increased precision and a realistic representation of the natural conditions and the resulting consequences, detailed input variables are essential for the modelling. This is exactly what imaging with the current methods can provide. The combination of imaging and modelling is therefore taken up in several studies on phosphate uptake (Fang *et al.*, 2009; Keyes *et al.*, 2013), water transfer (Doussan *et al.*, 2006; Javaux *et al.*, 2008), inverse modelling of root hydraulic conductivity (Zarebanadkouki *et al.*, 2016), or influence of root-induced compaction on hydraulic properties in the rhizosphere (Aravena *et al.*, 2011).

Image-based modelling enables the direct application of numerical methods (*e.g.* finite element method) on the images by converting the segmented structures (*e.g.* soil matrix, pore space and pore network) into a textural mesh. This allows the characterisation of the physical properties of the pore space (Koebernick *et al.*, 2017; Daly *et al.*, 2018a; Daly *et al.*, 2018b).

Furthermore, the imaging enables the associated modelling of the root system, *e.g.* with the hydraulic tree approach (Landsberg and Fowkes, 1978; Doussan *et al.*, 1998). This allows dynamic modelling of growing root systems that can, for instance, change their hydraulic properties as the roots mature (Koebernick *et al.*, 2015), which can be parameterised by image analysis.

3.5 Future developments

Root system architecture is measured indirectly because classical imaging normally is dependent on the radiation of the sample and the reception of the remaining beam on a detector. For this reason, imaging usually takes place on the laboratory scale. The application on the field

scale as a standard procedure is not yet in sight. For MRI, there are initial approaches for use outside the laboratory (Pohlmeier *et al.*, 2018).

The technological progress of the methods described in this chapter has been rapid in the last decades and will likely continue. New instruments with better detectors and faster measurement methods are under development. The reduction of the measuring time is an essential aspect for the future. This is associated with a lower radiation exposure of the samples and a possibility of increasing the frequency of measurements or the number of samples measured in order to make more precise statements about the values of dynamic variables based on improved statistical certainty.

Fast phenotyping of root system architecture is of great interest for many scientific and breeding questions. This is slowly becoming more realistic, but still hindered by time-consuming post-processing and analyses. Recent advances in developing more automated workflow to run the analyses in a batch mode are promising (Gao *et al.*, 2019b). The research community needs to define standards for root scanning and method comparison concerning the image analyses. The training data sets for algorithm development are now available. The huge amounts of data generated during acquisition and the complex steps of image processing and analysis are not only very time-consuming, but also require appropriate computing power and equipment to handle the input data. Therefore, image analysis is now increasingly recognised as “the new bottleneck in plant phenotyping” (Minervini *et al.* (2015), and the faster and more standardised segmentation protocols are needed (Pfeifer *et al.*, 2015). Major hurdles that need to be overcome are manual user interaction and combinations of different software packages in the image processing workflow as well as the computational costs of large three-dimensional datasets (Mairhofer *et al.*, 2012; Mairhofer *et al.*, 2013; Flavel *et al.*, 2017).

The machine learning approaches have a huge potential to revolutionise image analysis in the future. However, this has been used seldom so far, with a paucity of case studies such as the "trainable WEKA segmentation" (Keyes *et al.*, 2017a; Koebernick *et al.*, 2017).

In addition, the scan time itself can be described as a further bottleneck because a single scan usually takes a relatively long time, multiplied by a large number of scans required. The scan time can be reduced at the expense of image quality (signal-to-noise ratio). The acquisition time for a typical X-ray CT scan is in the range of 10-120 minutes (Tracy *et al.*, 2012b; Pfeifer *et al.*, 2015; Ahmed *et al.*, 2016b; Koebernick *et al.*, 2018). With a reduction to 8.5 min, it was still possible to detect successfully the rather thick *Vicia faba* root network at an image

resolution of 40 µm, whereas the detection of *Hordeum vulgare* roots failed under these conditions (Blaser *et al.*, 2018). Hence, the optimal trade-off between scan time and image quality depends on the plant species, as well as on pot-size dependent image resolution, X-ray CT hardware and reconstruction software. There are also approaches for faster data acquisition for other methods, for example ultra-fast Neutron Tomography with a total acquisition time not exceeding 60 seconds (Totzke *et al.*, 2017).

Methods and algorithms from the other specialised areas such as materials research and of course the medical sector could be useful here, *e.g.* spiral CT (Kalender *et al.*, 1997) or helical differential X-ray phase-contrast CT (Fu *et al.*, 2014; Marschner *et al.*, 2016). With these methods, an object often no longer has to be irradiated from all 360 angles in order to generate the 3D data set and, therefore, a lot of time can be saved during data acquisition. Furthermore, there are new iterative reconstruction algorithms that require a smaller number of X-ray radiographs to achieve the same image quality in the 3D scans than the conventional filtered-back projection algorithms (Beister *et al.*, 2012; Kazantsev *et al.*, 2016).

Fast-throughput CT scanning is available in the industrial sector with in-line inspection systems that are fully unmanned and equipped with automatic defect recognition (Brunke *et al.*, 2012). Hence, objects can be scanned in a few seconds, but much higher energies in the range of kW are used, which would not be applicable in root research because the dose would be harmful to plants (Johnson, 1936; Blaser *et al.*, 2018). Approaches with lower doses are more likely to come from the clinical and biological CT applications.

4 How much is too much? Influence of X-ray dose on root growth of faba bean (*Vicia faba*) and barley (*Hordeum vulgare*)

4.1 Introduction

X-ray CT is a very powerful technology to study root growth in soil *in-situ*. Due to its non-invasive nature, the same plant can be scanned multiple times during plant development to study root growth dynamics. By this, plants receive a cumulated dose of X-ray radiation. Depending on plant species, growing period, scanning frequency (and duration), scanning settings (energy and current, but also source to sample distance) and used filters, this dose might be high enough to influence root growth. Early studies have shown that X-ray radiation can have a destructive influence on auxin and affects meristematic cells, as cytological changes like mitotic cycle delay, chromosome aberrations or loss of proliferative capacity were observed (Skoog, 1935; Johnson, 1936; Gray and Scholes, 1951; Hornsey, 1956; Davidson, 1960; Clowes and Hall, 1963; Clowes, 1963; Evans, 1965).

Studies using X-ray CT to analyse root growth over time, show large differences in total X-ray dose received by plants (Kaestner *et al.*, 2006; Mooney *et al.*, 2006; Tracy *et al.*, 2010; Flavel *et al.*, 2012; Schmidt *et al.*, 2012; Carminati *et al.*, 2013; Koebernick *et al.*, 2014). Several authors try to keep the dose very low, being aware that the dose may affect root growth (Gregory *et al.*, 2003) or use older plants (*e.g.* (Kaestner *et al.*, 2006; Perret *et al.*, 2007)), assuming a reduced sensitivity of older plants to radiation exposure. Only two recent studies working with X-ray CT included a control treatment without scanning to evaluate potential harmful effects of X-ray dose (Flavel *et al.*, 2012; Zappala *et al.*, 2013) – both have been working with cereals.

Therefore, as summarised in a recent review by Zappala *et al.* (2013), there is a lack of information on the influence of the method itself on the parameter in question. The dose of 33 Gy which according to the conclusion of Zappala *et al.* (2013) should not significantly influence growth provides a first orientation. In the present paper we will show that this threshold does not hold true for all plant species.

It is expected that apart from X-ray CT system specific parameters (energy settings, filter and distance) and the soil used, also plant age and especially root system and root architecture patterns are important. The early and comprehensive work of Johnson (Johnson, 1936) on seventy species (35 families) of flowering plants and their exposure to X-rays gives a broad overview, but is difficult to interpret, as CT settings, number of replicates and plant age differ

strongly and almost exclusively aboveground parts of the plants were considered for evaluation of the results. Johnson (1936) grouped the studied families in three divisions, being i) apparently unaffected, ii) slightly affected and iii) species noticeably affected by the X-rays. All plant species investigated by Johnson (1936) were dicotyledonous. Hence, no comparison between dicots and monocots can be derived from these results. Moreover, these results show that even within dicots, large differences exist regarding sensitivity against radiation.

Apart from the overall influence of cumulative X-ray dose, the question is whether there is a specific effect on initiation of root primordia and hence lateral root formation. This would lead to problems when X-ray CT is used to visualize and quantify root system changes in response to different stimuli like nutrient patches, water distribution or mechanical impedance.

The aim of this study was to evaluate the influence of cumulated X-ray dose (< 8 Gy) on two plant species in a given experimental setup. One species was faba bean (*Vicia faba*), a tap rooted dicot plant with a rather coarse root system, with large root diameters also for first and second order laterals. Due to this root phenotype *Vicia faba* is particularly suitable for X-ray CT studies, overcoming in part the trade-off between sample size and image resolution. The second plant species was barley (*Hordeum vulgare*), a monocot with an adventitious root system, having smaller root diameters than *Vicia faba*. One control treatment was established in both experiments and not scanned until one day before harvest and is compared to the two treatments being scanned every two and four days, respectively. These frequencies are typical for X-ray studies on root growth dynamics under controlled conditions.

4.2 Material and methods

Soil preparation, plant material and growth conditions

Acrylic columns (250 mm height, 35 mm radius, 5 mm wall thickness) were filled with a sieved (< 2 mm) and homogenised silty clay loam and packed to a bulk density of 1.2 g cm^{-3}. For more details of the soil, see Vetterlein *et al.* (2013b). The soil was N-fertilised with five urea-granules per sample, containing a nitrification inhibitor (ALZON46, SWK Piesteritz) at a rate of 100 mg N kg^{-1} soil in one layer 6 cm below soil surface. This was done as these granules could be visualised in the CT and served as a spatial orientation. The soil was initially watered by capillary rise with 239 ml distilled water, resulting in a water content of about 27 vol.% (= pF 2.65). Three treatments with five replications were set up for *Vicia faba*. Three treatments with at least six replications (two of the treatments with seven replications) were set up for *Hordeum vulgare*. Each column was placed on a weighing cell to record total transpiration and to enable

keeping water content as stable as possible. Soil columns were wrapped with aluminium foil to prevent algae growth. Seeds of faba bean (*Vicia faba* L., *cv.* 'Fuego') and barley (*Hordeum vulgare*, *cv.* 'Marthe') were surface sterilised with H_2O_2 (10%) and soaked in saturated $CaSO_4$ for four hours before one seed was placed per column about 1.5 cm below soil surface. Coarse gravel was placed on top of the soil to reduce evaporation from soil surface. Plants were grown for 17 days in a climate chamber under controlled conditions (12/12 h day/night cycles at 19 and 16°C, respectively. Photosynthetic active radiation was 350 µmol m^{-2} s^{-1}).

CT scanning configuration and image analysis

X-ray tomography was performed with an industrial µCT (X-TEK XTH 225, Nikon Metrology) with 140 kV, 286 µA (equals 40 Watts) and 500 ms exposure time. Each scan was performed with 1000 projections and one frame per projection, resulting in an exposure time of 8.5 minutes per scan. A copper filter with 0.5 mm thickness was used to reduce beam hardening artefacts. Distance between X-ray source and sample was about 13 cm. The spatial resolution of the X-ray tomogram is 40 µm. The calculated dose rate for these settings is 480 R h^{-1} (calculated with the Rad Pro Calculator for Desktop PCs Version 3.26 from http://www.radprocalculator.com/RadProDownloads.aspx). This equals 421 rad h^{-1} (calculated with the conversion factor 1 R = 0.877 rad (to air) from Cember and Johnson (2009)), equal 4.2 Gy h^{-1}. The Rad Pro Calculator tool calculates the dose in air, not in soil. Soil properties (or growth media in general) and soil moisture is neglected in the calculator. Therefore the comparability of different doses is only warranted for the external dose at the wall of the soil container, but not for the dose that reaches the plant roots in the soil column. In the course of the paper, the calculated doses therefore refer to the *maximum* dose acting on the container wall.

The first tomograms were performed 4 days after planting (DAP) for both treatments. The control (C) treatment was only scanned on the last day before harvest for comparison. The other two treatments were scanned every two ('frequent scanning' (FS)) and every four days ('moderate scanning' (MS)), respectively. From 6 DAP onwards, two tomograms, one above the other, had to be conducted to capture the major part of the root system (equivalent to the top 14 cm of the soil column). The remaining part of the soil column was not shielded during the other scans. Moreover a small overlap between both scans was required to enable the concatenation of both scans per sample for subsequent image processing. In this 1-2 cm large region the received dose for the roots is expected to be even higher. Moreover, the remaining part outside the region of interest during a scan is expected to receive further radiation from the X-ray beam and additional scattered radiation. During the growing period of 17 days, this

scanning procedure results in 7 scans for the treatment ‘moderate scanning’ and 13 scans for the treatment ‘frequent scanning’. The cumulative scanning times and doses per sample are 60 and 111 minutes, or 4.2 and 7.8 Gy, respectively.

All X-ray scans were performed during the night phase in the climate chamber. The X-ray tomograph and the climate chamber are located next door to each other.

For *Vicia faba*, a detailed analysis of CT-images was conducted. Main points are stated here, for more details see the Method in Appendix 1 (pages 106-107). Raw data was filtered to reduce image noise. Root systems were segmented with semi-automated region growing in VG Studio Max 2.1. Based on the idea of Flavel *et al.* (2012), binarised root systems were skeletonised and analysed with the plugin BoneJ (Doube *et al.*, 2010) in Fiji (Schindelin *et al.*, 2012). The resulting information was used to quantify root length and to distinguish between tap root, first and second order lateral roots. CT-images of segmented roots from consecutive time steps were spatially aligned with the elastix image registration software (Klein *et al.*, 2010; Schlüter *et al.*, 2016).

Due to the small diameters of *Hordeum vulgare* roots, short scanning times and therefore weak image contrast between root tissue and water-filled pores, analysis of *Hordeum vulgare* roots was not possible in the same way as for roots of *Vicia faba*. Therefore, for *Hordeum vulgare* only the results for the washed out roots analysed with WinRHIZO are presented.

Shoot and root analysis

At 17 DAP, the plants were harvested. Shoot fresh weight and leaf area were measured directly after harvest. Roots were washed out and analysed with WinRHIZO 2009b (Regent Instruments, Canada). WinRHIZO analysis was performed for total root length and three functional diameter classes. For *Vicia faba*, thresholds were set at 0.75 and 1.25 mm with the intention to separate first and second order laterals from tap roots, based on root diameters. The smallest class below 0.1 mm was discarded, as it is error-prone due to root hairs causing misclassification.

For *Hordeum vulgare*, thresholds were set at 0.20 and 0.50 mm with the intention to separate first and second order laterals from seminal roots based on root diameters. The smallest class below 0.05 mm was discarded, as it is error-prone due to root hairs causing misclassification.

Leaf area was also measured by WinRHIZO 2009b. Plant samples were oven dried afterwards at 65°C for 48 hours.

Statistics

Statistics (T-test and Scheffé post-hoc-test) were performed with SPSS 22 (IBM) at $p<0.05$. Standard errors are given in all figures as error bars.

4.3 Results

1. *Vicia faba*

Root growth development can be visualised by X-ray CT with a very high degree of detail for *Vicia faba* (Figure 4.1 and Figures A1.1-A1.5 for all replicates). The spatial resolution of 40 µm is sufficient to visualise all root orders of *Vicia faba*, emerging during the first 16 days of root system development, except for those growing along the container walls. It was thus possible to follow initiation of first order laterals starting to appear at day 8. Second order laterals were only observed at day 16.

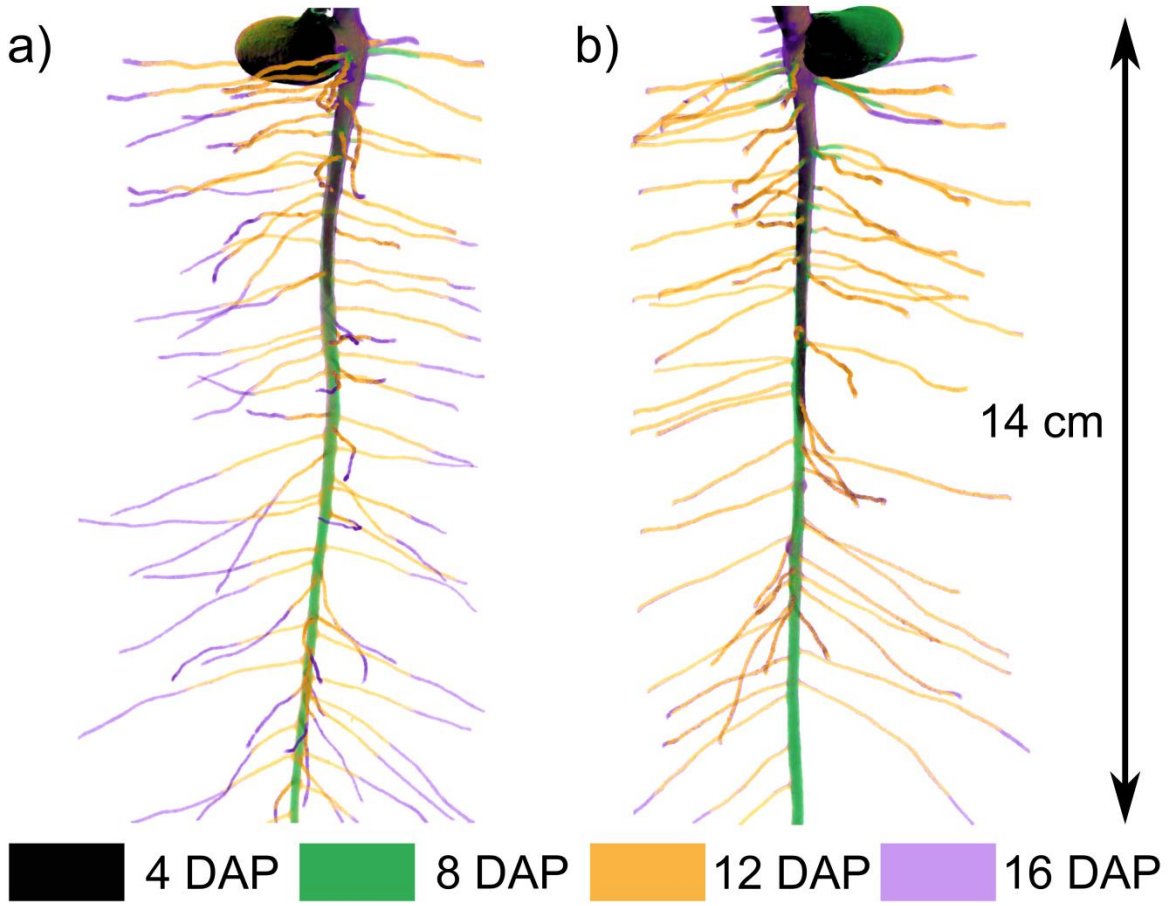

Figure 4.1: Time series of root system development of *Vicia faba*, acquired by X-ray CT. Representative 2D projections for both scanned treatments: a) frequent scanning (FS) and b)

moderate scanning (MS). Root age is colour coded for 4 (black), 8 (green), 12 (orange) and 16 (purple) days after planting (DAP). Changes in position are also recorded; this is the reason for the green shade at the seed in b). Secondary thickening can also be seen by the purple shade around the upper part of both tap roots. Illustrating videos for those two samples are available in the supporting information of this paper (links in Appendix 1).

CT data

As expected, the mean length of *Vicia faba* taproots did not differ among the treatments. Likewise, number of first order laterals showed no significant difference between both scanned treatments (Figure 4.2). For length of laterals, this is not the case. At 8 DAP the difference is not yet significant, but at 12 DAP the first order lateral roots of the 'moderate scanning' (MS) treatment are significantly longer than those scanned every second day (frequent scanning; 'FS', Figure 4.2). The difference between both treatments is 66.4%, very close to the overall difference in total root length at the end of the experiment, measured by WinRHIZO (66.7%). For the last point in time, again no significant differences were observed. Root length captured by X-ray CT at day 16 accounted for only 27-47% of total root length measured by WinRHIZO analysis at day 17. Roots growing below the CT's field of view (14-22 cm depth of soil columns) and those, growing at the container wall could not be observed.

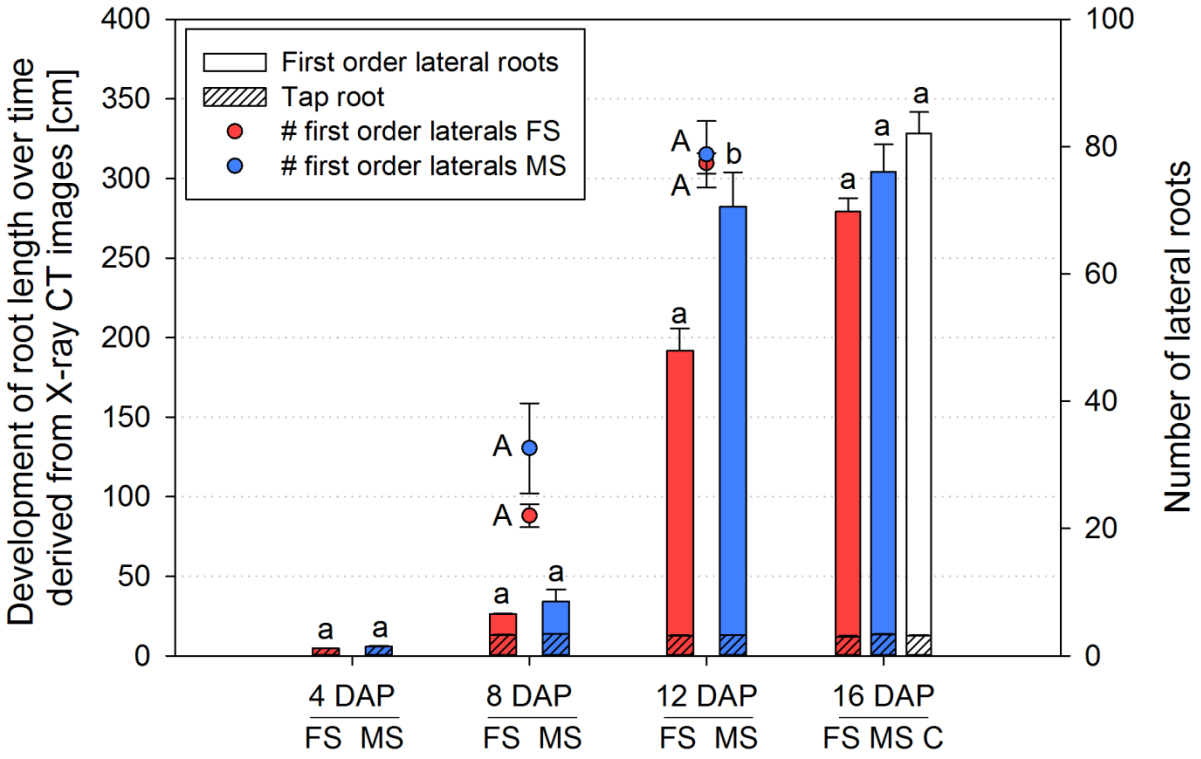

Figure 4.2: Root length (bars) and number of first order laterals (circles) of *Vicia faba* over time measured by X-ray CT; FS = frequent scanning; MS = moderate scanning; C = no scanning (control); Data is only shown for time steps when both treatments were scanned. Small letters refer to significant differences in root length and capital letters in number of laterals. Standard errors are given as error bars.

Apart from taproots and first order laterals, also number and length of most second order laterals was determined. This analysis was made for the data at 16 DAP, one day before harvest, comprising all three treatments (Figure 4.3). Both scanned treatments showed very few second order laterals (4.0 ± 2.0 for frequent scanning and 2.8 ± 0.7 for moderate scanning) with a small total length of 1.4 (± 0.8) and 0.9 (± 0.3) cm for frequent and moderate scanning, respectively. A high standard error for treatment 'frequent scanning' arose from one plant, having more second order laterals than all other replicates. For the control treatment, length and number of second order laterals were significantly larger (Figure 4.3).

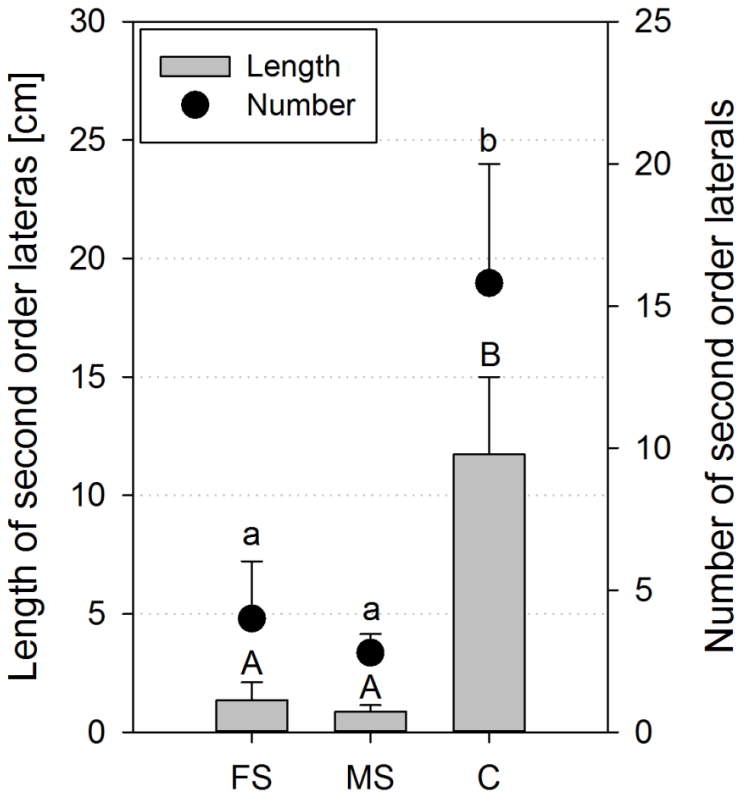

Figure 4.3: Length and number of second order laterals of *Vicia faba* measured with X-ray CT at 16 DAP; Standard errors are given as error bars.

Total root length at 16 DAP was reduced by X-ray scanning with high and moderate frequency (Figure 4.2). This result is confirmed by WinRHIZO analysis (Figure 4.4). Reduction was significant in comparison to control, but also both scanned treatments differed significantly. The mean reduction of scanned treatments in comparison to control is 25% for moderate scanning and 51% for frequent scanning.

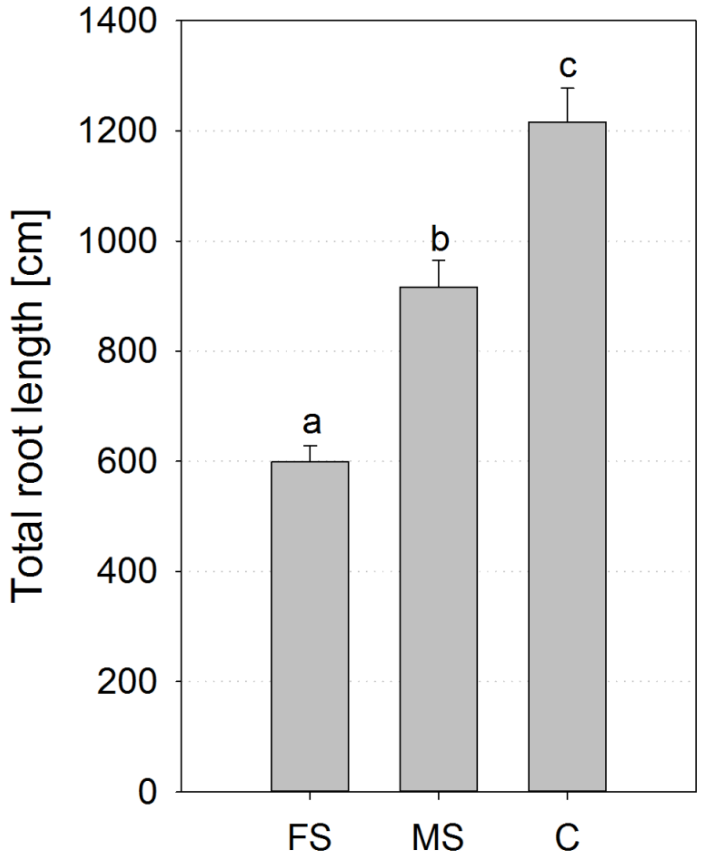

Figure 4.4: Mean total root length of *Vicia faba* at the end of the experiment (17 DAP) measured with WinRHIZO; FS = frequent scanning; MS = moderate scanning; C = control (no scanning); Standard errors are given as error bars.

Functional diameter classes, defined in WinRHIZO to distinguish between tap root, first and second order laterals, did not show clear differences between treatments (Figure 4.5). Length of first order laterals was significantly higher in the control, compared to both scanned treatments. However, root length in the smallest diameter class – which was supposed to represent second order laterals – did not match with the results from X-ray CT. Comparison of root length in this class obtained with both methods clearly indicates that threshold setting with WinRHIZO is not an adequate metric to derive root orders, because of similar root diameters for first and second order laterals and the conical shape of roots towards the root tip.

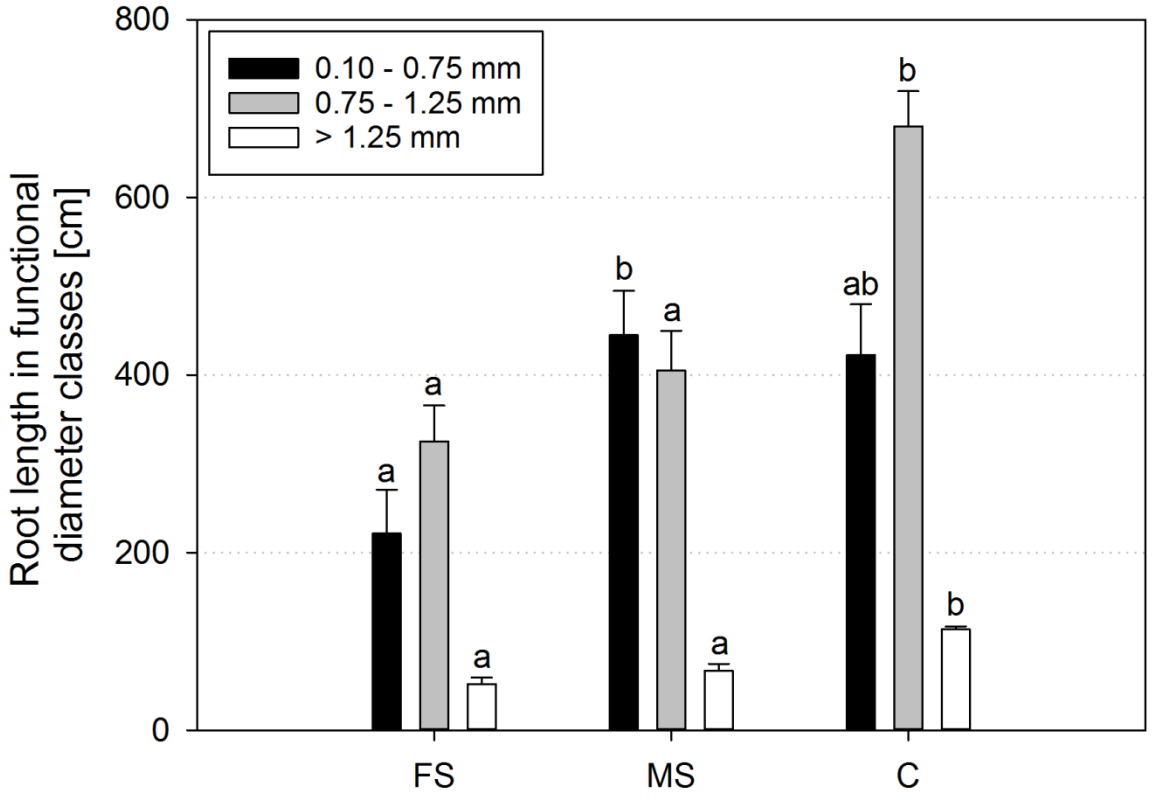

Figure 4.5: Mean root length in functional diameter classes of *Vicia faba* measured with WinRHIZO at the end of the experiment (17 DAP); FS = frequent scanning, MS = moderate scanning, C = control; Smallest diameter class < 0.10 mm is discarded due to distorted values by influence of root hairs; Standard errors are given as error bars.

Changes in root growth are not only apparent from root length data but also from visual inspection of the root system after destructive sampling at the end of the experiment. Root systems from control treatment show many elongated lateral roots with several lateral roots of second order at the oldest laterals (Figure 4.6). Most of the root systems from treatment 'moderate scanning' show smaller elongation of first order lateral roots and still some 2nd order laterals. For the treatment receiving the highest X-ray dose ('frequent scanning'), root system architecture reveals short laterals, especially in the upper third of the root system. This part of the root system received the highest total dose as it has been in the focus of scanning from day four after planting and also received further X-ray radiation during each scan of the lower part, as it was not shielded during the other scans. Overall, laterals are very short compared to the other treatments. Additionally, only very few 2nd order laterals can be found for most of the replicates. Moreover, roots were more brownish and brittle for the treatment of frequent scanning.

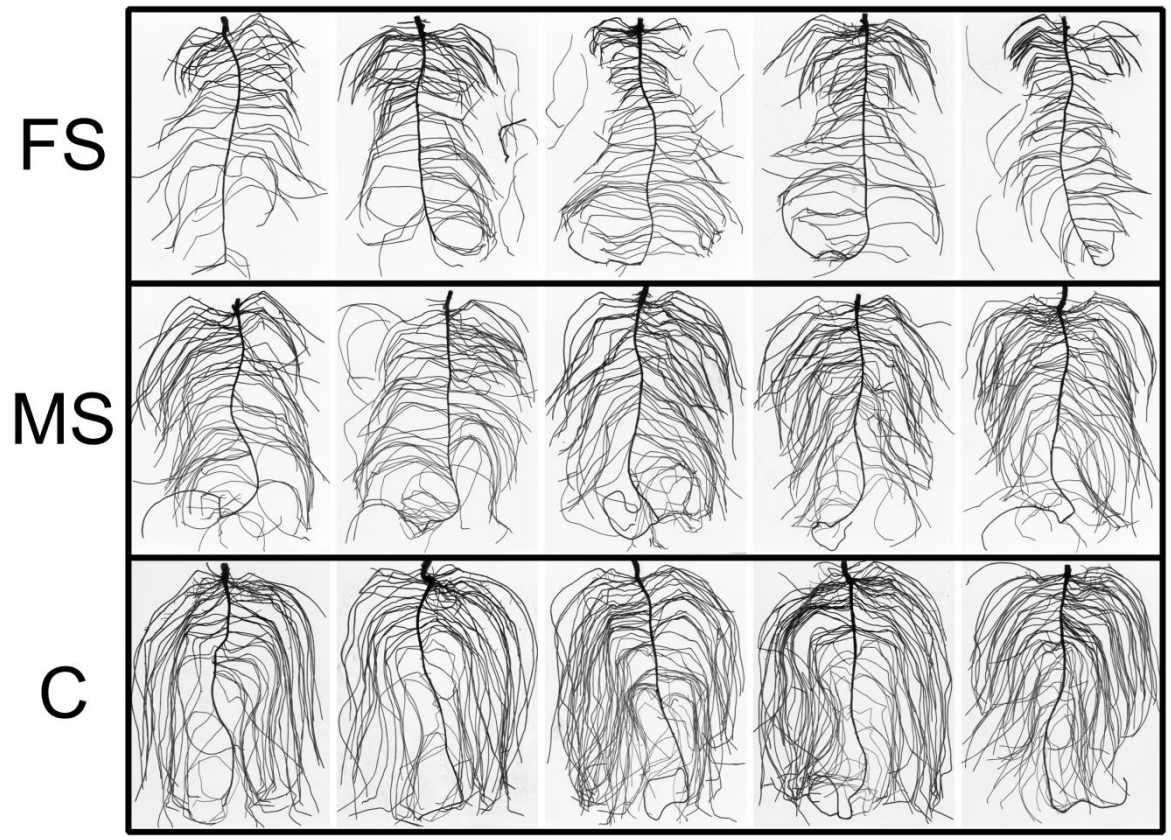

Figure 4.6: Washed out root systems from *Vicia faba* of all treatments (first row: frequent scanning (scanning every second day), middle row: moderate scanning (scanning every fourth day), bottom row: control without scanning) at the end of the experiment (17 DAP).

Leaf area and shoot weight

Mean fresh weight and mean leaf area of *Vicia faba* plants were significantly lower for both scanned treatments in comparison to the control. On average, mean shoot fresh weight was reduced by 25-29%, mean leaf area by 41-51% for both scanned treatments (Figure 4.7).

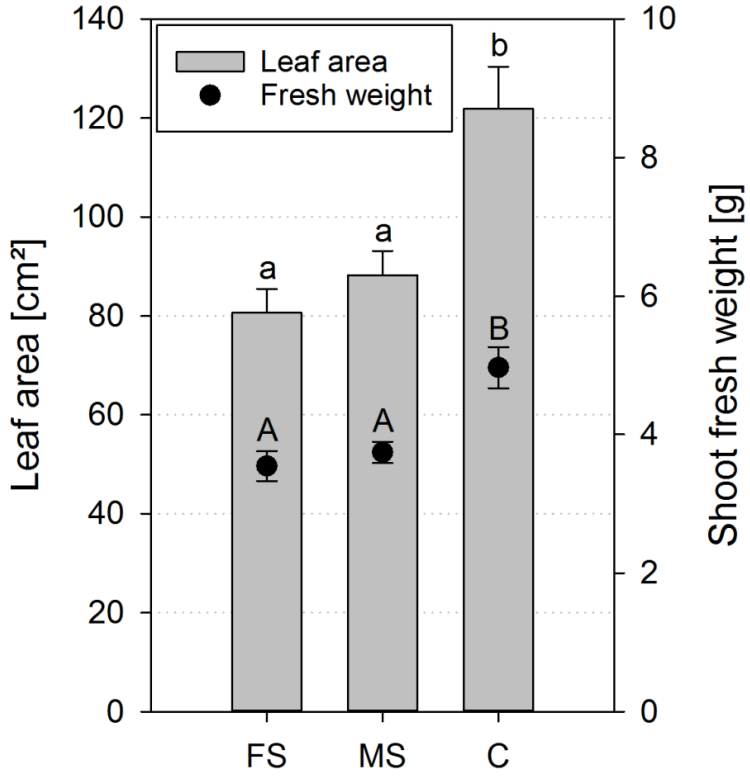

Figure 4.7: Mean leaf area and fresh weight of *Vicia faba* for frequent scanning (FS), moderate scanning (MS) and control treatment (C) at the end of the experiment (17 DAP); Standard errors are given as error bars.

2. *Hordeum vulgare*

For *Hordeum vulgare*, analysis of CT data was not possible due to very small root diameters and insufficient image quality in terms of image resolution and contrast. Hence, only washed out roots (Figure A1.6) were analysed with WinRHIZO at the end of the experiment. Results show, that both treatments of X-ray radiation had no influence on root growth for *Hordeum vulgare* in comparison to the un-scanned control treatment (Figure 4.8).

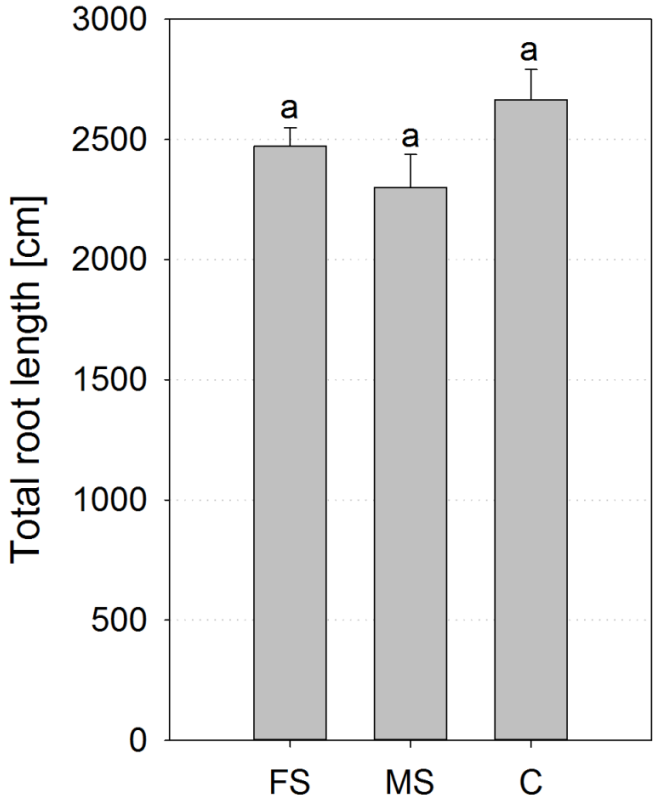

Figure 4.8: Mean total root length of *Hordeum vulgare* measured with WinRHIZO at the end of the experiment (17 DAP); FS= frequent scanning, MS = moderate scanning, C = control; Standard errors are given as error bars.

Like for *Vicia faba*, functional diameter classes were also chosen for *Hordeum vulgare*. Results are very similar for all treatments. There is a tendency towards a higher length of finest roots in the control treatment, but the absolute differences are small (Figure 4.9).

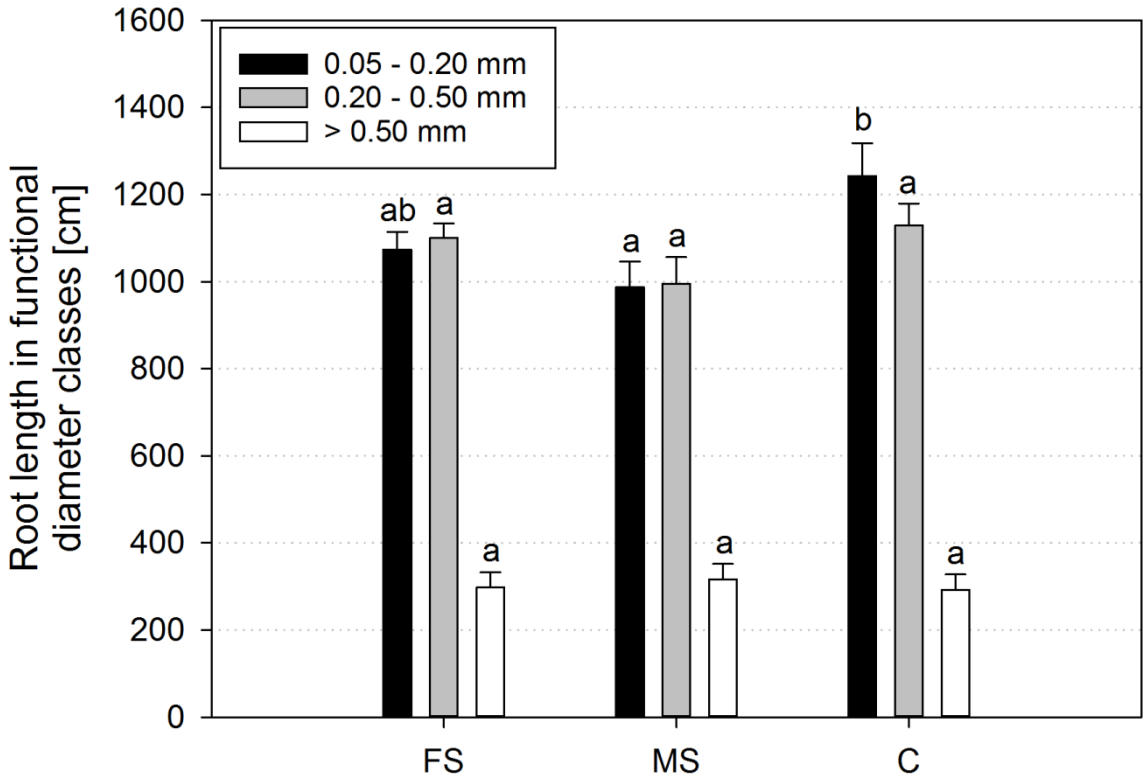

Figure 4.9: Mean root length of *Hordeum vulgare* in functional diameter classes measured with WinRHIZO at the end of the experiment (17 DAP); FS = frequent scanning, MS = moderate scanning, C = control; Smallest diameter class < 0.05 mm is discarded due to distorted values by influence of root hairs; Standard errors are given as error bars.

As for roots, no impact of X-ray scanning on shoot growth was detected. Neither leaf area nor shoot fresh weight was different for the scanned treatments in comparison to the control treatment (Figure 4.10).

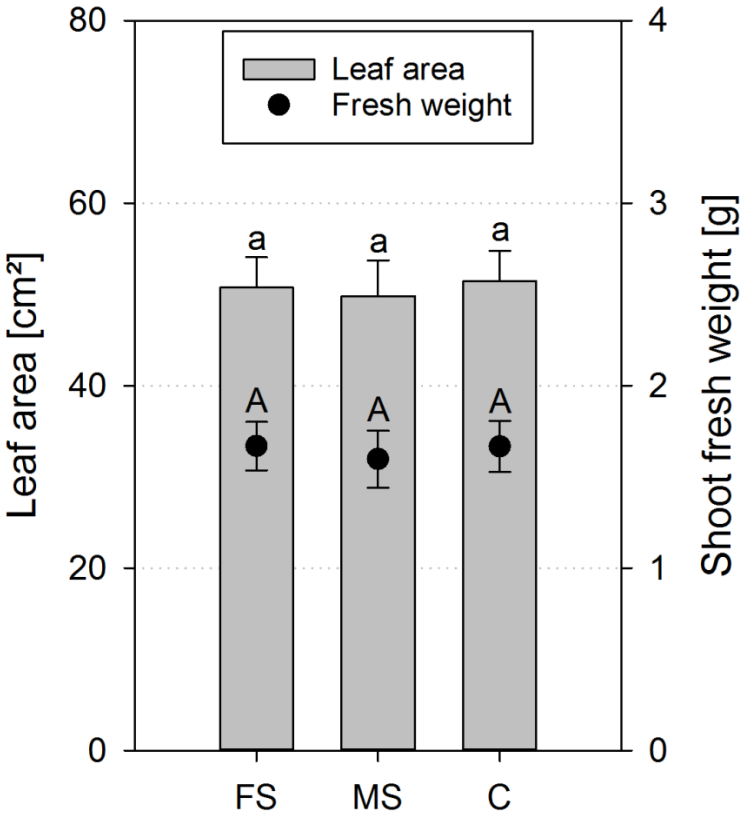

Figure 4.10: Mean leaf area and shoot fresh weight of *Hordeum vulgare* for frequent scanning (FS), moderate scanning (MS) and no scanning control treatment (C) at the end of the experiment (17 DAP); Standard errors are given as error bars.

4.4 Discussion

Root growth development and the influence of cumulated X-ray dose on *Vicia faba* and *Hordeum vulgare*

X-ray CT enables studies of root growth development in a very high quality. The chosen scanning interval revealed essential steps of root system development of *Vicia faba* (Figure 4.1, Figures A1.1-A1.5). At 4 DAP only the tap root had developed. At 8 DAP already several first order laterals had emerged and further initials could be recognised along the tap root. At 12 DAP first order laterals had developed and elongated along the whole length of the tap root; elongation of individual laterals could be accurately followed over time. The last scanning date (16 DAP) showed initiation of second order laterals (Figures A1.4 and A1.5).

For *Hordeum vulgare* with most of the roots smaller than 0.5 mm (Figure 4.9), visualisation of root system development by X-ray CT was not possible with the chosen X-ray CT settings.

These had to be the same for both plant species in order to enable a direct comparison of their susceptibility to a certain X-ray dose. Image quality for *Hordeum vulgare* could be improved by choosing smaller soil column diameter to achieve a higher resolution and by increasing the number of projections per scan. However, first this would reduce the soil volume available for root development and second this would increase scanning duration and therefore X-ray radiation dose. New algorithms like the one suggested in (Flavel *et al.*, 2017) can help to segment roots of *Hordeum vulgare* in general. But in the case of this study, assuring the comparability through identical scan settings was more important than an optimised detection of *Hordeum vulgare* roots.

In this study, we found a significant impact of cumulated X-ray radiation on plant development of *Vicia faba*. The influence was more pronounced for root traits, but also leaf area and shoot biomass was significantly smaller for plants exposed to X-rays in comparison to the control. Analysis with WinRHIZO revealed a clear difference of total root length between all three treatments of *Vicia faba*, but not for *Hordeum vulgare*. We have derived quantitative information about root length and number of first and second order laterals from the CT data for *Vicia faba*. Here, especially number and length of second order laterals was obviously affected by X-ray radiation.

Elongation of first order laterals was delayed by frequent application of X-ray CT. At MS the majority of first order laterals reached the container wall at 12 DAP. At FS, this was only the case at 16 DAP, indicated by the shorter orange segments in Figure 4.1, Figure A1.5 and two videos (link can be found in Appendix 1, page 113).

Between 12 and 16 DAP most of the root growth in the moderate treatment occurs outside the scanned region of interest (ROI), which gives the high-dose treatment the chance to catch up with respect to root length densities within the ROI. We would not ascribe the observed behaviour to a bonsai effect due to confined growth in a limited pot volume, but simply to limiting our observation to the ROI.

The reduction in root elongation might be related to phytohormones like cytokinins, ethylene and especially auxin, essential for cell division and root elongation. Skoog (1935) used different setups to study the effect of X-ray radiation on auxin and plant growth. Most experiments were performed with 900 kV and 3-4 mA, but with a layered filter consisting of lead, steel and aluminium to remove soft radiation. They either irradiated auxin directly in agar blocks or solutions, or seedlings and very young plants of *Vicia faba* and *Pisum sativum*. For example, at

50 Röntgen (600 kV and 3-4 mA) per minute, about 30 percent of uncovered auxin in agar blocks was destroyed after 30 seconds. The applied dose rate equals 0.44 Gy per minute. This is about 4 times higher compared to our study with less than 0.1 Gy per minute. Moreover, they could show that 30-40% of the auxin diffusing out from terminal buds and stem sections from *Vicia faba* were lost after irradiation of the plants in comparison to the control. In general they stated that by X-ray radiation, auxin is inactivated in solutions because of oxidation, that the formation of auxin is inhibited by moderate dosages, that decrease in growth is a function of dosage and that the mechanism of auxin transport is not affected. Given the fact, that auxin is not directly exposed to radiation in our case, as it is embedded in the root cells that are surrounded by soil that scatters and attenuates the radiation to some extent, it is likely that the influence is smaller. But still a considerable part of auxin may be destroyed by the accumulated dose of X-ray radiation during the scanning times. As auxin is also a key player in initiation of second order laterals, this is in line with the result that *Vicia* plants of both irradiated treatments had significantly less and shorter second order laterals (Figure 4.3) compared to the control. Unfortunately we could not find more recent literature investigating this auxin hypothesis for root development in a setting comparable to ours.

Evans (1965) cited a work from Gray and Scholes (1951), working with 143 R of X-rays. This equals about 1.3 Gy. In Gray and Scholes (1951), a 80% reduction of the growth rate of the primary root of *Vicia faba* five days after irradiation was found. Moreover, it was shown that the reduction in root growth was the same if only the root tips received radiation. When the root tip was shielded, no reduction in root growth was found. This underlines the essential role of meristematic cells regarding sensitivity against X-ray radiation.

In particular young tissue with a high share of meristematic cells was sensitive to radiation. Cytological changes were observed in irradiated plant meristem cells (Evans, 1965). Evans (1965) reported that the mitotic cycle delay is transitory and full recovery was possible within 24 h. This recovery effect is also reported by (Hornsey, 1956; Clowes and Hall, 1963; Clowes, 1963). Also our treatment with low scanning frequency had more time to recover compared to the treatment with high scanning frequency, but our experimental setup does not allow for separation of total dose and scan frequency effects. Evans (Evans, 1965) also stated that chromosome aberrations and influences on nuclear volume and DNA synthesis are crucial for growth inhibition. Similar results were achieved by Davidson (1960), reporting viable atypical chromosome complements in cells of primary roots of *Vicia faba* by exposure to a dose of 600

r (equals 5.3 Gy), 24 hours after germination. These effects are expected to be even more pronounced, when the same plant is irradiated with a high frequency.

Zappala *et al.* (2013) found no influence of X-ray radiation on root length and number of tips in rice (*Oryza sativa* spp. Azucena). X-ray settings were 110 kV, 320 µA, 0.2 mm Cu filter and a source to sample distance of 21.5 cm. This means lower kV, higher µA, thinner Cu filter and higher source to sample distance compared to our settings. Over a total of 9 scans of 73 minutes each, this resulted in a total dose of ~13 Gy per column (stated by the authors). This is about 5 Gy more than at the frequent scanning treatment in our study. The results of Zappala *et al.* (2013) for *Oryza sativa* are similar to our results for *Hordeum vulgare*, showing no influence of X-ray radiation on root growth. The contrasting results for *Vicia faba* may be related to the very coarse and compact taproot system architecture of the dicotyledonous plant in comparison to the large and adventitious root systems of *Oryza sativa* and *Hordeum vulgare*, or cereals in general.

This is also confirmed by the results from Flavel *et al.* (2012). They reported that no effect of exposure to X-rays on *Triticum aestivum* L. *cv.* 'Gregory' was detected. They used 100 kV, 270 µA, a 0.5 mm Cu filter and very short scanning duration (4 minutes and 10 seconds per scan, maximum scanning duration was 20 minutes 50 seconds). Dose in Flavel *et al.* (2012) cannot be quantified precisely, as information regarding distance between X-ray source and sample is missing but due to low energy settings and short scanning duration dose is estimated as rather low compared to our study.

Adventitious root systems have more root tips and meristematic cells than taproot systems. Therefore, statistically there are more cells available to respond to radiation. In turn, if a certain percentage of cells are affected by X-ray radiation, more meristematic cells in root tips stay intact to compensate and maintain root growth and by this plant development.

Moreover, plant age during the first exposure might have a strong relevance regarding sensitivity to radiation. Very often plants are only a few days old when they are placed in the tomograph for the first time (*e.g.* Gregory *et al.* (2003): "few days"; Hargreaves *et al.* (2008): 3-7 days). In our case, plants were scanned initially 4 days after planting. This is an early stage of development, but this is quite usual for many studies dealing with root development, measured by X-ray CT. In Zappala *et al.* (2013), plants were older at the first scan (19 DAP). This might also be an additional reason why there was no influence on root growth of *Oryza sativa* in that case, but more research is needed to clarify the relevance of plant age.

The review of Zappala *et al.* (2013) and in particular the comprehensive study of Johnson (1936) covering 70 plant species provided first evidence for differences in sensitivity against radiation between species. For instance, Johnson (Johnson, 1936) found changes of flowering time, change of flower number, varying average plant height at maturity for some species and stated a reduction in number and length of roots for at least one species (*Ricinus communis*). No general plant response can be derived, especially as some species were apparently completely unaffected by the X-ray radiation. Experimental conditions were not identical for all plant species and plant age varied between 8 and 74 days in the study of Johnson (Johnson, 1936). However, there is a need for more experiments with same settings comparing different plant species to increase understanding if the degree of sensitivity is a function of plant species (or families) root diameter, cell size, number of root tips or a combination of all parameters. This is especially true, as all studied plants in Johnson (1936) were dicotyledonous, *i.e.* just representing one group of flowering plants, and even within this group considerable variation regarding sensitivity to radiation was found, *e.g.* a varying average plant height at maturity from -79% to +77%.

In one preliminary experiment focused on optimizing the CT parameters for best image quality and short scanning duration we applied a dose of >16 Gy per single scan, calculated with a 0.2 mm Al filter in the Rad Pro Calculator, as Al reduces dose the least among the available materials in the Rad Pro Calculator (further CT settings were: 810 µA, 140 kV, no filter, 8.5 minutes duration, same column size and similar substrate). In that case, *Vicia faba* plants died immediately after the first two scans (4, 8 DAP), having a tap root of less than 4 cm in length (Figure 4.11).

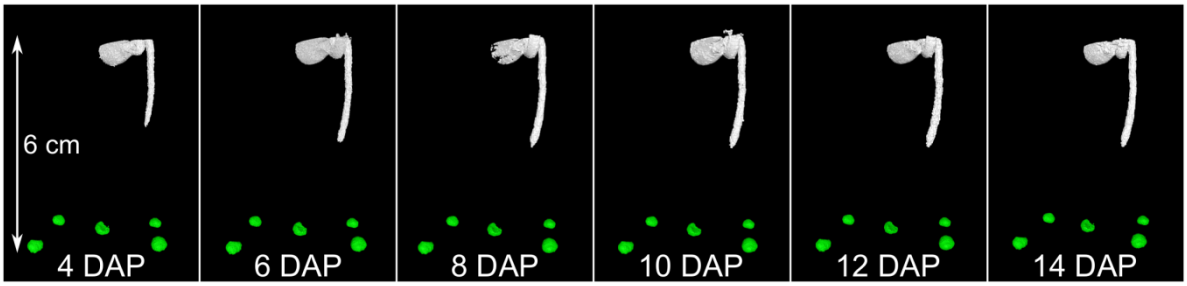

Figure 4.11: Time series of a taproot from *Vicia faba* visualised every second day by X-ray CT; Scanning settings were chosen for best image quality; Lethal influence on root tissue can be seen, as the root did not grow further within 14 days.

Strengths and limitations of dose estimation

Estimation of cumulative dose through X-ray CT enables a certain comparability between different studies. This is important as a direct measurement of the cumulative dose received by the roots is not a standard procedure at the moment. This localised dose measurement in soil as a function of wall distance should be addressed and implemented in future research. Moreover, root segments receive location-dependent dose rates as the soil shields radiation. Roots directly at the container wall receive a dose rate comparable to the estimated maximum dose rate in air, whereas X-ray attenuation is highest in the centre of the soil column. This attenuation depends on bulk density, soil moisture and sample diameter. But since the CT scan settings are typically adjusted to these soil properties, the actual dose that reaches the centre of the column rather depends on the signal-to-noise ratio that one is willing to accept or able to achieve with a given detector panel and reconstruction software. In the course of the growth experiment the root explores the entire range of wall distances which impairs a good estimate of the actual, cumulative dose experienced by individual root segments and the whole root system, respectively.

Despite these shortcomings in assessing the actual dose, reporting estimates of the maximum dose in air has some merits, as it enables a quick comparison between X-ray CT studies. Yet, there are also some limitations and uncertainties in dose estimation, as the current version of the Rad Pro Calculator includes only the basic settings like voltage, current, distance between source and sample, filter material and filter thickness. Hence, not all specifics of the X-ray source are included. Moreover, effects like scattering, attenuation and beam hardening are not included. Furthermore we experienced changes within the calculation method in different versions of the calculator that resulted in different calculated doses. Finally, applying the same total dose as one single scan or several scans with lower dose per scan is not distinguished in the calculator. In clinical radiobiology the concept of “biologically equivalent doses” (Joiner and Bentzen, 2009) of different scan frequencies is well established, but it is uncertain whether this also applies to root tissue.

Recommendation on best practices

We have shown that *Vicia faba* and *Hordeum vulgare* have a very contrasting susceptibility to X-ray radiation. In our case all experimental conditions were kept the same and the plants also had the same age when irradiated. Further research is needed to investigate in detail why different plant species react differently to X-ray exposure. Moreover, additional studies are

required to clarify the impact of *e.g.* plant age, soil properties, X-ray spectra and other parameters determined by the X-ray settings that may influence the susceptibility of plant roots to X-ray radiation.

In light of the 1) uncertainties in estimating the actual dose received by plants in pot experiments, 2) the different response of the two investigated species to the same dose and 3) the harmful irradiation effects in *Vicia faba* at relatively low dose for a typical growth experiment, we strongly recommend to always use an un-scanned control (*i.e.* only scanned once at the end) to adequately assess radiation effects. Moreover, the number of scans and dose per scan should be reported rather than the cumulated dose, to assess potential recovery effects. Finally, care should be taken to minimise the applied dose to the roots, especially when working with young tap rooted plants. This could also be done by shielding the part of the soil column that is not in the region of interest during the scan.

4.5 Conclusion

We conclude that X-ray CT is a powerful method to observe root system development in the soil *in situ* with high quality of information. But there is a lack of information regarding the influence of X-ray radiation on root growth. As we could show, *Vicia faba* was affected significantly by X-rays, especially when scanned at a frequent temporal resolution (every 2nd day in this case). In comparison, *Hordeum vulgare* showed no influence of X-ray radiation for the exact same sample conditions and scanning parameters. This information is very important to adjust experimental setups and scanning parameters in the future. When X-ray CT is used to study root dynamics as a response to *e.g.* nutrient availability, water distribution or soil mechanics it is essential to know about the effect by the method itself to enable reduction of this influence or to distinguish between method-associated reaction and dynamic response of the roots to soil-physical and chemical conditions. This is especially true for cumulative scanning setups for time-lapse analysis of root growth. Having an un-scanned control treatment is a viable option to estimate potential influence of the X-rays.

Further research is needed to investigate why different plant species have a different susceptibility to X-ray radiation and to elaborate on the impact of *e.g.* plant age and soil properties, as well as CT scanning parameters on the influence of X-rays on root growth.

Acknowledgements

We thank SKW Piesteritz for provision of the fertilizer granules. We also thank Ina-Maria Zickenrott and Bernd Apelt for their help during preparation and harvest of the experiments.

5 When Drew meets soil - Dynamics of localised supply of nitrogen-species in soil and their relevance for root system morphology – A comparison between faba bean (*Vicia faba*) and barley (*Hordeum vulgare*)

5.1 Introduction

Root system architecture (RSA) is an important property that can be influenced by the availability of nutrients and their concentration and distribution in the growth medium (Robinson, 1994; Forde and Lorenzo, 2001; Hodge, 2004). This is a well-known phenomenon for phosphorus (Drew and Saker, 1978; Flavel *et al.*, 2014; Ahmed *et al.*, 2016b), but also of high interest for nitrogen (N) not least due to the agricultural practice of local fertiliser application.

As stated by Nacry *et al.* (2013), a "typical" root growth response to N that is applicable for all species and conditions is almost impossible to define, as many biotic and abiotic factors are responsible for the final shape of the RSA. Nacry *et al.* (2013) highlighted two very general aspects that hold true for most circumstances: 1) high N status of the plant leads to a systemic repression of lateral root growth and 2) exogenous NO_3^- or NH_4^+ stimulates lateral root growth locally.

Earlier studies from Drew and colleagues (Wiersum, 1958; Hackett, 1972; Drew *et al.*, 1973; Drew, 1975; Drew and Saker, 1975; Drew and Saker, 1978; Granato and Raper, 1989) were conducted to analyse root growth response to local availability of nutrients. Especially Drew (1975) has shown a marked response of barley roots to NO_3^- and NH_4^+ in column experiments. In Drew (1975) quartz sand was used as growth medium and wax membranes were installed to separate the columns in three layers percolated with nutrient solution differing in concentration of the respective nutrient. The best-known illustrations are from Drew's treatment with increased concentration in the middle layer, compared to N-deficient conditions in the layers above and below.

Other studies were carried out addressing root growth response to different distributions or ratios of NO_3^- and NH_4^+, using approaches with nutrient solution (Maizlish *et al.*, 1980; Granato and Raper, 1989; Sattelmacher and Thoms, 1989; Gerendás and Sattelmacher, 1990; Thoms and Sattelmacher, 1990; Bloom *et al.*, 1993; Schortemeyer *et al.*, 1993; Caba *et al.*, 2000; Bloom *et al.*, 2006; Tian *et al.*, 2008; Ogawa *et al.*, 2014) or agar plates (Zhang and Forde, 1998; Zhang *et al.*, 1999; Remans *et al.*, 2006; Lima *et al.*, 2010; Gruber *et al.*, 2013). These

studies pointed out that RSA can be modulated in an intense manner, given the right experimental conditions. Taken together with Drew's results, one general conclusion from these studies was that NH_4^+ nutrition results in a higher number of lateral roots, while NO_3^- nutrition results in increased elongation of laterals. The results vary considerably and one has to keep in mind that in these systems, applied concentrations are typically low and constant, as is the pH due to added buffers or frequent exchange of the nutrient solution. Furthermore, sorption is not a factor there and the root surface is usually in direct contact with the nutrient solution, which is quite different from conditions found in soils.

While it is uncontroversial that the artificial conditions in the laboratory do not adequately represent natural conditions in soil, the vast majority of studies on this topic are still carried out in artificial systems like agar or nutrient solution. In soils, organic matter is always an additional source of N. Also, microbial activity in soils is highly diverse and dynamic resulting in a high variability of turnover rates for hydrolysis of urea and for nitrification (Vitousek *et al.*, 1982; Heil *et al.*, 2016; Beeckman *et al.*, 2018). Also, NO_3^- and NH_4^+ normally co-exist in soils at the same time but concentration and mobility may differ by orders of magnitude (Blume *et al.*, 2015). Moreover, NO_3^- is highly mobile but NH_4^+ can be adsorbed to the soil matrix, with sorption depending strongly on many factors, *e.g.* clay mineral composition, clay content and amount of competing cations (Nommik and Vahtras, 1982; Nieder *et al.*, 2011).

There are studies that have been performed in soil (Maizlish *et al.*, 1980; Anghinoni and Barber, 1988; Anghinoni *et al.*, 1988; Bloom *et al.*, 1993; Zhang and Barber, 1993; Van Vuuren *et al.*, 1996; Hodge *et al.*, 1998; Hodge *et al.*, 1999a; Hodge *et al.*, 1999b; Maestre and Reynolds, 2006; Ogawa *et al.*, 2014; Xu *et al.*, 2014; Pan *et al.*, 2016; Rabbi *et al.*, 2017), which found widely differing results of plant growth response to different N applications, covering the whole spectrum from no response to stimulation of root growth to inhibition and toxicity symptoms. However, measurement of N-dynamics in soil and soil solution was largely neglected, as was root growth analysis over time in most of these studies.

With this paper we enhance the understanding of temporal and spatial dynamics of root response *in situ*, in the soil to different N forms. For this purpose we used commercial urea fertiliser granules with and without a nitrification inhibitor (NI) in order to create treatments varying in NO_3^- and NH_4^+ concentrations. Urea was used as it is the most widely applied nitrogen fertiliser on a global scale according to the Food and Agriculture Organization of the United Nations (FAO) statistics (http://www.fao.org/faostat/en/#data/RFB). In soil, urea is rapidly hydrolysed to NH_4^+ which is subsequently adsorbed to clay particles and organic matter

or oxidised to NO_3^- if the relevant bacteria are present. This oxidation process is delayed by the use of NI (Slangen and Kerkhoff, 1984; Abalos *et al.*, 2012; Zaman *et al.*, 2013). As a consequence, NH_4^+ is more stable in the soil and its importance as a nutrient has increased.

The work from Drew (1975) and especially the resulting striking figures are well-known. It should be clear that these results cannot be directly transferred to the soil system, as the conditions in soils differ significantly from those in Drew's studies or related experiments in nutrient solution or on agar.

Drew's work clearly was the inspiration for our experiments, but it was not our goal to replicate Drew's results. On the one hand we adopted some of the methodological approaches from Drew's work, *e.g.* the subdivision of the sample in three segments with higher nutrient concentrations in the middle one and the comparison between NO_3^- and NH_4^+. On the other hand we have deliberately chosen a modified approach that is more likely to mirror situations that are experienced by crops growing in fertilised systems in soil or other agroecosystems with heterogeneous nutrient distributions.

Hence, we used soil instead of quartz sand, refrained from the physical barrier between the three segments (wax membranes in Drew's work) and applied commercial urea fertilisers (100 mg N/kg soil) with and without nitrification inhibitor to create different NO_3^- and NH_4^+ concentrations and ratios in the soil and soil solution, instead of a continuous in- and outflow of nutrient solution with constant concentration and pH. Moreover, soil solution chemistry and plant growth development were monitored over time.

The well-known issue that roots growing in soil cannot be observed directly is overcome by application of X-ray computed tomography (CT). X-ray CT is currently one of the best non-invasive imaging techniques for visualising and quantifying plant roots in soil *in-situ* over time (Metzner *et al.*, 2015). *Vicia faba* was chosen as with its large root diameters the challenge of following the development of individual laterals could be addressed (Koebernick *et al.*, 2014). In this way, development of RSA for different root orders was captured (Blaser *et al.*, 2018; Schlüter *et al.*, 2018). However, as *Vicia faba* is not relevant for N fertilisation purposes in agricultural practice, we added *Hordeum vulgare* as a second species, the same plant that was used in Drew's experiments. For *Hordeum vulgare*, use of X-ray CT was not practicable for the given column size and the very fine root diameters. Therefore, harvests after 8, 12 and 16 days were carried out for *Hordeum vulgare* to obtain information about root growth dynamics over time.

As in Drew, we use columns as growth containers for the plants. This also has limitations, because a column does not provide the same conditions as the field. We carefully designed an experiment that allowed the simultaneous imaging of root architecture, measurement of soil solution chemistry and the influence of plant nutrient uptake during a growth period of 16 days. The resulting pot size with 7 cm in diameter is certainly a compromise that comes with a number of restrictions, but it allows determining the relevant parameters in the system for this study.

With this work, we show that responses to naturally occurring heterogeneities in N supply after fertilisation may also affect the root architecture of major crops albeit to a different extent compared to studies with very steep gradient in nutrient concentration and without a genuinely nutrient-deficient zone the roots have to grow through first before reaching the patch with higher nutrient content, which is found in other studies. The literature is controversial about the reason for the morphological root reactions to N, as it is generally a transient nutrient and morphological reactions are often slow and energetically expensive.

5.2 Material and methods

Experiment 1 – test of inhibitor functionality and fertiliser turnover under standardised laboratory conditions

For all experiments in this study a silty clay loam soil, originating from the subsoil of a haplic Luvisol (40-65 cm depth) was used. This was done, as the background concentrations of NO_3^- and NH_4^+ in soil solution were rather low in this subsoil, *i.e.* 2-4 mM NO_3-N and 0.2-0.3 mM NH_4-N, respectively (Beuters *et al.*, 2014). Total N content of the soil without addition of fertilisers was 690 mg kg^{-1} (Beuters *et al.*, 2014). CEC of the soil was 170.6 (± 9.1) mM_c kg^{-1} and C_{org} was low with 0.5 (± 0.01) % (Vetterlein *et al.*, 2013a). For more details of the soil, see Vetterlein *et al.* (2013a).

From this sieved (2 mm) and homogenised soil material, 300 g were mixed with 30 mg N in form of ground urea granules (treatment "U", product name PIAGRAN® 46, SKW Piesteritz) or ground urea granules containing the nitrification inhibitors 1H-1,2,4-triazol (0.09%) and DCD (0.91%) (treatment "U+NI", product name ALZON® 46, SWK Piesteritz), both of which are commercial and approved EU-fertilisers. Water was added to obtain 50% of maximum water holding capacity (180 ml kg $soil^{-1}$, equal to 18% w/w). This mixture was split into three replicates at 100 g of soil each, filled in bottles and analysed separately. Closed bottles (loosely closed by plastic foil and rubber band) were kept at constant temperature of 20°C over a period of 67 days. Samples were taken at days 0, 1, 2, 3, 7, 10, 14, 21, 28, 35, 43, 49, 56 and 67,

subsequently extracted with 1 M KCl by headlong shaking for 1 h (VDLUFA, 1991). Concentration of NH_4^+ was measured photometrically (AA3, Seal Analytical) to test the functionality of the nitrification inhibitor and to monitor urea turnover under controlled laboratory conditions without plant influence in order to give a rough estimation of the temporal dynamics for the subsequent plant experiments.

Experiment 2 – fertiliser turnover under controlled climate chamber conditions, measured in soil solution over time and soil extraction after 38 days

Acrylic columns (250 mm height, 35 mm radius, 5 mm wall thickness) were filled with 1050 g of sieved (< 2 mm) and homogenised silty clay loam described before and packed to a bulk density of 1.2 g cm^{-3}. The uppermost 2-3 cm of the columns were left unfilled to enable addition of coarse gravel. The coarse gravel served as a capillary barrier to prevent capillary water movement to the soil surface and by this to prevent excessive evaporation from the soil surface.

Three treatments were set up: 1) control = "C" without any fertiliser input; 2) urea without any additives = "U"; and 3) urea with nitrification inhibitor = "U+NI". Both N-fertilised treatments received five fertiliser granules of similar size per sample. Urea granules are very easily soluble: in water they dissolve within minutes and in moist soil within a few hours at most. Urea is known to be hygroscopic and hence, forced to dissolve even by limited water availability. Due to these properties, a similar dissolution behaviour as with the ground granules in experiment 1 can be expected. The rate was 100 mg N kg^{-1} soil, placed in one layer 5-6 cm below soil surface and arranged equidistantly. These 100 mg N per kg account for about 13% of total N related to the background N of 690 mg kg^{-1} soil. Two replicates per treatment were set up, as no considerable variation was expected.

The soil was initially watered with 177 ml distilled water, resulting in a water content of about 27 vol.-% (= pF 2.65, equal to -0.45 kPa). First, 137 ml were supplied from below by capillary rise and, as soon as the water level reached the fertiliser layer, 40 ml were carefully supplied from above to prevent excessive transport of the very mobile urea out of the fertiliser layer towards the soil surface. Coarse gravel was placed on top of the soil to reduce evaporation. Each column was placed on a weighing cell to keep water content in each column as stable as possible by rewatering every second day. Soil columns were wrapped with aluminium foil to prevent algae growth. Soil columns were set up in a climate chamber under controlled conditions (12/12 h day/night cycles at 19 and 16 °C, respectively; photosynthetic active

radiation was 350 µmol m^{-2} s^{-1}, relative air humidity was 65%). Samples were incubated for three weeks before the micro suction cups were installed.

Soil chemical conditions in soil solution were measured over time with micro suction cups ('MicroRhizons', Rhizosphere Research Products B.V., The Netherlands), installed in all six columns in two different soil depths. Soil solution was only extracted from the experiment with *Vicia faba*, due to technical problems during the experiment with *Hordeum vulgare*. The upper layer was located in the plane of fertiliser placement. The second layer was installed 5 cm below, as a control for monitoring potential N transport. In each layer, three suction cups were installed. All suction cups were connected to thin Teflon tubes, attached to a chamber equipped with 2 ml vessels for each tube. These chambers were set to negative pressure (-40 kPa) for about one hour per sampling date to collect soil solution. Sampling was conducted on day 20, 28, 32 and 36 after start of incubation. In relation to the plant growth experiment these dates correspond to -1, 7, 11 and 15 DAP (days after planting – this is the reference time scale used in all figures). Soil solution samples from individual suction cups in the same layer were combined to one mixed sample representative for the respective soil layer. Soil solution was analysed for pH (IQ240, I.Q. Scientific instruments, Inc., USA), osmotic potential (Osmomat 030, Gonotec, Germany), NO_3^--N and NH_4^+-N (measured photometrically, AA3, Seal Analytical).

After 36 days, soil columns were cut with a ceramic knife in horizontal layers of 3 cm thickness, resulting in 7 layers per sample. These samples were analysed for mineral N (NO_3^- and NH_4^+). The mineral N fraction extracted by 1 M KCl (VDLUFA, 1991) comprises N in solution and extractable N at the exchange sites of the soil like clay minerals and organic matter. Strongly bound or fixed NH_4^+ in the interlayers of clay minerals are not completely extracted with this method. This fraction is henceforth referred to as the sum of readily available and adsorbed N species, while N species in soil solution are referred to as readily available fractions.

Experiment 3 – plant growth and root response of Vicia faba and Hordeum vulgare to different soil chemical conditions and N-forms in soil, resulting from urea fertiliser with and without nitrification inhibitor, over time

Experiment 3 was conducted with the same treatments and conditions as Experiment 2 regarding soil preparation, fertiliser application, climate chamber conditions and extraction of soil solution. The major difference was the presence of plants.

Not inoculated seeds of faba bean (*Vicia faba* L., *cv.* 'Fuego') and barley (*Hordeum vulgare*, *cv.* 'Marthe') were surface sterilised with H_2O_2 (10%) for 10 minutes and soaked in saturated $CaSO_4$ for about four hours before one seed was placed per column about 2 cm below soil surface. Seed planting was performed at day 21 after start of soil incubation (= 0 DAP). Plants were grown for 17 days.

Four replications were set up for *Vicia faba*, 12 replications for *Hordeum vulgare*. The reason for different numbers of replicates was the chosen method to acquire data about root growth dynamics over time. For *Vicia faba*, X-ray computer tomography (CT) was used (Blaser *et al.*, 2018). For *Hordeum vulgare*, having much finer root diameters, X-ray CT at the given column diameter would have captured only seminal roots and first adventitious roots in the soil utilised in the present experiment. The soil was characterised by small aggregates and hence high heterogeneity in grey values caused by the mix of small intra-aggregate pores and larger inter-aggregate pores would have hindered segmentation of roots from X-ray CT images. Hence, in the same frequency as X-ray CT was performed for *Vicia faba*, four replicates of *Hordeum vulgare* treatments were harvested destructively. Roots were washed out and analysed with WinRHIZO 2009b (Regent Instruments Inc., Canada). Destructive harvest was also conducted for *Vicia faba* at the end of the experiment (17 DAP).

CT scanning configuration and image analysis

X-ray tomography was performed with an industrial µCT (X-TEK XTH 225, Nikon Metrology) with 140 kV, 286 µA (equal to 40 Watts) and 500 ms exposure time. Each scan was performed with 1000 projections and one frame, resulting in an exposure time of 8.5 minutes per scan. A copper filter with 0.5 mm thickness was used to reduce the beam hardening artefact. Distance between X-ray source and sample was about 13 cm. The spatial resolution of the X-ray tomogram was 40 µm in the field of view. The calculated dose rate for these settings was 480 R h^{-1} (calculated with the Rad Pro Calculator for Desktop PCs Version 3.26 from http://www.radprocalculator.com/RadProDownloads.aspx). This equals 421 rad h^{-1}, or 4.2 Gy h^{-1}. Two tomograms one above the other were performed per sample in order to visualise and analyse the uppermost ~14 cm of the root systems in the soil in its true spatial arrangement. X-ray CT was performed on 8, 12 and 16 DAP. The cumulative scanning times and X-ray doses per sample are 51 minutes and 3.6 Gy, respectively. This dose is lower than in the low radiation treatment in Blaser *et al.* (2018).

A detailed analysis of CT-images was conducted for *Vicia faba*. Main points are stated here, for more details see supporting information in Blaser *et al.* (2018) or Appendix 1 (pages 106-107). Raw data was filtered (Gauss, kernel size 5) to reduce image noise. Root systems were segmented with semi-automated region growing in VG Studio Max 2.1. Based on the idea of Flavel *et al.* (2012), binarised root systems were skeletonised and analysed with the plugin BoneJ (Doube *et al.*, 2010) in the software Fiji (Schindelin *et al.*, 2012). The resulting information was used to distinguish between tap root, first and second order lateral roots.

A distance map was performed for the X-ray data, according to Schlüter *et al.* (2018). Main steps are given here, for more detailed information see Schlüter *et al.* (2018). 3D Euclidian Distance Transform was performed in Fiji, as suggested in Koebernick *et al.* (2014). For each slice, the histogram for the soil-root-distance was retrieved. The cumulated histograms depict the relative frequency of distances from the soil towards the nearest root voxel. With this parameter we can describe the exploration of the given soil volume by the root system. The distance map was performed for three separate layers within the X-ray CT stack, referring to the layers of destructive harvest for *Hordeum vulgare* (see below).

Shoot and root analysis

Shoot weight and leaf area were measured directly after harvest. Leaf area was measured with WinRHIZO. Roots were washed out and also analysed with WinRHIZO. For *Hordeum vulgare*, soil columns were cut in three parts during each harvest. The first layer represented the part above the fertiliser plane and was 0-4 cm below soil surface. The second layer represented the fertiliser plane and was 4-9 cm below soil surface. The third layer represented the part below the fertiliser plane and consisted of the remaining 9-23 cm in depth. Roots were washed out carefully and analysed separately for each layer with WinRHIZO.

WinRHIZO analysis was performed for total root length and three functional diameter classes. For *Vicia faba*, thresholds were set at 0.75 and 1.25 mm with the intention to separate first and second order laterals from tap roots, based on root diameters. The smallest class below 0.1 mm was discarded, as it was error-prone due to root hairs causing misclassification.

For *Hordeum vulgare*, thresholds for functional diameter classes (seminal roots, first and second order lateral roots) were selected individually for each sample. This was necessary, as the root diameters changed during growth and therefore two fixed values for all points in time and soil depths would have led to low quality of analysis. Thresholds between seminal roots and laterals ranged between 0.175 and 0.425 mm. Thresholds between first order laterals and

second order laterals ranged between 0.080 and 0.180 mm. Segments with diameters below 0.04 mm were discarded, as they were distorted due to root hairs. Additionally, the number of lateral roots was counted manually by eye for all layers and points in time, apart from the C-layer at 16 DAP due to the very high number of roots in those samples.

Statistics

For all experiments, statistics were performed with SPSS 22 (IBM). The urea turnover experiment in experiment 1 and the influence of the plants on the pH at the end of experiment 3 were analysed with the T-test for independent samples taking into account the Levene's test.

The temporal dynamics of the pH values and N-forms in the soil solution were analysed with a one-way ANOVA with repeated measurements with regard to the four sampling dates and Bonferroni adjustment of the confidence interval taking into account the Mauchly's test for sphericity and if necessary the Greenhouse-Geisser correction method. All remaining analyses were evaluated with one-way ANOVA and post-hoc Bonferroni tests to compare the treatments. This was always done separately for all time points and soil depths. All ANOVA performed were one-way without interaction term.

For the pH values, the statistical tests were carried out using the proton concentration. Standard errors are given in all figures as error bars. Significant differences ($p<0.05$, unless stated otherwise) are indicated by different letters or asterisks (*) in the figures. No significant difference is indicated by "ns".

5.3 Results

Experiment 1 – Characterisation of urea hydrolysis and nitrification activity of the soil material under standardised conditions without plants

Experiment 1 revealed NH_4^+ formation by hydrolysis of urea and the subsequent depletion of NH_4^+ by nitrification. Both treatments were characterised by the same hydrolysis velocity, as the applied inhibitor selectively influenced nitrification. NH_4^+ concentration reached the highest level at day 7 for both treatments with a concentration of about 60 mg NH_4^+-N kg^{-1} soil (Fig. 5.1).

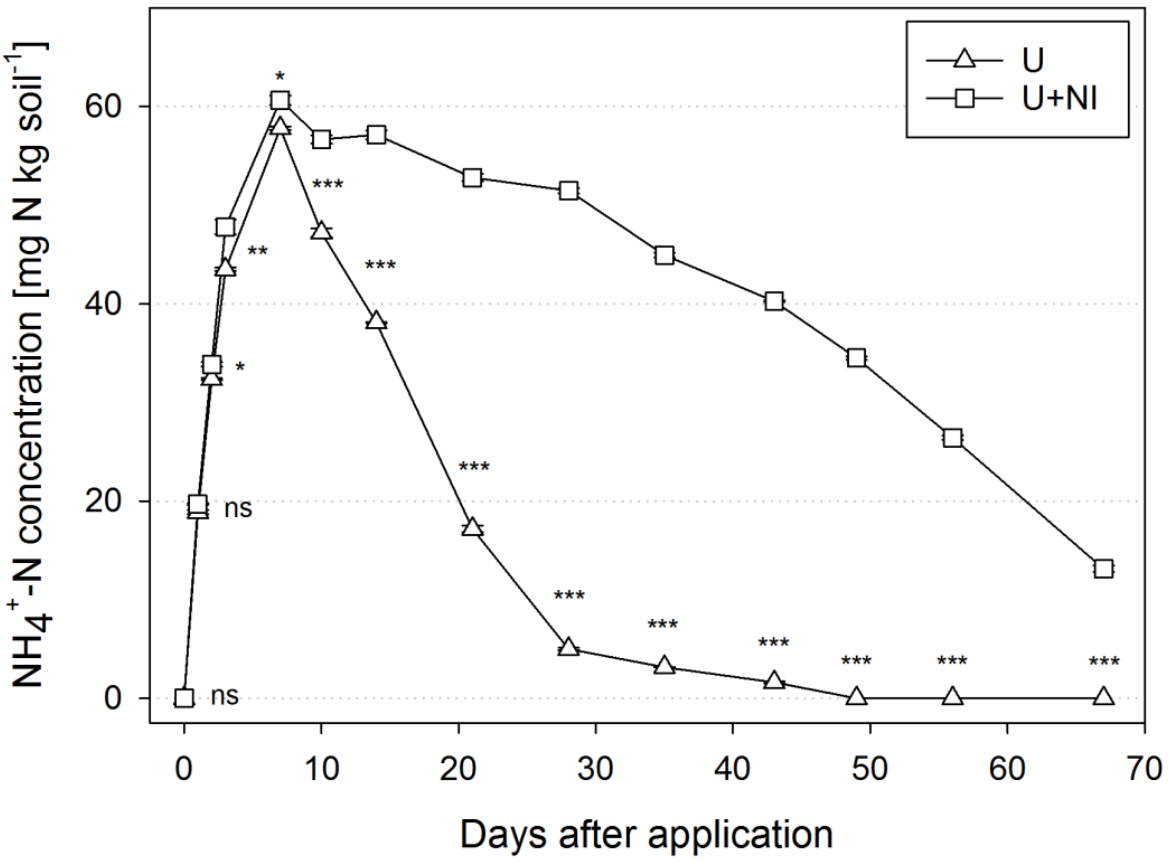

Figure 5.1: Temporal change of NH4+-N concentration under standardised conditions for treatments urea ("U", open triangle) and urea with nitrification inhibitor ("U+NI", open square) in soil extract (1 M KCl). Asterisk indicates significant difference between treatments at p<0.05 (*), p<0.01 (**) and p<0.001 (***), ns = not significant. Error bars are masked by treatment symbols in most cases.

Urea hydrolysis is followed by nitrification provided that the relevant bacteria are present. The effect of the nitrification inhibitor was clearly shown, as NH_4^+ concentration decreased at a much slower pace in U+NI, compared to the pure urea treatment (U). The greatest differences regarding NH_4^+ concentration between both treatments were observed between days 20 to 30. Therefore, the incubation period for the later plant experiments was set to 21 days for a good trade-off between maximum difference in N-speciation, and high level of NH_4^+ in one of the treatments. The corresponding values for NO_3^- are given in the Appendix 2 (Fig. A2.1).

Experiment 2 – fertiliser turnover under controlled climate chamber conditions, measured in soil solution over time and soil extraction after 38 days without plants

Soil solution

Dynamics of pH values (Fig. 5.2) reflected the turnover of applied urea-based fertilisers in experiment 2. In the control treatment (C), initial pH was close to 7 and did not change significantly over time ($p>0.05$). In both fertilised treatments (U and U+NI) initial pH was higher (around 8) due to preceding urea hydrolysis. Thereafter, both treatments showed completely different developments. In U, pH steadily decreased over time to values around 6.5 at 12 DAP ($p<0.05$). In U+NI, pH was stable around pH 8 and did not change over time ($p>0.05$).

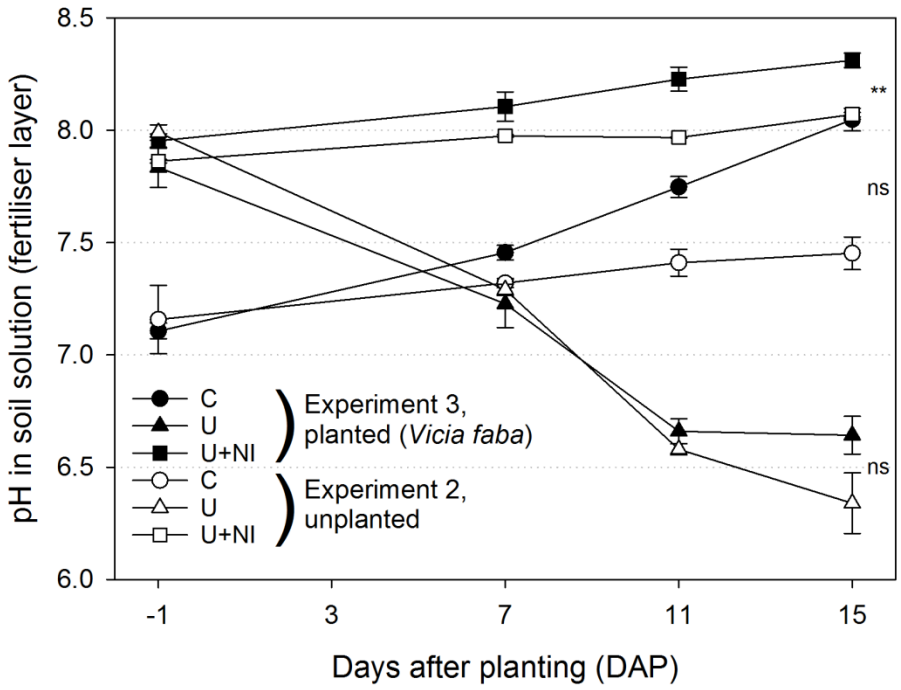

Figure 5.2: Change of soil solution pH with time in the fertiliser layer in experiments 2 (open symbols) and 3 (closed symbols). First sampling point (-1 DAP) defines starting conditions for root growth in experiment 3. Asterisk indicates significant difference between planted and unplanted treatments at p<0.01 (**), ns = not significant. Error bars indicate standard error.

The pH-dynamics were similar to those found in the fertiliser layer in the treatments C and U+NI in the layer 5 cm below the fertiliser. A decreasing pH in the treatment U was not observed 5 cm below the fertiliser placement (Appendix 2, Fig. A2.2).

Independent if planted or not, nitrification was the most striking transformation process in U, resulting in release of protons and decrease of pH. This is also reflected in the NO_3^- concentrations in soil solution (Fig. 5.3). C and U+NI showed very similar and stable NO_3^- concentrations around 30 mM. In contrast, NO_3^- concentration in U increased throughout the duration of the experiment, reaching a maximum concentration of about 100 mM in the fertiliser layer by the end of the experiment. Linear correlations were found in U with ($r^2 = 0.99$) and without plant ($r^2 = 0.98$).

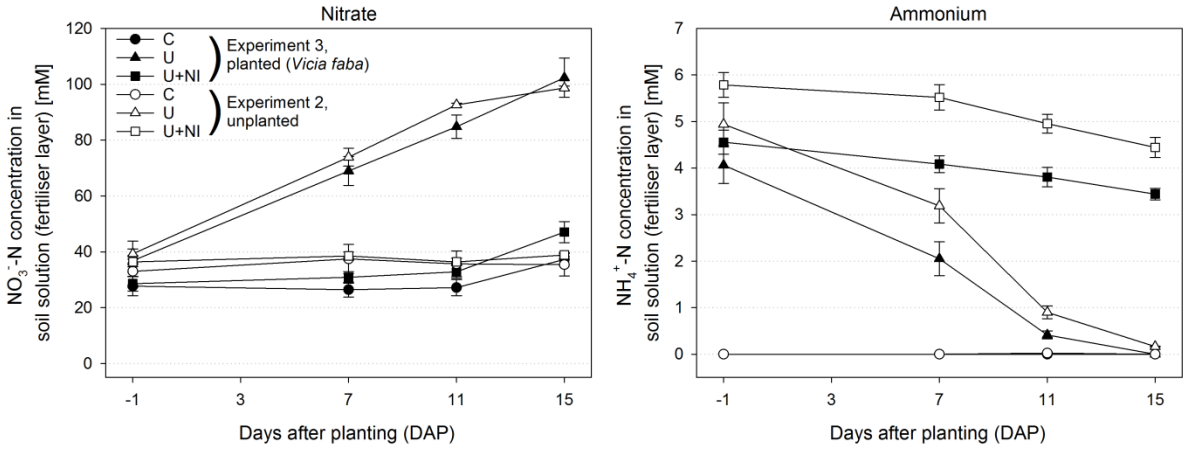

Figure 5.3: Temporal change of NO_3^- (left) and NH_4^+ (right) concentration in soil solution in the fertiliser layer from experiments 2 and 3 after three weeks of incubation for treatments C (circles), U (triangles) and U+NI (squares). Filled symbols indicate plant presence (experiment 3), open symbols indicate plant absence (experiment 2). Starting point is one day before plants were introduced in experiment 3.

The NH_4^+ concentrations in soil solution confirmed the ongoing nitrification process in U. NH_4^+ concentration in the fertiliser layer decreased over time from about 4.5 mM to almost zero at the end of the experiment (Fig. 5.3). In U+NI, NH_4^+ concentration was stable around 4.5 mM during the first 7 days, reflecting the successful NH_4^+-stabilisation by the nitrification inhibitor. Subsequently, NH_4^+ concentration decreased slightly. In C, NH_4^+ was barely detectable. Linear correlations were found in both fertilised treatments, independent if planted ($r^2 = 0.98$ in U and $r^2 = 0.99$ in U+NI) or not ($r^2 = 0.96$ in U and $r^2 = 0.90$ in U+NI).

NH_4^+ concentration in soil solution below the fertiliser layer was below 1 mM. NO_3^- concentrations were the same in C and U+NI as in the fertiliser layer, and much lower in the U treatment (Appendix 2, Fig. A2.3). The results of the osmotic potential were very similar to the results of NO_3^-, showing the same slope in U and a constant pattern in the treatments C and U+NI (Appendix 2, Fig. A2.4).

Absorbed and readily available N species in soil extraction

Analysis of N species in the soil extract showed the distribution of mineral NO_3^- and NH_4^+ within the soil columns, separately for layers with 3 cm in height. The maxima of both N forms were found in the layer in 3-6 cm depth, representing the fertiliser layer. Also the adjacent layers revealed higher concentrations than the remaining parts of the soil columns (Fig. 5.4).

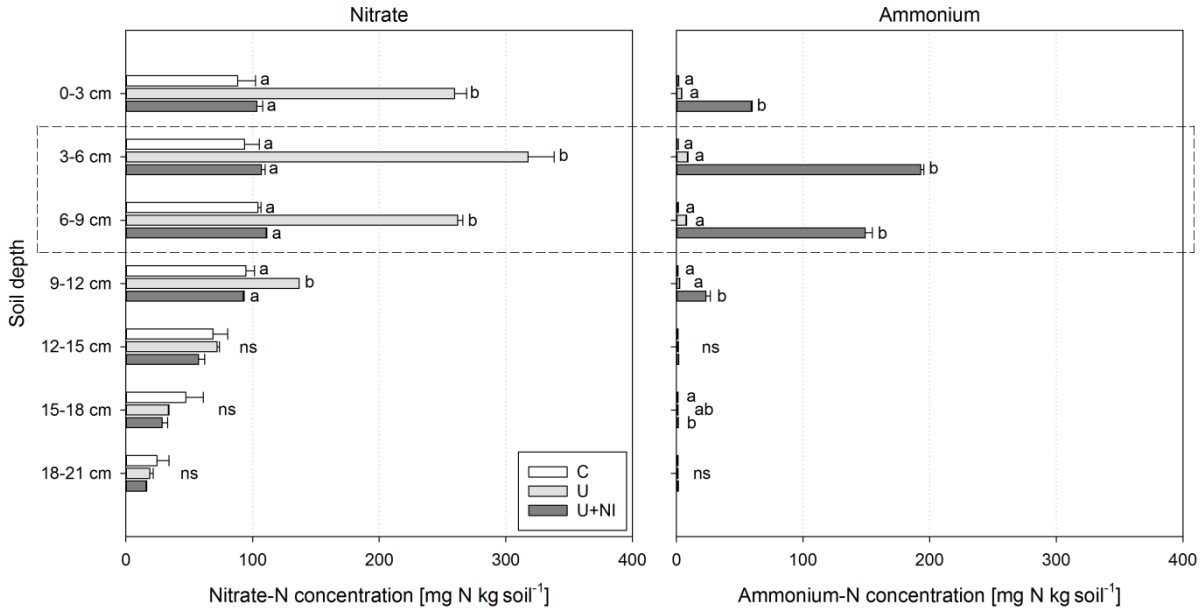

Figure 5.4: Depth distribution of readily available N and adsorbed N species NO_3^- (left) and NH_4^+ (right) in unplanted soil columns (experiment 2) for the treatments control (C), urea application (U) and urea application + nitrification inhibitor (U+NI) on day 17, corresponding to 17 DAP in experiment 3. Significant differences between treatments within each layer ($p<0.05$) are indicated by different letters, n.s. = not significant. Dashed box indicates area of fertiliser placement.

NO_3^- concentration in C and U+NI was almost identical and much lower than in U. In contrast, NH_4^+ concentration in C and U was similar and much lower than in U+NI. NO_3^- was available in all treatments and all soil depths. NH_4^+ was more restricted to the fertiliser layer and only in U+NI considerable concentrations were detected. As for NO_3^-, significant and relevant differences for NH_4^+ concentration were found in soil layers down to a depth of 12 cm.

Experiment 3 – influence of plants on soil chemical conditions, plant growth development and root response of Vicia faba and Hordeum vulgare over time

For all treatments, initial pH values were statistically the same for planted (experiment 3) and unplanted samples (experiment 2), even though Fig. 5.2 reveals distinct differences. With time, differences started to develop within the treatments C and U+NI, depending on plant presence

or absence. Planted samples tended to have higher pH values compared to their unplanted counterparts, but only for the treatment U+NI a difference at $p<0.05$ was found (Fig. 5.2).

In C, the pH increased by about 0.9 units with plant presence within 16 days ($p<0.01$). In U+NI, the pH increase was smaller (about 0.4 units, $p<0.01$) compared to C. In contrast, the initial pH in the soil solution from U was the same as in U+NI but decreased about 1.2 units ($p<0.01$) within 12 days of plant development. Both graphs for planted and unplanted samples in U showed a similar development.

NO_3^- and NH_4^+ concentrations from the planted columns were very similar compared to the unplanted columns from experiment 2 (Fig. 5.3). No differences ($p<0.05$) were found between planted and unplanted columns apart from U at 15 DAP, where the treatment with plant presence was already depleted, while 0.2 mM NH_4^+ was left in the unplanted counterpart ($p<0.05$). In all planted treatments, NO_3^- concentrations were greater at 15 DAP compared to -1 DAP ($p<0.01$). Decreasing NH_4^+ concentrations were found in U and U+NI at 15 DAP compared to -1 DAP ($p<0.01$).

Aboveground biomass and N-uptake by *Vicia faba* and *Hordeum vulgare*

For *Vicia faba*, no significant differences were found between all treatments regarding leaf area (Fig. 5.5, left) and shoot fresh mass (Appendix 2, Fig. A2.5, left). A tendency towards larger values in U+NI was observed for leaf area compared to C ($p<0.1$). N-concentration in the shoot of *Vicia faba* showed the same tendency as shoot mass and leaf area with the highest values in U+NI (Fig. 5.6, left).

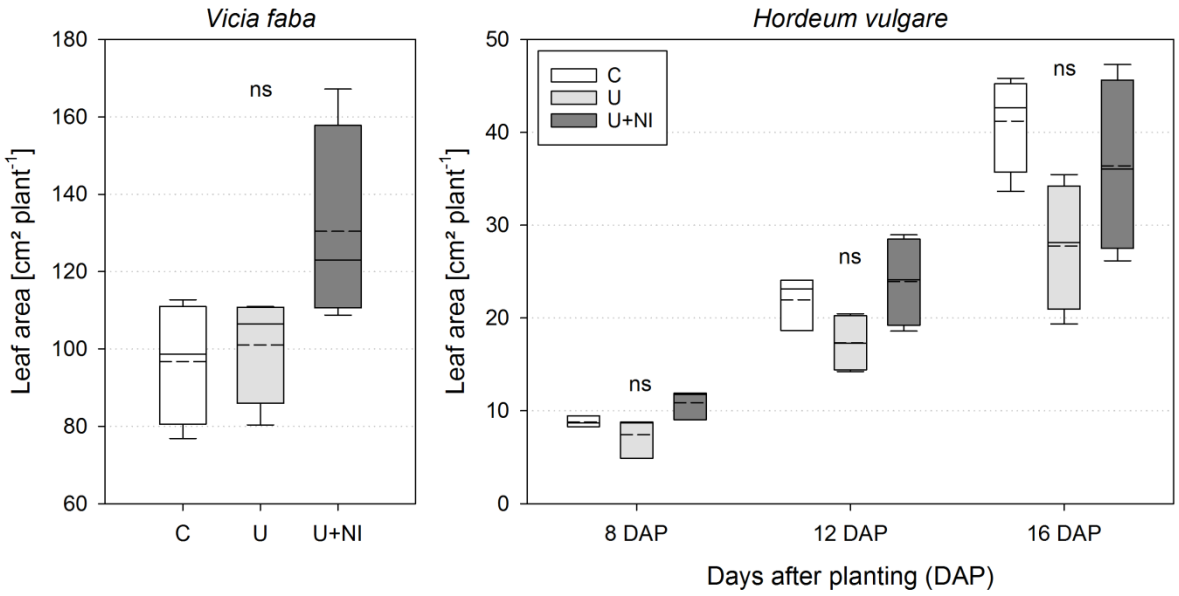

Figure 5.5: Leaf area for *Vicia faba* (left) after 16 days and for *Hordeum vulgare* (right) over time. Significant differences (p<0.05) between treatments for each point in time are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

For *Hordeum vulgare*, leaf area was also not different for all treatments and all time steps (Fig. 5.5, right). Shoot fresh weight was affected by the different fertiliser treatments (Appendix 2, Fig. A2.5, right). After 16 days, mean shoot fresh mass in U was reduced by 42% compared to C ($p<0.05$).

Similar results were found for shoot N concentration. Shoot N concentrations in *Vicia faba* and *Hordeum vulgare* ranged from 4.9 to 7.4 % dry mass. For *Vicia faba*, no difference was found (Appendix 2, Fig. A2.6, left). For *Hordeum vulgare*, mean shoot N concentration on day 12 was 0.5% smaller ($p<0.05$) in U compared to C and U+NI (Appendix 2, Fig. A2.6, right).

Root development

1. *Vicia faba – root growth dynamics measured by X-ray CT and root growth analysis with WinRHIZO at the end of the experiment (17 DAP)*

Visualisation of root growth development over time by X-ray CT and soil-root distances (Euclidian distance map transform)

Examples of segmented root systems of *Vicia faba* are shown in Fig. 5.6 as time series from one representative replicate for each treatment. The most representative replicate was chosen,

with individual root length closest to the mean value of the respective treatment. All other projections can be found in the Appendix 2 (Fig. A2.7).

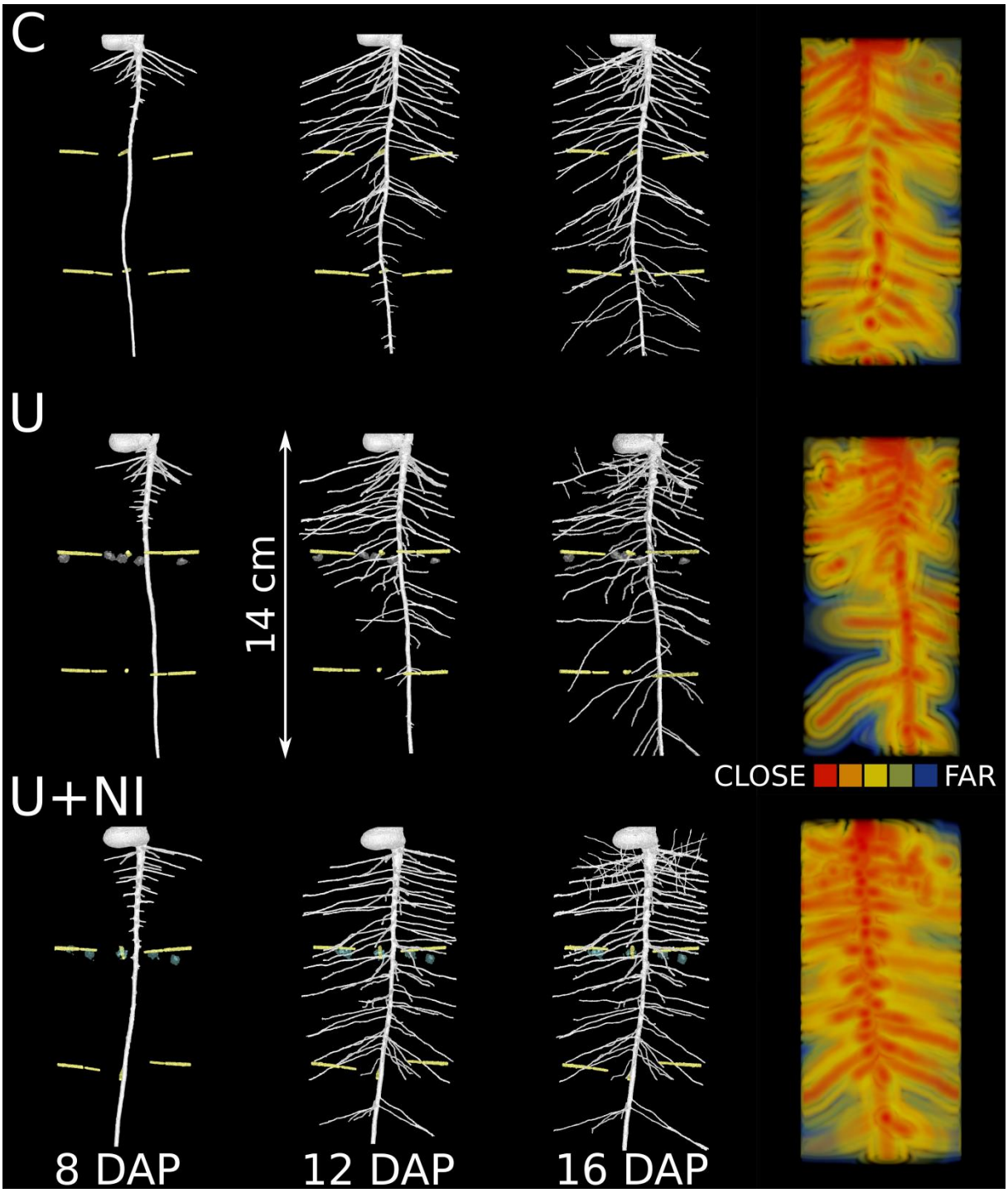

Figure 5.6: Time series of root growth development of *Vicia faba* for all three treatments (characteristic representatives) and visualisation of relative frequencies of soil-root-distances (representative layers) in 2 mm steps. Micro suction cups are represented in yellow. Remaining voids from fertiliser granules are indicated in grey (middle row) for urea and turquoise (bottom row) for urea with NI.

Distance maps – relative frequencies of soil-root-distances

Information on the spatial extension of the root system and the three-dimensional exploration of the soil volume by the roots is insufficiently represented in the quantification of root lengths or number of roots. The true potential of 3D data is only revealed when geometric or topological measures are used for quantification. Relative frequencies of soil-root distances for the examples shown in Fig. 5.6 are given in Fig. 5.7 for the final state at 16 DAP for 3 layers. Presence of second order laterals in 0-4 cm compared to the other layers is reflected in a shift of the maximum to shorter distances. The larger number of second order laterals in the representative sample chosen for U+NI (Fig. 5.6) is reflected in the maximum for U+NI being higher than for the representative samples selected for C and U. The direct comparison of segmented CT root system architecture in Fig. 5.6 and the relative frequencies of soil-root distances illustrate how sensitive changes in root architecture can be captured by this new measure. Deriving relative frequencies of soil-root distance is less time consuming than deriving the number of second order laterals. Relative frequency curves for all individual samples can be found in the Appendix 2 (Fig. A2.8). According to Schlüter *et al.* (2018), the frequency distribution of root distances integrated over all soil voxels can be denoted as root distance histogram (RDH). From the RDH, the mean soil-root-distance ⟨RDH⟩ can be derived, which is closely related to the classical parameter half mean distance between roots (HMD) for equidistant distributions (Schlüter *et al.*, 2018). However, it must be noted that this parameter can be well determined and statistically compared, but does not describe the actual shape of the curve sufficiently. All ⟨RDH⟩ values are provided in the Appendix 2 (Fig. A2.9).

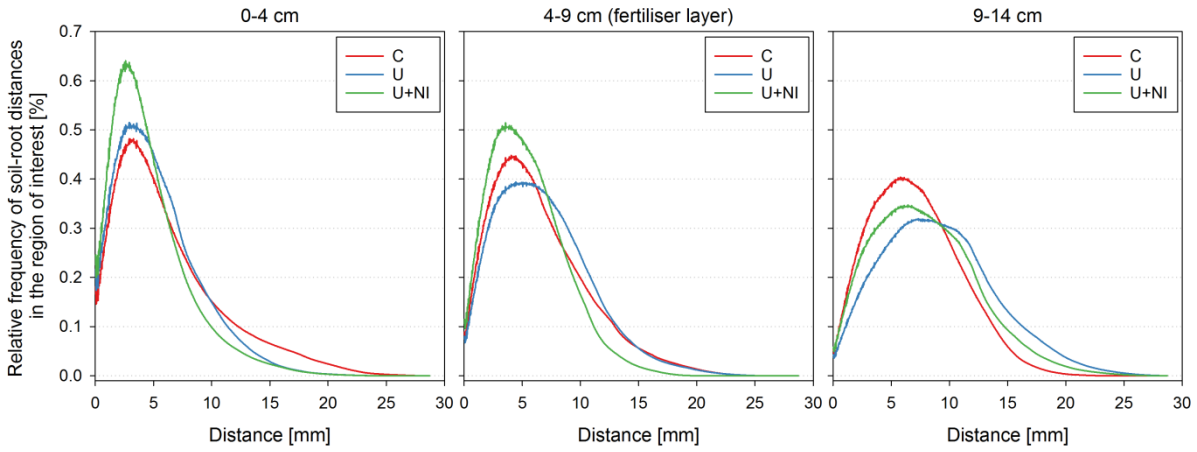

Figure 5.7: Relative frequencies [% of all soil voxels in the respective layer] of soil-root distances at 16 DAP for representative samples given in Fig. 5.6, separated in 3 layers, representing the fertiliser layer (middle) and both parts above (left) and below (right) the fertiliser layer. C is given in red, U in blue and U+NI in green.

X-ray CT analysis

Root length and number of first and second order laterals showed no differences between treatments. This is true for all three points in time (8, 12 and 16 DAP) and soil depths (Appendix 2, Figs. A2.10-13).

WinRHIZO analysis

Total root length as well as root length per functional diameter classes showed no differences between treatments (Appendix 2, Fig. A2.14).

2. *Hordeum vulgare root growth dynamics analysed with WinRHIZO over time and in 3 separate soil layers*

Total root length of *Hordeum vulgare* in U was considerably smaller compared to C and U+NI throughout all harvests (Fig. 5.8). Compared to C, mean total root length in U was 75% smaller at 8 DAP ($p<0.05$), 68% at 12 DAP ($p<0.05$) and 44% smaller at 16 DAP ($p<0.05$). Because of large deviation, the difference of 902 cm in mean total root length between U and U+NI at 16 DAP was barely not significant ($p = 0.06$).

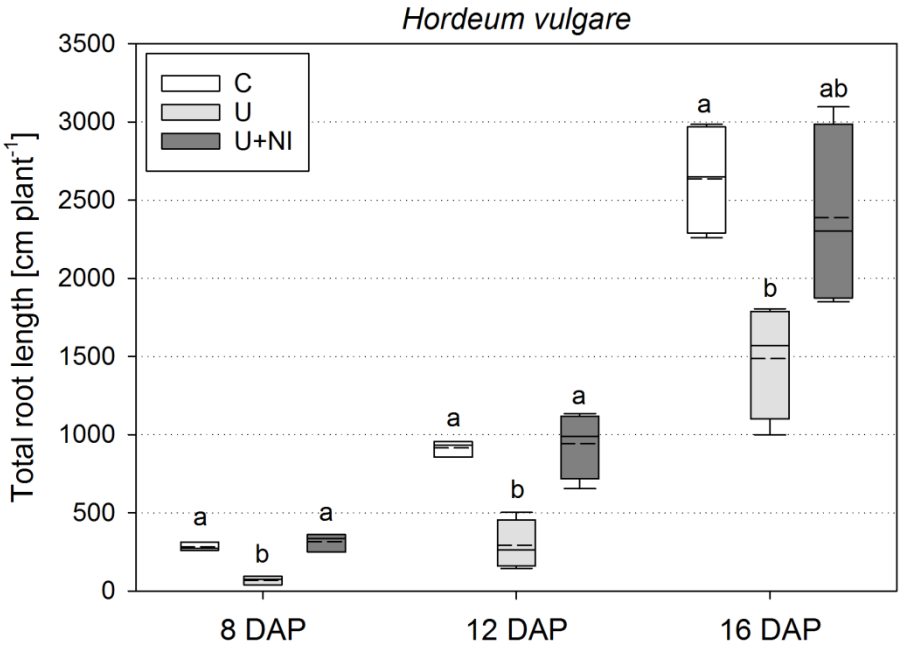

Figure 5.8: Total root length of *Hordeum vulgare* plants. Significant differences ($p<0.05$) are indicated by different letters; dashed lines in the boxplots represent mean values while solid lines represent the median.

Root length per soil depth and diameter class

The analysis of the root lengths per soil depth and functional diameter classes (Fig. 5.9) underlined the results of the total root length (Fig. 5.8). Furthermore, it becomes apparent that the inhibition of root growth in U took place particularly in the area of local fertilisation. This applies to all root diameter classes, namely seminals (upper row), first order laterals (middle row) and second order laterals (bottom row). For second order laterals this is only the case at 16 DAP, where the smallest length was found in U (1.4 cm), followed by U+NI (51 cm). The root length of both treatments was much smaller ($p<0.05$) compared to C (136 cm).

There was no influence on the seminal roots observed above the fertiliser layer. After 8 days, the length of first order laterals in U was only 63% of the mean length in C ($p<0.05$), but this difference disappeared over time. After 8 days, second order laterals were found only in C. The treatments did not differ from each other later on.

Below the fertiliser layer, clear differences were found regarding the length of seminal roots and first order laterals. The latter were almost non-existent after 8 days in this layer (< 1 cm). This difference became smaller over time and was no longer detectable after 16 days ($p>0.05$).

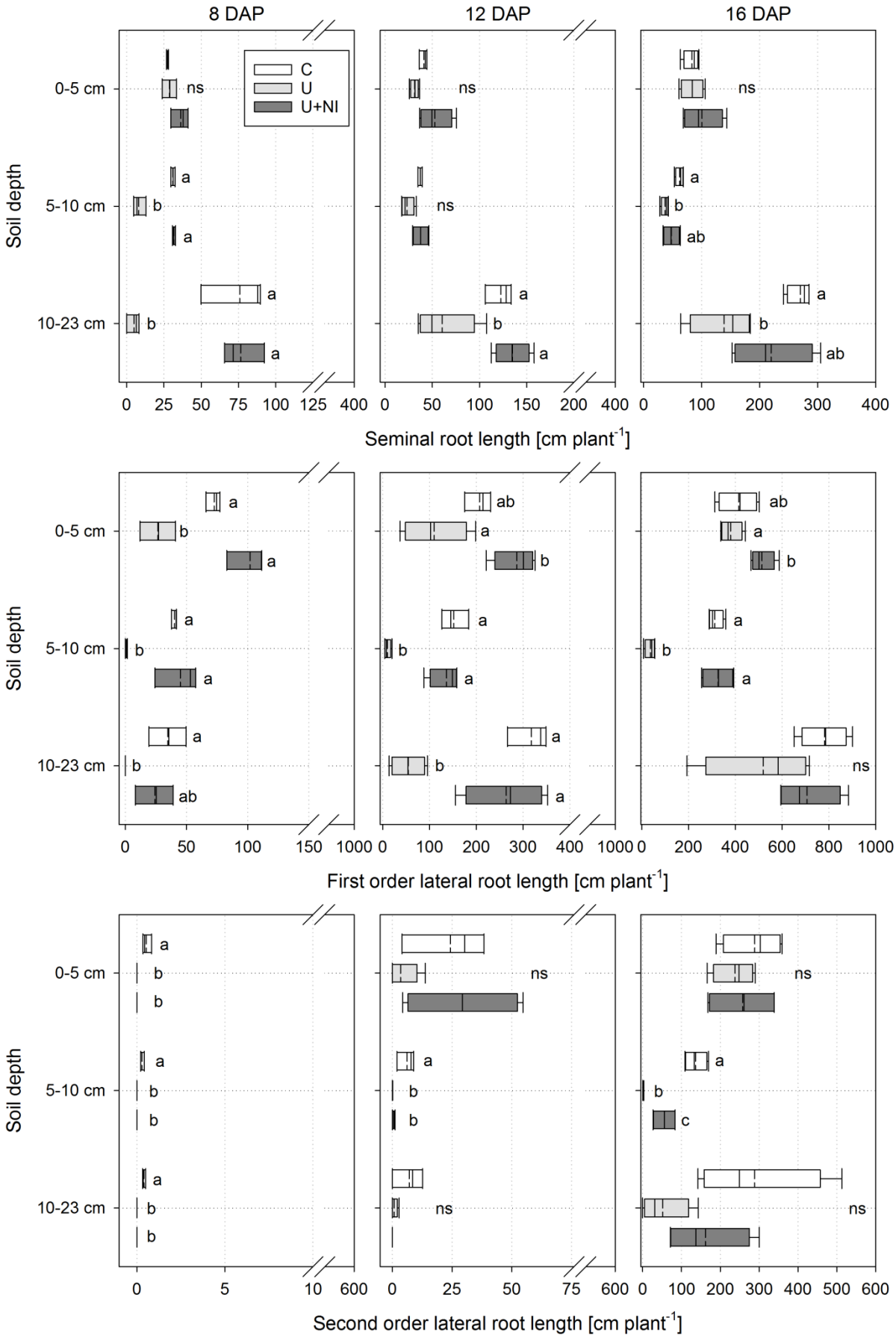
8 DAP
12 DAP
16 DAP
C
U
U+NI
Soil depth
0-5 cm
5-10 cm
10-23 cm
Seminal root length [cm plant-1]
First order lateral root length [cm plant-1]
Second order lateral root length [cm plant-1]

Figure 5.9: Root length of *Hordeum vulgare* plants, separated for three depths and root orders for each sampling point. Top layer (0-5 cm) is above fertiliser zone, bottom layer (10-23 cm) is below fertiliser zone. Top row = seminal roots, middle row = first order laterals, bottom row = second order laterals. Statistical comparison of root length is performed per layer between treatments. Significant differences ($p<0.05$) are indicated by different letters; ns = no significant difference for $p<0.05$; dashed lines in the boxplots represent mean values while solid lines represent the median.

Number of first order laterals

Length of laterals was not significantly different between U+NI and C (Figs. 5.8 & 5.9), but morphologic appearance of the root systems showed differences. Number of first order laterals was highest in the U+NI treatment. This is the case for almost all sampling dates and soil depths and especially in the fertiliser layer (Fig. 5.10). A very distinct difference was present as early as 8 DAP (Fig. 5.10, left). Whereas only 8 (± 5) first order laterals were counted on average in U, 36 (± 8) first order laterals were found in C and 87 (± 5) in U+NI ($p<0.05$).

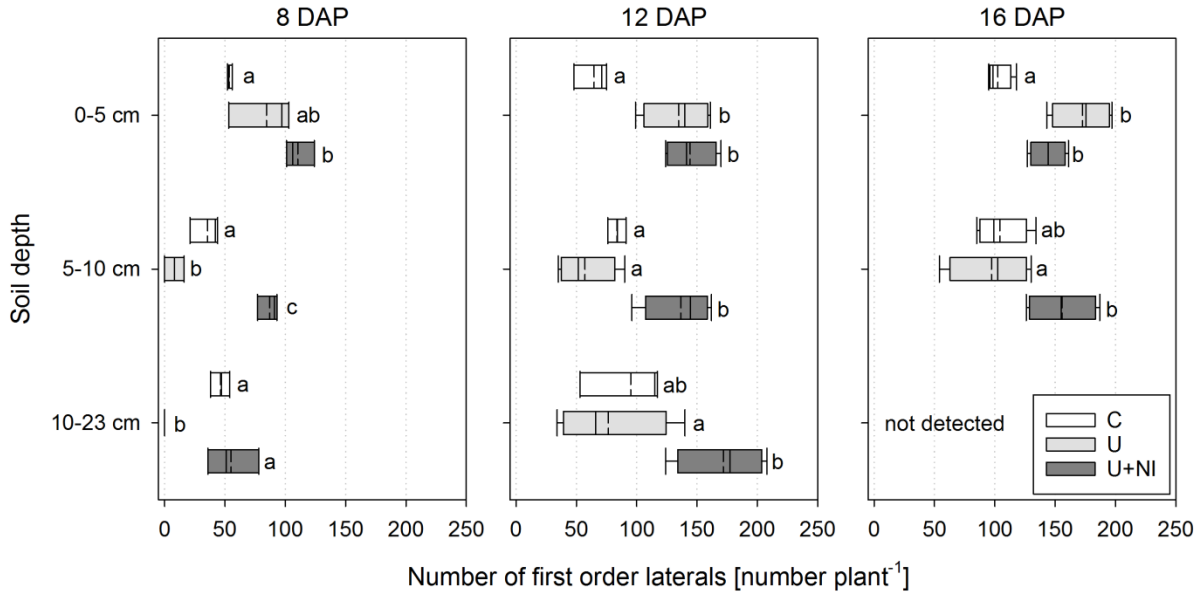

Figure 5.10: Number of first order laterals of *Hordeum vulgare* plants, separated for three depths for each harvest. Top layer (0-5 cm) is above fertiliser zone, bottom layer (10-23 cm) is below fertiliser zone. Statistical comparison is performed for number of first order laterals per layer between treatments. Significant differences ($p<0.05$) are indicated by different letters. Bottom layer at 16 DAP was too complex for a reliable analysis; dashed lines in the boxplots represent mean values while solid lines represent the median.

5.4 Discussion

Methodological approach

The aim of the experimental setup was to study the local impact of NH_4^+ and NO_3^- on root architecture as in the classical work of Drew (1975), but stimulated by the application of urea fertiliser with and without a nitrification inhibitor under field relevant soil conditions. While Drew (1975) used coarse sand percolated with nutrient solution separated with wax membranes, we conducted the study in a natural soil with a high CEC. The limitations which Drew (1975) faced at his time for soil studies, *i.e.* to follow soil solution N speciation dynamics over time and simultaneously observe the response of root morphology *in situ*, were overcome in the present study by soil solution sampling with micro suction cups and X-ray CT scanning associated with detailed, supervised image analyses. CT-scanning of *Vicia faba* roots every 4 days enabled to follow the initiation of first and second order laterals over time and to analyse this temporal development separately for all root orders. To enable this combination of investigations, a column (7 cm in diameter) experiment was carried out. It is undisputed that columns are not able to reproduce field conditions exactly, but they offer the best compromise for the objectives set in this study.

The temporal and spatial resolution chosen for soil solution collection was sufficient to illustrate the change in NH_4^+ to NO_3^- ratio and absolute concentrations over time as well as the change in pH associated with N transformation in soil and plant N assimilation. Although the absolute numbers are smaller, similar tendencies for N-dynamics were found in a field study by Kirschke *et al.* (2019), using similar types of urea fertilisers. The large discrepancies between NH_4^+ and NO_3^- ratios in soil solution compared to soil extraction nicely illustrate the problem of transferring concentration related results from hydroponics and gel plates to soil systems and will be discussed in more detail below.

By splitting the watering between top and bottom we avoided NO_3^- leaching. Background NO_3^- from mineralisation of organic matter was moved with irrigation water to the fertiliser layer (treatment C in Fig. 5.4). In U+NI, NH_4^+ was indeed the dominant N form in soil extract (KCl), reflecting the fraction adsorbed to the soil matrix. However, in soil solution NO_3^- dominated, although concentrations of NH_4^+ were substantial and much higher than in U (Fig. 5.3). This is also due to the fact that the mobility of NO_3^- is considerably higher than that of NH_4^+, since the latter is adsorbed to clay minerals and organic matter. Therefore, a much larger proportion of total NO_3^- is reflected in the soil solution concentration compared to NH_4^+. Nitrate production

is considered as a two-step reaction, first desorption of adsorbed NH_4^+ into the solution and second, nitrification of dissolved NH_4^+ to NO_3^-. Under normal conditions nitrification in soil is a rather quick process. NH_4^+ concentration in soil solution would be constant over time as long as the desorption rate (*i.e.* NH_4^+ supply to the solution) is equal to the consumption rate (*i.e.* NO_3^- production by nitrification of dissolved NH_4^+). This means that NH_4^+ concentration in solution remains constant over time, while NO_3^- concentration increases significantly. In the case of this study, NH_4^+ concentration in U decreased, since nitrification rate exceeded desorption rate and therefore adsorbed NH_4^+ started to deplete (*i.e.* decreasing NH_4^+ concentration in soil solution towards zero). In U+NI nitrification was severely restricted for a while (as visible in the missing nitrate production in Fig. 5.3) and hence, NH_4^+ is only removed from the solution towards the end of the experiment.

This is also reflected in the change of soil solution pH with time in the fertiliser layer (Fig. 5.2). In U+NI, plant N nutrition was a mixture of NH_4^+ assimilation originating from the fertiliser and assimilation of background NO_3^- from the soil. Both processes have an influence on pH due to release or consumption of H^+ or OH^-, respectively. In C, NO_3^- nutrition of the plants caused the pH increase in the planted treatment (Experiment 3). In contrast, soil biochemical metabolism had a stronger impact on soil pH than plant uptake in U. This revealed the strong impact of nitrification and the associated release of protons.

Regarding root growth, we expected similar results as in Drew (1975) with an increased root length in U and an increased initiation of lateral roots in U+NI as a response to higher NO_3^- and NH_4^+, respectively – and especially in the layer of fertiliser placement. In part, this is the case and our results are in line with those from Drew (1975), but only in terms of higher initiation of laterals due to NH_4^+ in *Hordeum vulgare*. However, we have not observed an increase in root length due to NO_3^-, but instead inhibition of root elongation in *Hordeum vulgare*.

The analysis of the relative frequency distribution of root-soil distances, based on Schlüter *et al.* (2018) was revealing. Small differences in root architecture are reflected in alteration of the relative frequency distribution (Figs. 5.6 & 5.7). To further exploit this tool the approach of Schlüter *et al.* (2018) to fit a triangular gamma model with just four parameters has to be developed further. This will then enable a statistical evaluation of changes in shape of the distribution function. The latter is not captured by just deriving the mean root-soil distance.

The less pronounced response to NH_4^+ in our experiment and an even negative one to NO_3^- in *Hordeum vulgare* may be explained by (i) differences in absolute concentrations, (ii)

differences in NO_3^-: NH_4^+ ratio, (iii) uncertainty about the impact of adsorbed NH_4^+, (iv) differences in temporal development of NO_3^- and NH_4^+ concentrations, (v) ratio of N placement volume to total volume (vi) N-status of control treatment and (vii) species preference for NH_4^+ or NO_3^- and susceptibility to NH_4^+ toxicity or inhibition by high NO_3^-.

As stated by Nacry *et al.* (2013), a typical root growth response to N that is applicable for all species and conditions is almost impossible to define, as many biotic and abiotic factors are responsible for the final shaping of the root system architecture. Nacry *et al.* (2013) highlighted two very general aspects that hold true for most circumstances: 1) high N status (in root and/or shoot) leads to a systemic repression of lateral root growth and 2) exogenous NO_3^- or NH_4^+ can have local effects on root growth.

The N status of the shoots was high but not exceedingly high given the very young age of the plants (Bergmann, 1986). Exogenous concentration of NO_3^- and NH_4^+ altered root growth during the early plant growth stage investigated. It is possible – in fact likely – that later season growth differs as plant demand changes with time.

Root growth response in different systems

Different systems of plant cultivation range from agar plates to nutrient solution to soil and may vary greatly in their response of root growth. Very pronounced root growth responses were found in studies using agar plates with extremely controlled conditions for growth of *Arabidposis* (Zhang and Forde, 1998; Zhang *et al.*, 1999; Remans *et al.*, 2006; Lima *et al.*, 2010; Gruber *et al.*, 2013). These approaches reveal the potential of root growth response to different N-forms and concentrations. But the utilised distributions and concentrations on agar are not realistic for soils, as applied concentrations in these studies are typically low and constant, and the pH is buffered to exclude potential influences.

An optimum curve for intermediate N-concentrations of both N-forms was determined in several studies, with lower and higher concentrations leading to smaller total root length, mainly explained by the negative response of lateral root growth (Zhang *et al.*, 1999; Lima *et al.*, 2010; Gruber *et al.*, 2013). Zhang *et al.* (1999) found a strong inhibition of lateral root growth of 50% for 50 mM NO_3^-. The described reaction of *Arabidopsis* with presence of many but very short laterals is very similar to what we observed for *Hordeum vulgare* in the fertiliser layer in the U treatment with even higher NO_3^- concentrations up to 100 mM (see Appendix 2, Fig. A2.15). Though, we did not observe the same for *Vicia faba.*

The problem of transferability similarly accounts for nutrient solution studies (Maizlish *et al.*, 1980; Granato and Raper, 1989; Sattelmacher and Thoms, 1989; Gerendás and Sattelmacher, 1990; Thoms and Sattelmacher, 1990; Bloom *et al.*, 1993; Schortemeyer *et al.*, 1993; Caba *et al.*, 2000; Bloom *et al.*, 2006; Tian *et al.*, 2008; Ogawa *et al.*, 2014). Typically, applied concentrations are rather low, absorption is irrelevant and chemical and nutritional conditions are kept constant by adding buffers and replacing the solution in a high frequency. Moreover, in solution nutrient mobility is high and a large solution volume is in direct contact with the root system, which is not the case under natural soil conditions.

Similar to results from agar plates, there is evidence in these studies for a growth stimulating effect at low NO_3^- concentrations, while higher concentrations result in growth inhibition (Sattelmacher and Thoms, 1989; Tian *et al.*, 2008). Other studies have also found that at low concentrations of both N-forms, NH_4^+ can be more beneficial for root growth than NO_3^-, *e.g.* for potato in Gerendás and Sattelmacher (1990) or maize in Bloom *et al.* (1993) and the citations therein. In contrast, regarding higher concentrations, NH_4^+ impeded root growth more than NO_3^-, *e.g.* for maize on 4 mM (Bennett *et al.*, 1964) or tomato on 8-10 mM (Ganmore-Neumann and Kafkafi, 1980; Magalhaes and Wilcox, 1983).

These results emphasise that plant species and even genotypes of the same species can be quite different regarding their susceptibility against N-form inhibition or even toxicity, or the other way round, their preferred N-form and concentration. This can also be derived from our data, as *Vicia faba* was not distinctly inhibited by high NO_3^-, while lateral root length was markedly reduced in *Hordeum vulgare*.

These results illustrate well the fact that plant's responses to localised nutrients fall on a spectrum (Einsmann *et al.*, 1999). Drew's barley plants were definitely at the upper end of responsiveness, albeit under contrived laboratory conditions. Other species and experimental setups usually produce small, zero, or even, as here, negative responses. Not all experiments with localised nutrient availability yield distinct results (Maestre and Reynolds, 2006; Rabbi *et al.*, 2017), and *Vicia faba* seems to be a relatively unresponsive species in general (Li *et al.*, 2014a; Gao *et al.*, 2019a).

Studies in soil, conducting pot experiments (Anghinoni and Barber, 1988; Anghinoni *et al.*, 1988; Zhang and Barber, 1993; Van Vuuren *et al.*, 1996; Hodge *et al.*, 1998; Hodge *et al.*, 1999a; Hodge *et al.*, 1999b; Maestre and Reynolds, 2006; Xu *et al.*, 2014; Rabbi *et al.*, 2017) or field studies (Maizlish *et al.*, 1980; Bloom *et al.*, 1993; Ogawa *et al.*, 2014; Pan *et al.*, 2016)

found more diverse results of plant growth response to different N applications, ranging from no effect to stimulation of root growth to toxicity. These differences are related to the fact that soils can have an extremely wide range of physical, chemical and biological conditions for plant growth compared to well-controlled conditions on agar plates or in nutrient solution.

To overcome these given uncertainties, we monitored both – N-status and pH in soil solution as well as root growth development – concurrently. By introducing the 3 week phase of incubation, reasonable N-input rates for pot experiments, together with a sufficient spacing between the urea granules themselves and to the seeds, we avoided NH_4^+ toxicity that was found in Xu *et al.* (2014) and Pan *et al.* (2016), as well as for the highest N-application rates in Anghinoni *et al.* (1988) and Anghinoni and Barber (1988).

Apart from the local root growth depression by high NH_4^+, Xu *et al.* (2014) found higher total root length in the treatment with local urea application without NI in comparison to the control without N input and to the +NI treatment. Similar to our results for *Vicia faba*, this cannot be explained by a local root foraging into the fertiliser patch, as the gain in root length was achieved in areas further away from the fertiliser in Xu *et al.* (2014). But one has to note that in Xu *et al.* (2014), NH_4^+ in soil extract from the fertiliser placement was very high in both treatments, almost twice the NH_4^+ concentration in our study so that their final result of root growth distribution may be a consequence of both, stimulation by NO_3^- and inhibition by NH_4^+.

In contrast, Anghinoni *et al.* (1988) and Anghinoni and Barber (1988) measured N-concentration in soil solution at the end of the growth experiment. They observed highest dry weight of shoots and roots for maize at a concentration of 1.33 mM NH_4^+ in soil solution, while higher NH_4^+ markedly reduced plant growth. In our study, both treatments C and U+NI are characterised by the same NO_3^- conditions but additional NH_4^+ in U+NI. Hence, differences between those two treatments can be attributed to NH_4^+. We found NH_4^+ concentrations in soil solution higher than 1.33 mM, locally in the fertiliser layer, but we have not observed toxicity symptoms even though *Fabaceae* and barley have been assigned to be NH_4^+ sensitive (Britto and Kronzucker, 2002). Anghinoni *et al.* (1988) cite further studies that emphasise the relevance of sorption, as 1 mM in sand culture was toxic to tomato (Wilcox *et al.*, 1985) and muskmelon (Elamin and Wilcox, 1986), while NH_4^+ toxicity was not observed in two corn genotypes grown in vermiculite with up to 25 mM NH_4^+ (Handa *et al.*, 1984). Hence, sorption but also the soil specific dynamic equilibrium between absorbed and NH_4^+ in solution needs to be taken into account to evaluate the potential for root growth responses to external NH_4^+, NO_3^- and the ratio of both N-forms. As shown here and recently reported by Kirschke *et al.* (2019) the soil solution

concentration of NH_4^+ is maintained at a considerable level by NI application, highlighting nitrification as a key process of *in situ* NH_4^+ concentration and thus, suggest a possible interrelation of soil nitrification rates and root architecture.

5.5 Conclusion

We conclude the following aspects:

- Response of root growth development was different for *Vicia faba* and *Hordeum vulgare*. For *Vicia faba*, no significant differences in root growth were observed, but the soil-root distances from the CT analysis indicate a difference regarding the three-dimensional shape of the root system in the soil shown by a higher frequency of shorter soil-root distances in the treatment with higher NH_4^+. *Hordeum vulgare* showed a strong inhibition by high nitrate concentration in the soil and a higher number of first-order lateral roots due to higher NH_4^+, especially in the fertiliser layer. Higher NH_4^+ concentrations had no negative influence on both species. Comparing two or even more different plant species is recommended to distinguish between general responses and responses that are species specific.
- We recommend to measure root length development over time and to also monitor number of roots of different orders additionally to total root length or per diameter class, as root growth response is not always reflected in total length.
- Experiments in soil are more realistic than studies carried out in nutrient solution or on agar plates, but differences are expected to be less pronounced and less significant due to the various buffering effects of real soil. The main reasons for this are the physical structure of the soil matrix, the adsorption of NH_4^+ to clay minerals and organic matter, dynamic turnover of N-forms by enzymes and microorganisms, as well as the background concentration of N in the soil, related to soil organic matter content, which prevents extremely steep concentration gradients. Soil solution studies at the same time scale as observation of root growth development is very valuable for the purposes of separating mechanistic drivers of root:soil interactions. In a natural soil material soil solution is normally dominated by NO_3^- and the concentration of NH_4^+ is small due to sorption processes and very quick nitrification (when sufficiently aerated, not dry and at temperatures of higher than 5 °C). Therefore additional analyses *i.e.* analysis of NO_3^- and NH_4^+ in soil extracts are recommended for future studies, *e.g.* to allow a distinction between absorbed and readily available NH_4^+.

Acknowledgements

We thank Ina-Maria Zickenrott and Gao Wei for their help during plant harvest. Moreover we thank Bernd Apelt for measurement of N-concentration in plant biomass.

6 Overarching discussion

Recently it came into focus that roots, as main organ responsible for the uptake of water and nutrients, have a central function in increasing the uptake and utilisation efficiency of the nutrients present in the soil and supplied by fertilisation (Garnett *et al.*, 2009; Kant *et al.*, 2011; Xu *et al.*, 2012). The adaptability of the root growth and the spatial arrangement and development of the root system architecture show a considerable plasticity (de Kroons and Hutchings, 1995; Hodge, 2004; Hodge, 2006; Gruber *et al.*, 2013). From the perspective of plants, this is an essential survival strategy to secure access to water and nutrients and to succeed in inter- and intraspecific competition with other plants. The essential adaptation strategy of the stationary organisms is therefore a resource-efficient exploration of the soil. The objective of this thesis was to image this exploration and to observe the plasticity of root system architecture with regard to N form, distribution and concentration. Furthermore, the evaluation of the imaging technology (X-ray CT) itself was another focus of this thesis.

6.1 Methodological discussion

The investigation of a plant's ability to explore a certain soil volume by its root system is of great interest in respect to resource acquisition. As described in chapter 3, there are several ways to achieve this, but all methods have their advantages and disadvantages. In general terms, the more realistic, that is, unrestricted the growing conditions of the plants are, the more difficult it is to obtain high quality data from the roots using imaging methods. On the field scale a temporally and spatially high-resolved recording of root growth is hardly possible, but only here the root system develops in the realistic way that is most relevant for the agricultural point of view. Only in the field the roots have enough space to explore the soil both vertically and horizontally. Here they react to the heterogeneity of the soil, from a physical as well as a chemical and biotic perspective. Influences on plants such as climate, precipitation and temperature fluctuations can only be marginally reproduced on the scale of the greenhouse or climate chamber. However, in order to utilise imaging techniques such as X-ray CT, conditions such as an appropriate sample size are necessary. It is also advantageous if the imaging device and location of the growth experiment are close together. In particular if growth dynamics are addressed by repeated scanning of the same sample, this is a way to minimise disturbance.

The overriding compromise in imaging techniques is the intrinsic relationship between sample size and spatial resolution of the data with the general rule: the larger the sample, the coarser the spatial resolution of the images. Depending on the plant species used in the experiments,

this critical point occurs sooner or later - depending on the root system and the associated root radius. As shown in the present studies in chapters 4 and 5, the root system of the faba bean could be very well captured under the present conditions of the soil used, the dimensions of the columns used (diameter: 7 cm) and the parameters of CT image acquisition. For barley, though, it was not possible to record a relevant proportion of the roots using the image processing and analysis options available. In this case, this is due to three criteria in particular: 1) the very small diameter of the roots of barley in comparison to faba bean, 2) the relatively low homogeneity of the soil used with regard to the grain size distribution and the associated characteristics of the soil structure consisting of soil matrix and rather large pores, and 3) the parameters used for image acquisition with relatively short exposure time and low number of projections per frame, so that the signal-to-noise ratio can be evaluated as unfavourable from the point of view of image processing and analysis (Gao *et al.*, 2019b).

The selection of the boundary conditions for the experimental set-up of the studies carried out within the framework of this thesis was the result of extensive considerations and numerous preliminary tests with the state of the art and knowledge. A larger column diameter would have provided for a larger soil volume, so that the root system could have developed in a more natural shape, allowing the extension of the experiment to gain information on more mature plants (maximum age of the plants in the present work was 17 days). However, this would have impaired the spatial resolution of the tomograms, resulting in dissatisfactory detection even for faba bean.

The selection of CT parameters was also a compromise solution. A first experiment conducted with parameters for best image quality led to the immediate death of the faba bean plants in that experiment (see Chapter 4, Figure 4.11, page 55). What initially started with a random discovery resulted in two specific experiments on the influence of X-rays on root growth of the two plant species *Vicia faba* and *Hordeum vulgare* (Chapter 4). The topic of the influence of X-rays on root growth was largely neglected in the recent literature up to this point and played hardly any role in the previous experiments using CT as an imaging technique. This is due to the fact that in normal cases when X-ray CT is the method of choice for root growth imaging, all experimental plants are irradiated with X-rays, so that an influence cannot be noticed at all due to a lack of a control treatment. In addition, no standards are known for the selection of CT settings, which are often based on certain rules of thumb, *e.g.* which minimum gray value the darkest areas in radiography should have. Also the choice of the exposure time and the number of projections from which the 3D volume file is reconstructed after the acquisition, resulted

from a compromise between sufficient image quality and scan time duration. Only with the mentioned coincidence discovery this topic got more attention and enabled a closer investigation of the influence of X-rays in specially designed experiments in the framework of this thesis.

The soil used was also chosen with care as a consequence of several preliminary tests. The first tests in the context of this work were carried out with a mixture of quartz sand composed of different grain sizes, as it was done in *e.g.* Koebernick *et al.* (2014). This type of growth media would have been very suitable in terms of homogeneity and hence the resulting image quality of the CT scans. Due to the lack of sorption sites for NH_4^+, various additions of illite and goethite were tested. By the addition of an emulsion of an active topsoil, inspired from Neumann and George (2005), the necessary microorganisms for the N-metabolism were introduced into the system in a later test. The work and time required for the preparation and composition of these experiments and the subsequent verification of applicability on the basis of actual plant development and chemical analysis of soil and soil solution was very high. However, the results were unsatisfactory and in view of the relatively artificial conditions this approach was rejected. The subsequent test using a homogenised topsoil material was also problematic, as there was a high background concentration of nitrate in this soil, which was further increased by the mineralisation of the present organic matter, so that observation of the plant response to the local N-fertilisation was severely impeded. The result of this lengthy process was the decision to use a subsoil originating from a Luvisol, which was already used in several publications, *e.g.* Vetterlein *et al.* (2013a) or Beuters *et al.* (2014). This subsoil is characterised by the fact that it has a relatively low background concentration of nitrate and ammonium compared to a topsoil, has a high cation exchange capacity, provides a natural composition of microorganisms and is therefore relatively close to natural conditions, at least in comparison to the quartz sand mixture. For use in the experiments listed here, only homogenisation and sieving to a certain grain size was necessary to exclude excessive structural heterogeneities, enabling the observation of the root reaction to the local application of urea fertilisers.

In column experiments of this type, irrigation is usually carried out from below by capillary rise, due to the lower influence on the soil structure compared to irrigation from the top. In addition, irrigation from above would result in soluble and mobile substances being rinsed into the lower areas of the soil column, so that they would become relevant for the plant relatively late. In the experiments carried out here on the reaction to placed fertilization, both approaches

- irrigation exclusively from below or above - were not practicable. This was also investigated in preliminary experiments, and the results showed that in these cases the nutrients were no longer to be found at the location of placement, but either in the upper or lower edge areas of the soil column. This is further attributed to the use of distilled water, as is common practice in plant growth experiments of this kind. From this knowledge the necessity was derived to carry out the initial irrigation of the soil columns to the desired water content from top and bottom. By this way of watering it could be achieved that a large part of the fertiliser N was present in the desired layer. In addition, this type of irrigation has led to the situation that a large part of the background N in the soil has also been shifted into the fertiliser layer by water transport.

The sampling of soil solution via extraction using micro suction cups ('MicroRhizons', Rhizosphere Research Products B.V., The Netherlands) was very successful. After the initial use of self-made suction cups with a ceramic as porous medium (according to Vetterlein and Jahn (2004)) in the very first experiments, they were replaced due to their fragility. The collection of soil solution by negative pressure provided sufficient sample volumes for the necessary analyses to be carried out in sufficient quality. The sampling directly in the layer of the fertiliser application and the temporal resolution of sampling in the same rhythm as the acquisition of the CT images allows for linking the chemical conditions of the soil solution with the development stage of the roots.

6.2 Discussion of experimental results

The study on the influence of X-ray dose on root growth carried out in the context of this thesis has shown that even a moderate dose of X-rays, well below a threshold value of 33 Gy (Zappala *et al.*, 2013), can have an influence on root growth. In the case of this study, faba bean showed a significant dose-dependent decrease of root length in response to radiation exposure, while barley showed no response to the radiation under the same experimental conditions. A further development of root classification was achieved based on the skeleton idea in Flavel *et al.* (2012). This enabled the distinction of different root orders (tap root, first order lateral roots and second order lateral roots) of faba bean. With this novel approach is was possible to show that in particular the length of the first-order lateral roots as well as the length and number of second-order lateral roots were reduced by the X-rays. This is particularly critical, as these roots of higher order are often responding more sensitively to locally available nutrients compared to the first order laterals or the primary root (Zhang and Forde, 2000; Lima *et al.*, 2010; Gao *et al.*, 2019a).

The physiological reasons for this reaction go beyond the scope of this work. However, there are many indications that phytohormones (especially auxin) which are essential for root growth and their branching behaviour were damaged by the X-rays. This is also evident from very early investigations (Skoog, 1935; Gray and Scholes, 1951; Clowes, 1963; Evans, 1965), even if the technique and approach used there cannot be directly compared with those used in this thesis.

The present study was therefore designed to demonstrate the influence of X-rays on root growth over time and to increase awareness for this topic, when experiments are conducted using CT as imaging technique. The formulation of a threshold value seems infeasible and the fact that one plant species has shown no reaction at all, while the other was significantly influenced, clearly argues against the applicability of such a threshold value in general.

Generally valid conclusions for all plant species cannot be derived with certainty from such a study. The number of potential influencing factors is far too great, such as the CT device itself, the selected settings, the soil used, the duration of irradiation, the rhythm of irradiation, the plant species and the age of the plants. Therefore, one of the central conclusions as a consequence of this empirical study is the recommendation to establish a non-irradiated control treatment in future experiments. In this kind of experiments, it may not always be possible to completely exclude the influence of X-rays on root and plant development, but knowledge about the extent of this influence is an important argument in the evaluation of the results.

The data set obtained in this publication was also used to enable a new way of quantifying root growth patterns from the soil perspective via root distance models (Schlüter *et al.*, 2018). Based on ideas from Koebernick *et al.* (2014), 3d time-lapse imaging and image registration was used to generate a parsimonious root distance model capable of describing root growth patterns of the first 3 weeks of growth of *Vicia faba*. These models are able to abstract the complex spatial root growth patterns that usually cannot be determined by traditional root system analysis. This approach can help to estimate the root age dependent rhizosphere volume or can be integrated into the modelling of water uptake for example.

For the investigation of the reaction of root growth to locally applied nitrogen forms, the application of X-ray CT has provided valuable insights, but only for faba bean. While the differences regarding absolute root lengths between the treatments were negligibly small, the distribution of soil-root distances derived from the CT data was able to visualise different root distribution intensities. Analysis of data indicates a greater exploitation of the soil volume with higher availability of ammonium. For the reasons mentioned above, the detection of barley

roots was not sufficient in this study, so that they were examined classically destructively and analysed with WinRHIZO only. Therefore, a much larger number of experimental plants was necessary in order to be able to derive temporal information - analogous to the assessment of root growth of faba bean using X-ray CT – another benefit of this technique. Despite the greater workload the investigations without the use of CT have yielded important insights into the reaction of barley root growth to NO_3^- and NH_4^+ over time and spatially discretised.

The generally formulated statement on the response of root growth to NO_3^- and NH_4^+ is that NO_3^- leads to an increased elongation of the roots, while NH_4^+ causes an increased branching of the roots. Although this statement may be true for some cases, it should be verified for specific conditions, concentrations, spatial and temporal distribution patterns as well as for different plant species. Furthermore, it should be noted that most studies from which this general conclusion was derived were not carried out in soil, but in nutrient solution (Granato and Raper, 1989; Bloom *et al.*, 2006; Tian *et al.*, 2008; Ogawa *et al.*, 2014) or on Petri dishes with a growth medium (mostly agar) with an applied nutrient solution in often very low concentrations (Zhang *et al.*, 1999; Remans *et al.*, 2006; Lima *et al.*, 2010; Gruber *et al.*, 2013). Studies in soil found widely differing results of plant growth response from no response to stimulation of root growth to inhibition and toxicity symptoms (Maizlish *et al.*, 1980; Anghinoni and Barber, 1988; Anghinoni *et al.*, 1988; Bloom *et al.*, 1993; Van Vuuren *et al.*, 1996; Hodge *et al.*, 1998; Hodge *et al.*, 1999b; Xu *et al.*, 2014; Rabbi *et al.*, 2017). However, in most of these studies N-dynamics measurement in soil and soil solution or root growth analysis over time was largely neglected.

As already described by Nacry *et al.* (2013), a general statement about a typical root growth response to N that is applicable for all species and conditions is almost impossible to define, but some very general aspects hold true for most circumstances. First, high N contents in the shoot and/or roots can lead to a systemic repression of (mainly lateral) root growth. In addition, exogenous NO_3^- and NH_4^+ can have local effects on root growth. It is known that excessively high nitrate concentrations in the nutrient solution can lead to local growth inhibition (Zhang *et al.*, 1999; Zhao *et al.*, 2007; Tian *et al.*, 2008) and in particular high ammonium concentrations can have a toxic effect on the roots (Britto and Kronzucker, 2002; Pan *et al.*, 2016). In this context, the question arises as to when a concentration is too high and if this is a universal reaction or has to be defined differently specifically for different plant species.

The results of the studies carried out here (Chapter 5) show that the reaction of the two plant species towards NO_3^- and NH_4^+ in the soil was different from each other and quite different

from many existing studies. Basically, the assumption in the experiments carried out within the framework of this thesis on the reaction of root and plant growth to locally available N-forms was to obtain results similar to those found in the literature. Especially due to the methodical proximity to the work of Drew (1975), a certain similarity of the results was expected, but was confirmed only to a limited extent. Only the more pronounced branching of the roots in barley, in particular the increased initiation of first-order lateral roots locally in the fertiliser layer, as a reaction to the greater concentration of NH_4^+ in the treatment of the nitrification-inhibited urea in this area, is consistent with the findings of Drew (1975). In contrast, increased NO_3^- concentration led to a clear inhibition of root growth in barley instead of an increased elongation of the roots. For faba bean it should be noted that no statistically reliable differences could be observed between the treatments, neither with regard to the potential promotion of lateral root formation by NH_4^+, nor with regard to growth inhibition of the roots by NO_3^-.

These results illustrate that plant's responses to localised nutrients cover a broad spectrum, as also stated by Einsmann *et al.* (1999). Drew's barley plants were definitely at the upper end of responsiveness to heterogeneous N supply, albeit under contrived laboratory conditions. Other species and experimental setups may produce negligible or even, as here, negative responses. This means that not all experiments with localised nutrient availability yield distinct results (Maestre and Reynolds, 2006; Rabbi *et al.*, 2017), and *Vicia faba* seems to be a relatively unresponsive species in general (Li *et al.*, 2014a; Gao *et al.*, 2019a).

In addition to the analysis of root growth, further insights concerning the chemical conditions of the soil solution were gained. By sampling the soil solution from the fertiliser layer and analysing it for NO_3^- and NH_4^+, but also for the pH value, it was possible to trace the fertiliser turnover very conclusively over time in a running experiment. The comparison with unplanted soil columns made it possible to distinguish between the impact of the fertiliser turnover and the influence of the plants. This temporal assessment of soil chemical conditions in the same temporal resolution as the analysis of root growth under relatively near-natural conditions *in-situ* has not been carried out in this form.

The combination of the used N-input rate of 100 mg N/kg soil and the type of irrigation as a mixture from above and below, and the resulting displacement of the background N, however, led to remarkably high nitrate concentrations in the fertiliser layer, especially in the non-inhibited urea treatment, of up to 100 mM. Even the control treatment contained about 30 mM NO_3^- in this layer. The comparison to other studies is difficult because methodological

approaches differ considerably, soil solution is rarely measured and hardly any studies work with high N concentrations.

It should be noted that the concentration of soil solution cannot be directly compared with the concentration in nutrient solution. There, a very large volume of solution is in direct contact with the root system. This solution is usually exchanged frequently in order to keep the concentration constant. In addition, the pH value is usually buffered. Sorption is not relevant in such systems and microorganisms, which are actually required for nutrient turnover, are usually not present. Due to these boundary conditions, there are no nutrient dynamics involved and the formation of gradients at the root surface is practically impossible. These conditions are substantially different from the conditions in soil and therefore limit the comparability considerably.

In contrast to the study presented here, other studies mostly focus on N-deficiency and hence, use very low concentrations in comparison to a region with a higher concentration. This circumstance was also present in the work of Drew (1975), as can be seen from the concentrations used in the experiments. In the layer with a "high" concentration 1 mM N was present in the form of NO_3^- or NH_4^+, while in the areas above and below this layer extremely low concentrations or no N at all were applied (0.1 mM NO_3^- or 0 mM NH_4^+). These conditions are very well suited to show a clear and local reaction of root growth. Therefore, the barley plants in Drew (1975) can be considered at the upper end of responsiveness.

However, such conditions do not occur in nature or in the agricultural context. Nitrogen, especially in the form of nitrate, is omnipresent in natural soil and currently NO_3^- concentrations in agricultural soils are rather too high, especially in Germany, which poses a problem for groundwater quality.

The results obtained here provide a contribution to the classification of the root response to locally available N-forms. It could be shown that the root reaction behaves in some aspects similar to studies in nutrient solution or on agar, but only for a few traits. In consideration of other studies, especially those using soil, it can be recognised that the reactivity of plants covers a broad spectrum. In addition, it has been shown that two different plant species with contrasting root systems behave very differently. The results obtained are directly related to the given experimental conditions. In particular, the soil type and composition, method of irrigation and fertiliser type, as well as concentration and spatial distribution of the fertiliser should be taken into account. Therefore, it is not possible to regard the determined plant reaction as a generally

valid "typical" reaction and conclusions about other soils, fertilising strategies and plant species must be drawn with caution.

In fact, the experiments and trials carried out here should be an incentive to conduct similar studies (as done by *e.g.* Gao *et al.* (2019a)) in the future in order to gain further knowledge and, in the long term, enable optimisations for the selection of plants and fertiliser strategies. This approach should serve to further increase nutrient efficiency and contribute to the improvement of environmentally sustainable agricultural practice.

7 Conclusions and Outlook

7.1 Conclusions

1) The study of root growth and the *in-situ* development of root system architecture in soil are of great interest, especially as it is a goal of modern and future agriculture to improve the nutrient use and uptake efficiency by plants. To achieve this, a sound knowledge of root growth and response to soil conditions and nutrient availability is necessary.
2) Studies in nutrient solution or on nutrient media (agar) have made an essential contribution to improving knowledge of root growth and its response to local heterogeneity of nutrient availability and concentrations. However, these artificial systems do not adequately reflect natural and agricultural reality, making it difficult to draw conclusions for soil based systems or even the field scale.
3) Imaging techniques are of great relevance for observing and analysing root growth and spatial exploration of soil volume by the root system *in-situ*. This allows - in contrast to destructive methods - to record the root growth in the true 3D geometry over time and to change perspective, as it was shown with the soil-root distances. Due to the opaque nature of the soil, direct imaging is not possible, requiring the use and further development of advanced techniques to visualise and measure the roots in the soil. For this purpose, methods that were originally developed for medical application are particularly suitable.
4) The main methods for *in-situ* observation of root growth are neutron tomography, magnetic resonance imaging and industrial X-ray computed tomography (with the special form of synchrotron CT). All these methods have decisive advantages and disadvantages, as well as typical fields of application. Essential criteria for the differentiation of the methods are the availability or accessibility, image quality, possible sample size and the disturbance of the sample by the measurement. Classical, destructive methods and the subsequent analysis with programs like WinRHIZO still remain the standard and are particularly suitable for a final comparison at the end of the experiments.
5) X-ray computed tomography has emerged as a widely used method for the dynamic recording of root growth in soil. This is due to the availability of the equipment, the fairly high image quality and resolution, and relatively quick measurements with only few restrictions, *e.g.* with regard to the soil used. In addition to root growth, the soil structure can also be analysed at the same time. Decisive disadvantages are the

limitation of the sample size, image artefacts, poor contrast between roots and water-filled pores and the potential influence of X-rays on root growth. Additionally, powerful computers and a good knowledge of how to evaluate the data are necessary, as no user-friendly commercial programs (such as WinRHIZO for root analysis from washed out roots) are available yet. The differentiation of the specific root orders in faba bean on the basis of their spatial orientation was a new approach developed in this thesis, added to the analysis of root system architecture using X-ray CT. In contrast, it was not possible to segment barley roots from the CT data in sufficient quality. To achieve this, a smaller sample size (as it is the case in many other studies using X-ray CT for root visualisation) and a soil with greater homogeneity would have been helpful. New algorithms, such as the recently published "Rootine" (Gao *et al.*, 2019b), are promising, but also here the outcome is dependent on many factors, especially the homogeneity of the soil.

6) This work has shown that irradiating young roots with X-rays can reduce root growth. This depends on the dose of radiation, which was varied in this study by changing the frequency of irradiation, and the plant species. In the case of faba bean (*Vicia faba*), the length of the higher-order roots in particular was reduced as a function of the radiation dose. This led to a significantly shorter total root length. Irradiated plants were also characterised by smaller leaf area and shoot weight. In contrast to faba bean, the same dose of irradiation with X-rays had no impact on root and shoot growth in barley (*Hordeum vulgare*).
7) Faba bean and barley behaved differently in terms of the response of root growth to local supply of different N-forms in soil. While faba bean showed no significant change in root system architecture, barley plants reacted to the different nitrogen supply in soil. Higher available concentration of ammonium in the soil and soil solution led to an increased initiation of first order lateral roots in the immediate vicinity of fertiliser application. Increased nitrate concentration in the soil solution led to a clear inhibition of root growth in barley. This was demonstrated already after 8 days of growth and continued over the experimental period. The respective reactions of the root growth of both plant species were also reflected in the aboveground biomass formation.
8) The use of micro suction cups and the regular sampling of soil solution enabled monitoring the turnover of urea via ammonium to nitrate and the associated influence on the pH value of the soil solution. By coupling the extraction of soil solution closely with the recording of root growth at each sampling time, both observations can be

combined effectively. Study of soil solution is of particular interest due to its direct contact with the root surface and the essential uptake and release processes of plant-soil-interaction take place here. It is in exchange with the soil matrix, in particular the ions adsorbed to clay minerals and organic matter, so that a dynamic equilibrium is expected. Nevertheless, complementary destructive analyses are useful, such as the extraction method for mineral N, in particular for soils with a high exchange capacity such as the one used in the experiments of this thesis.

9) The studies presented here have provided valuable new insights into the impact of X-ray radiation on root growth in soil and root growth responses to local supply of different urea-based N-forms. The methodology that was developed here may serve as a useful benchmark for future studies analysing the interplay between root growth and soil solution chemistry. It was clearly shown that a broader knowledge base on the influence of X-rays on root development is needed, particularly when plants are repeatedly scanned. The reaction of root growth to heterogeneous nutrient availability should be further investigated under conditions close to natural field conditions, *e.g.* for different soils and plants. Care should be taken with the conditions of the experimental setup (*e.g.* type of irrigation, soil type, plant species, sample size and radiation dose) as these can also have a substantial influence on the results.

7.2 Outlook

7.2.1 Root growth visualisation and analysis, especially with the use of X-ray CT

Despite the growing importance of imaging methods, the classical method of measuring root growth in soil, *i.e.* root washing, scanning and subsequent analysis and evaluation with programs such as WinRHIZO or similar will still be an integral part of root growth studies in the future. This tool continues to provide robust and comparable results as shown in this thesis. This approach is widely used and offers a variety of rapid and practical evaluations, such as the classification of roots by diameter. However, defining appropriate thresholds for diameter classes can be difficult. Analysis with WinRHIZO is often regarded as the standard and it is also very suitable for comparing different acquisition and evaluation methods. The software itself is constantly being refined and is used by many researchers. Nevertheless, there is also potential for improvements for this established software, such as supporting the user in the search for suitable root diameter classes and a better way to deal with root hairs.

Still, one major disadvantage remains, as this method normally requires that the roots are removed from the soil and washed properly to allow for analysis. This requires the destruction

of the soil sample to access the roots. This means that all information describing the spatial architecture of the root system is lost. Furthermore, it is not possible to track the root growth of individual plants over time. To obtain information on the temporal development of root growth, the amount of work required is much higher, since a multiple of replicate plants is necessary. This number cannot be increased arbitrarily either, as it is limited not only by the increased working time but also by the amount of soil needed, the size of the climate chamber and the number of connections for *e.g.* suction cups and all the attached tubes needed for sampling.

Occasionally, approaches are pursued in which, for example, an inclined box is scanned including the soil and the image is then analysed with WinRHIZO or similar tools. This has several disadvantages, *e.g.* only a certain number of roots grows along the plate and can therefore be detected. Moreover, it is more difficult to distinguish the roots from the soil than from a transparent background. In addition, condensation drops on the plate make it difficult to obtain a sufficiently good image quality.

Many of these disadvantages or shortcomings can be overcome by the use of non-destructive and non-invasive imaging techniques. Therefore, their relevance, availability and use will continue to increase in the future. This is particularly the case for X-ray CT, which currently has major advantages over other methods such as MRI or neutron radiography (see Chapter 3). Even if the basic principle remains unchanged, further technical improvements are conceivable. For example, the resolution of the detectors is constantly improving. In addition, devices are already available in which the sample no longer has to be irradiated from all angles of a full rotation in order to enable a high-quality reconstruction (Beister *et al.*, 2012; Kazantsev *et al.*, 2016; Marschner *et al.*, 2016). This is particularly important because one part of this thesis (Chapter 4) has shown that the cumulative irradiation of the roots with X-rays itself can have an influence on root growth. This raises the question if and to what extent the measured results are influenced by artefacts generated by the method itself. Apparently, this is particularly relevant for lateral roots of higher order. There is a strong demand for approaches to reduce the irradiation duration while maintaining or even improving the image quality. The further possibilities to investigate the interaction between X-rays and root growth are discussed in the following chapter 7.2.2.

Qualitative imaging by X-ray CT allows the estimation of different root growth patterns, *e.g.* in Nwankwo *et al.* (2018) or information and predictions about the root age at which certain root orders are developed. Of course, the quantitative evaluation of the data has an increased significance and there has been a rapid development in image processing algorithms.

Due to the general increase in the overall computing power of high-performance computers, image processing is easier and faster than ever before. This was a necessary development to evaluate the increased amount of data and the requirement for greater detail in the analysis. Nevertheless, these existing algorithmic sequences are not very user-friendly and no commercial software (analogous to WinRHIZO for the analysis of washed out roots) has been established in this area. Most approaches are individually composed sequences of processing steps. Examples are “RooTrak” from the University of Nottingham (Mairhofer *et al.*, 2012) and “Root1” from the University of New England (Flavel *et al.*, 2017). The underlying approaches and algorithms mostly come from the medical sector, for which many of the popular plugins for the free software ImageJ have been developed. These are constantly being expanded and thus the possibilities of evaluation are successively increased as well.

Methods such as "region growing" have proven successful for some experimental conditions. For the segmentation of faba bean in the studies of this thesis, but also in other publications (Tracy *et al.*, 2012b; Helliwell *et al.*, 2019), this approach enables high-quality evaluations. It requires a strong user interaction, can be very time-consuming and can only be automated to a small extent. However, as soon as the soil structure becomes more heterogeneous and the roots have a very small diameter, this method reaches its limits relatively quickly.

Approaches that are primarily based on the analysis of the gray value histogram and related parameters can be automated much better, but are also strongly influenced by soil conditions. Since a water-filled pore and a root in the CT image have a very similar grey value, this can lead to a considerable misclassification, which considerably reduces the quality of the evaluation.

The latest developments combine region growing and histogram-based approaches and extend them with shape recognition, as can be seen from the title of the publication by Gao *et al.* (2019b): "A shape-based method for automatic and rapid segmentation of roots in soil from X-ray computed tomography images: Rootine". This concept is currently being further developed at the Helmholtz Centre for Environmental Research GmbH - UFZ. Given a relatively homogeneous soil material and high quality CT images, the root system of *e.g.* maize was extracted in high quality from relatively large soil columns (ø = 7 cm), while many other studies deal with much smaller sample sizes, *e.g.* 2.5 cm in Helliwell *et al.* (2019) or 3 cm in Flavel *et al.* (2012).

The analysis of the extracted root system enables a number of high-quality evaluations, most of which cannot be obtained by many classical methods. This applies primarily to spatial parameters, *e.g.* local root length density in extremely high depth discretization.

A further type of analysis has been developed and applied in the framework of this thesis in the form of soil-root distances, based on a parsimonious root distance model, which is able to describe the root growth patterns of faba bean during the first 3 weeks based on X-ray CT data (Schlüter *et al.*, 2018). By this the so-called "distance map" is derived. The implementation of the distance map adds yet another factor to the approach of the classical root length density. Other methods of this kind are essential to obtain more information from the spatial distribution of the roots in the soil. This allows enhanced evaluation of the development of the soil, the combination with physiologically important parameters, such as the characteristics of the rhizosphere, areas of influence for diffusion and mass exchange, and the quantification as a function of root development over time. For *Vicia faba* it was possible to distinguish between the different root types. In other species this might prove difficult due to the much more complex root architecture. Nonetheless, this type of analysis would be useful since individual root segments perform different tasks or differ in their contribution to relevant processes, primarily water and nutrient uptake and transport.

7.2.2 Influence of X-ray radiation on root growth

Further studies are necessary for a holistic understanding of the interaction between X-rays and the root growth of plants. These studies are necessary to better understand the interaction between X-rays and plants, to define and minimise the influence in future experiments. A review on the effect of ionising radiation on physiological and molecular processes in plants was published recently (Gudkov *et al.*, 2019) with the conclusion that ionising radiation affects almost all aspects of plant vital activity. This concerns the whole spectrum of *e.g.* genome regulation, balance of reactive oxygen species, long distance regulation and signals, as well as intracellular signals and the interactions between all of them.

The establishment of a specific threshold value would have little use, since the present work and the corresponding publication Blaser *et al.* (2018) has shown that two plant species with different root system architectures behave extremely differently. Despite the large number of plant species, the investigations in Johnson (1936) hardly allow any superordinate conclusions to be drawn, as only flowering plants were investigated and the experimental conditions of the individual experiments were too different. A threshold value by plant family or type of root architecture might be appropriate. From a statistical point of view (*i.e.* absent other plant-

specific mechanisms), it is conceivable that dicot plants with a tap root are particularly susceptible to X-rays. This could be justified by the fact that their relatively compact root system has fewer possibilities for compensating damaged cells. In contrast, monocotyledonous plants with their extensive and highly branched root system have potentially more possibilities to compensate damaged cells. An indication of this is the unaffected root system of rice in Zappala *et al.* (2013). Further experiments with the specific comparison of plants with contrasting root system architecture would be helpful to verify this hypothesis. Plant species of particular interest are pea, tomato, lupin and rapeseed compared to wheat, maize and rice as they are often used in root development studies.

Early studies from the 1930-60s (Skoog, 1935; Gray and Scholes, 1951; Clowes, 1963; Evans, 1965) have shown a clear relationship between X-rays and the phytohormone auxin. This is to be regarded as conclusive, since auxin is a major contributor to the elongation and branching of roots and lateral roots (Bari and Jones, 2009; Vanneste and Friml, 2009). Moreover, it would be helpful to know to what extent auxin and also the other phytohormones such as ethylene, cytokinin or abscisic acid are influenced in detail by radiation. For particularly far-reaching statements, an experimental approach can be used which compares the scanning frequency as well as the cumulative dose or high single dose and the recovery effect. This recovery effect was shown in Evans (1965).

In most studies, a copper filter is placed directly in front of the X-ray source during CT imaging. Primarily, this filter was used to reduce the so-called "beam hardening" artefact and thus improve the image quality. The “beam hardening” artefact is reduced because the filter attenuates the lower energy photons that would cause the artefact (Brooks and Di Chiro, 1976). This attenuation of lower energy photons has another advantage apart from improving the image quality, because the low-energy radiation is suspected to have a particularly high damage potential for tissue.

A comparative investigation of different filter thicknesses or the comparison between different metals has apparently not been carried out in the field of industrial CT applications so far. It would have enormous advantages if a filter would be available that preferentially filters the part of the radiation spectrum that is particularly harmful for the roots, but nevertheless permits a sufficiently good image quality. A systematic comparison of filter materials and thicknesses would be recommended here.

Other parameters such as soil type, plant age, recreational ability and water content serve more for a better holistic understanding of the topic than for adapting the experimental conditions. This is due to the fact that because of the limitation of the sample size only very young plants are scanned anyway and that the boundary conditions of the soil are usually not arbitrary. Nevertheless it could be a decisive difference if the first scan is done after 2, 4 or 8 days. Moreover, a systematic variation of the composition of the soil could indicate whether and to what extent the scattering and absorption of X-rays is related to the soil type and bulk density.

Following the publication of Blaser *et al.* (2018), a frame was designed with a metal plate and a square recess. This is then positioned directly in front of the X-ray source during image acquisition and used to prevent some of the scattered radiation from directly penetrating the sample outside the field of view, including the aboveground part of the plants. To better describe the effect of this device, it is necessary to measure the X-rays arriving at the sample. Due to the low availability of suitable measuring instruments, which are appropriate in terms of size but also relevant dose ranges, such measurements have not yet been performed. The first measurements are currently being prepared at the Helmholtz Centre for Environmental Research GmbH - UFZ.

7.2.3 Nitrogen nutrition and fertilisation

Nitrogen is and remains an essential nutrient for plant nutrition and the supply of plants with N will continue to be an important component of agricultural practice in the form of fertilisation in the future. However, an increase in N application is not possible in many areas, although the world population continues to grow. On the contrary, in many regions a reduction of N inputs will be necessary. In Europe, and especially in Germany, nitrate pollution of groundwater is still a major problem. This issue has only recently attracted major attention again, as the European Court of Justice in Luxembourg has condemned Germany for violating existing EU law due to increased nitrate concentrations in groundwater. This issue is therefore relevant at many levels, from politics to the fertiliser industry, from agricultural practice and environmental protection to the population and its legitimate concern for good drinking water quality.

As a result, the innovation pressure on the fertiliser industry is always present, leading to a constant change in the product market. Due to scientific knowledge, political decisions and related legal regulations, but also economic interests, older products disappear from the market and are replaced by new ones. This also applies, for example, to the urea fertiliser without inhibition used in this thesis. According to the German fertiliser ordinance, farmers must use urea with urease inhibitors from 2020 onwards, if the urea cannot be incorporated into the soil

within four hours. As shown in the present study, urea can be rapidly turned into nitrate. Nitrate is extremely mobile in the soil and if it is not immediately assimilated by the plants via the roots, it can easily be transferred to groundwater and surface waters. This problem may even be exacerbated in the context of climate change due to the expected increase of heavy rainfall events.

Therefore, the relevance of urea fertilisers with inhibitors will continue to increase in the future. In addition to the fertilisers with nitrification inhibitor used here, products with urease inhibitor or a combination of several inhibitors are also available. This is intended to reduce N losses due to leaching as well as losses in the form of climate-relevant gases. These losses also have economic consequences, as these are expenses that do not reach the plant and thus do not contribute to yield and consequently food security for the population. The use of modern and advanced fertilisers should help in both respects - the environment and farmers through cost-effective use of fertilisers through an increased nutrient use efficiency and nutrient uptake efficiency.

The scientific community should continue to monitor this development and use the knowledge gained to help improving the efficiency of nutrient uptake and use. This can be achieved, for example, by ensuring that the type and composition of the fertiliser ensures that the nutrients are available to the plant in accordance with its needs, in terms of concentration, N form, spatial and temporal distribution. As shown by the example of barley in this thesis, high nitrate concentrations in a limited soil volume can be very detrimental to root and plant development. Excessive ammonium concentrations near roots are also harmful, if not toxic (Gerendas *et al.*, 1997; Britto and Kronzucker, 2002; Xu *et al.*, 2014; Pan *et al.*, 2016). Nevertheless, there is a conviction that in particular a balanced plant nutrition with both mineral N-forms enables the best root growth and shapes the lateral root structure in a complementary manner (Lima *et al.*, 2010). This is plausible because a combination of decent root elongation with a high number of branches should allow for the best exploration of the soil volume. This should achieve a high degree of flexibility with regard to the response to heterogeneous distribution of water and nutrients, but also to physical soil properties such as compaction or the availability of biopores. In addition, such a balanced ratio is also beneficial from the environmental point of view, as it reduces the risk of losses via the paths mentioned above. New urea fertilisers with various inhibitors, encapsulations and other conditioning agents such as in combination with P and other nutrients, nanoparticles (Milani *et al.*, 2015; Morales-Díaz *et al.*, 2017) or "plant biostimulants" (Rouphael and Colla, 2018) should therefore be used in similar comparative trials. In addition

to the type of fertiliser, the application rate used is another variable factor. This should be investigated in future studies in order to derive optimised fertilisation strategies depending on soil, climate and plant species. This is also of great interest because local fertiliser placement is of particular importance in agricultural practice. Therefore, the investigation and analysis of the spatial and temporal distribution of N forms in the soil and the reaction of root growth to this distribution is of great relevance to improve the NUE.

Experiments such as those presented in this thesis provide valuable insights for the topic of the root reaction to heterogeneous distribution of nitrogen in soil. Although the significance and applicability for the agricultural context is probably far greater than that of studies in nutrient solution or on nutrient media (agar), these studies are also limited in their significance for the field and landscape scale. A combination with studies on a larger scale is strongly recommended here. To a certain extent this could be achieved within the framework of this work, *e.g.* in cooperation with Kirschke *et al.* (2019). A consistent investigation of a situation on the various scales would lead to the best results and the most relevant conclusions. From the very small scale, such as in experiments on gene expression (Raghuram and Sopory, 1999; Wang *et al.*, 2000; Araki and Hasegawa, 2006; Miller *et al.*, 2007) or identification of transcription factors and affinity transporters (Zhang and Forde, 1998; Remans *et al.*, 2006; Lima *et al.*, 2010), to the small scale, such as the column experiments presented in this thesis, to the middle scale in greenhouses and *e.g.* Mitscherlich vessels, to the field scale. None of these approaches alone provides sufficient or holistic knowledge about the entire topic. Close cooperation between groups with different research focuses is therefore recommended.

The results obtained here are strongly dependent on the boundary conditions and characteristics of the experimental setup. In this context the investigation of further plant species using the same boundary conditions would be interesting. The results in this thesis corroborate the statement that plant's responses to localised nutrients show a broad spectrum (Einsmann *et al.*, 1999). Some relevant cultivated plants were evaluated with regard to their robustness and susceptibility to ammonium toxicity (Britto and Kronzucker, 2002). A similar classification of the plants in regard to their root plasticity towards heterogeneously distributed N-forms in the soil and the resulting effect on plant growth would therefore be useful.

In addition to the plant species, the type and composition of the soil is another major influencing factor. The soil used in the present studies is characterised by a relatively high cation exchange capacity. This means that a large proportion of NH_4^+ is adsorbed to the exchange sites (mainly clay minerals and organic matter). Although this NH_4^+ pool is to some extent in a dynamic

equilibrium with the concentration in the soil solution (Avnimelech and Laher, 1977; Wang and Alva, 2000), adsorption and desorption also depend on other factors, such as the concentration of other cations, especially divalent cations (Nommik and Vahtras, 1982; Nieder *et al.*, 2011). The relevance of sorption has been exemplarily demonstrated in some studies (Handa *et al.*, 1984; Wilcox *et al.*, 1985; Elamin and Wilcox, 1986). In order to address the question of how strongly the root reaction depends on the amount of NH_4^+ in the soil solution compared to the adsorbed NH_4^+ systematically, an experimental design can be used in which the exchange capacity of the soil is modified by a stepwise "dilution" with quartz sand (and silt etc.) and the respective reaction of the root growth is observed over time together with chemical analysis of soil solution.

7.3 Closing remark

This work has shown that it can be enlightening to re-examine familiar issues with new approaches and in the context of new circumstances and changing goals of a changing world. The development of new concepts can be laborious and setbacks are certainly not uncommon. And this is exactly where new discoveries can be made. Many methods, such as X-ray CT, have great potential, but also certain disadvantages. Technological improvements will help to make even better use of these methods. In the context of localised fertilisation and the related issue of nutrient use efficiency, further studies are needed to meet the demands of modern and environmentally sound agriculture in relation to a growing world population and changing climate. This work has only been able to cover a small part of this major topic and I hope that this work will be an inspiration for future research.

Appendix 1

Supporting information corresponding to the article

"How much is too much? - Influence of X-ray dose on root growth of faba bean (*Vicia faba*) and barley (*Hordeum vulgare*)"

Method: Image processing of CT data and root analysis for *Vicia faba*.

For *Vicia faba*, a detailed analysis of CT-images was conducted. Raw images were filtered with a 3D Gauss-filter with kernel size 5 to reduce image noise. The Gauss-filter was chosen, as it is fast, robust and sufficient enough for the high diameters of the *Vicia faba* roots. Root systems were segmented with dynamic semi-automated region growing in VG Studio Max 2.1. The segmented root volumes were exported as .RAW files from VG Studio and processed with Fiji (Schindelin *et al.*, 2012), a distribution of ImageJ (Schneider *et al.*, 2012). Root volumes from the top and bottom CT-images were merged (either with pairwise stitching or manually by canvas size, translate and concatenate), binarised and filtered (3D Median-filter kernel size 5) to smoothen the outer surface to prevent that surface roughness is detected as false branches in the next step. Based on the idea of Flavel *et al.* (2012), root volumes were skeletonized and analyzed with the Fiji plugin 'BoneJ' (Doube *et al.*, 2010). The resulting information about all detected 'branches' (in the following denoted as segments) was used to distinguish the tap root from first and second order laterals by the three-dimensional extension of every segment and their position in the 3D space. False segments were discarded, when either of the following criteria was met: (a) length < 25 voxel (= 1 mm), (b) overlay of the complete detected segments with the extension of the seed from *Vicia faba* (detected manually) or (c) ratio between geodesic length and Euclidian distance > 2. All queries and summations are performed in Excel.

Segments with maximum extension in direction of the z-axis are referred as taproot. The sum of all segments defined as tap root results in the total tap root length. This is true for the first three points in time 4 DAP, 8 DAP and 12 DAP, as only taproots and first order laterals (for 8 and 12 DAP) existed. Later on, this procedure would overestimate the length of the tap root, as most of the second order laterals were also oriented vertically. Hence, the corresponding coordinates in x- and y-direction were used as additional information for proper assignment. This is valuable, as it refers as a query to detect lateral segments having a connection with these tap root coordinates. These junction points enable the detection of the number of first order laterals emerging from the tap root. It is important to note that in the skeletonize algorithm in Fiji, every segment is defined from one junction to the next junction or to the end point of the

segment. Therefore, number of laterals would be overestimated, if every single segment with mainly horizontal expansion would have been counted as a single lateral root. For 16 DAP, the coordinates from 12 DAP were used and 100 voxels were added for the maximum extension and subtracted for the minimum extension of the tap root in both, x- and y-direction, respectively. This is necessary, as ImageJ works with relative coordinates, depending on the total spatial expansion of the stack. Therefore, coordinates are not exactly the same for 12 and 16 DAP. Because of the widening of the tap root coordinates, the detection of number of first order laterals is distorted for 16 DAP, as too many segments would be defined as having a junction to the tap root. Hence, number of first order laterals is not analyzed for 16 DAP. Moreover, no crucial further increase was detected visually after 12 DAP. Values for taproot extension at control treatment were selected manually within Fiji, as no information from earlier points in time exist. Second order laterals were detected as being vertically oriented, but not defined as tap root by comparison with the coordinates of the known tap root from 12 DAP. By this, some small fragments and some second order laterals are not captured, in case they were to short or oriented more horizontally, but still this approach leads to a very high level of detail and results are promising and plausible. Number of second order laterals can be verified by eye, because of the small and compact root system of *Vicia faba* and results match largely, with only few underestimations. In summary, the combination of root segmentation by a region growing algorithm with the analysis of root architecture by a skeleton analysis is not yet fully automatic, but results in a robust estimation of the morphological changes during root growth.

CT-images of segmented roots of the same plant at different time steps are not spatially aligned, since it is impossible to relocate the exact position and orientation of the columns at each scanning date with microscopic precision. To overlay all time steps in one time series (Figure 4.1, Figure A1.5, Video 1 and Video 2) we registered the images with the software elastix (Klein *et al.*, 2010) through an Euler transform (translation and rotation). An optimal registration was achieved by simultaneously maximizing the correlation coefficient between co-located voxels and minimizing the Euclidean distances between manually chosen corresponding points in both images. Convergences was accelerated with image pyramids, that allow for fast optimization at a coarse resolution and subsequent refinement at higher resolution. More information about the chosen parameters can be found in (Schlüter *et al.*, 2016).

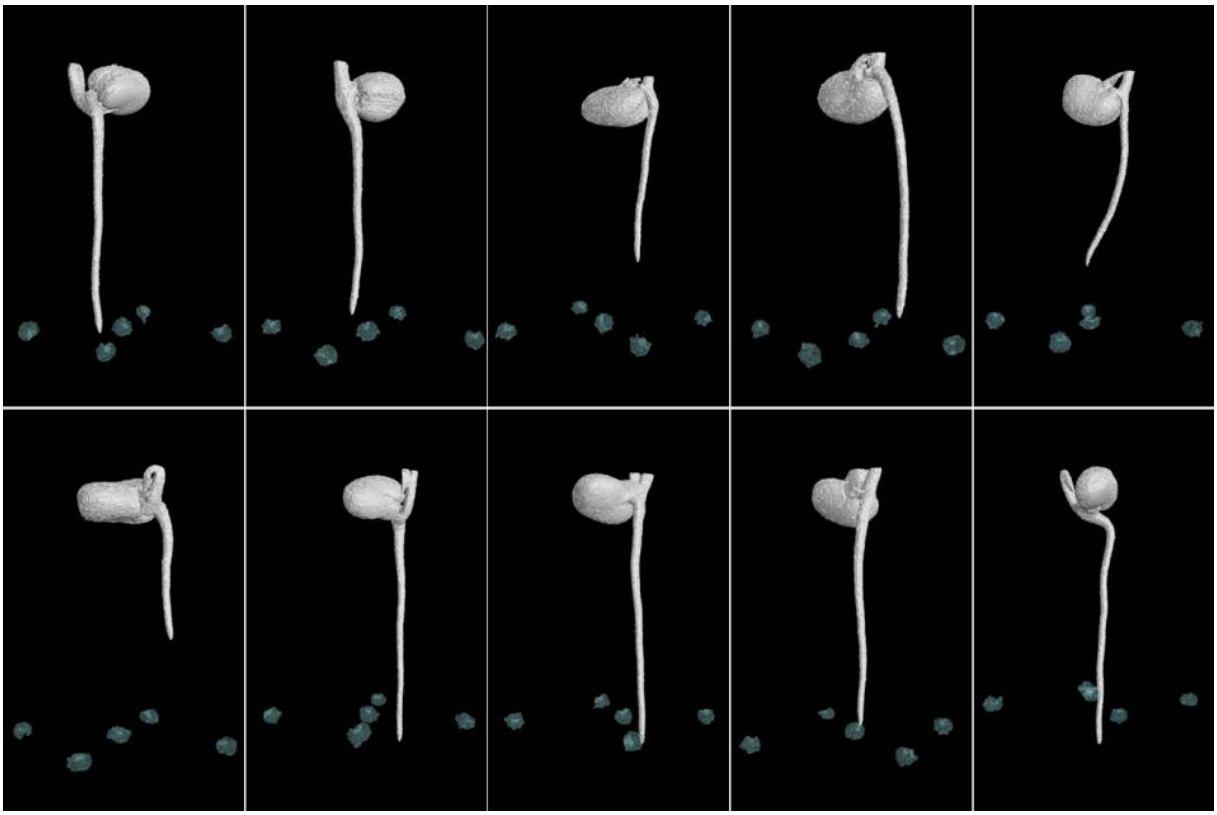

Figure A1.1: All root systems of *Vicia faba*, acquired by X-ray CT (representative 2D projections) at 4 DAP. Top row = frequent scanning (FS); bottom row = moderate scanning (MS). This was the first CT scan for both treatments.

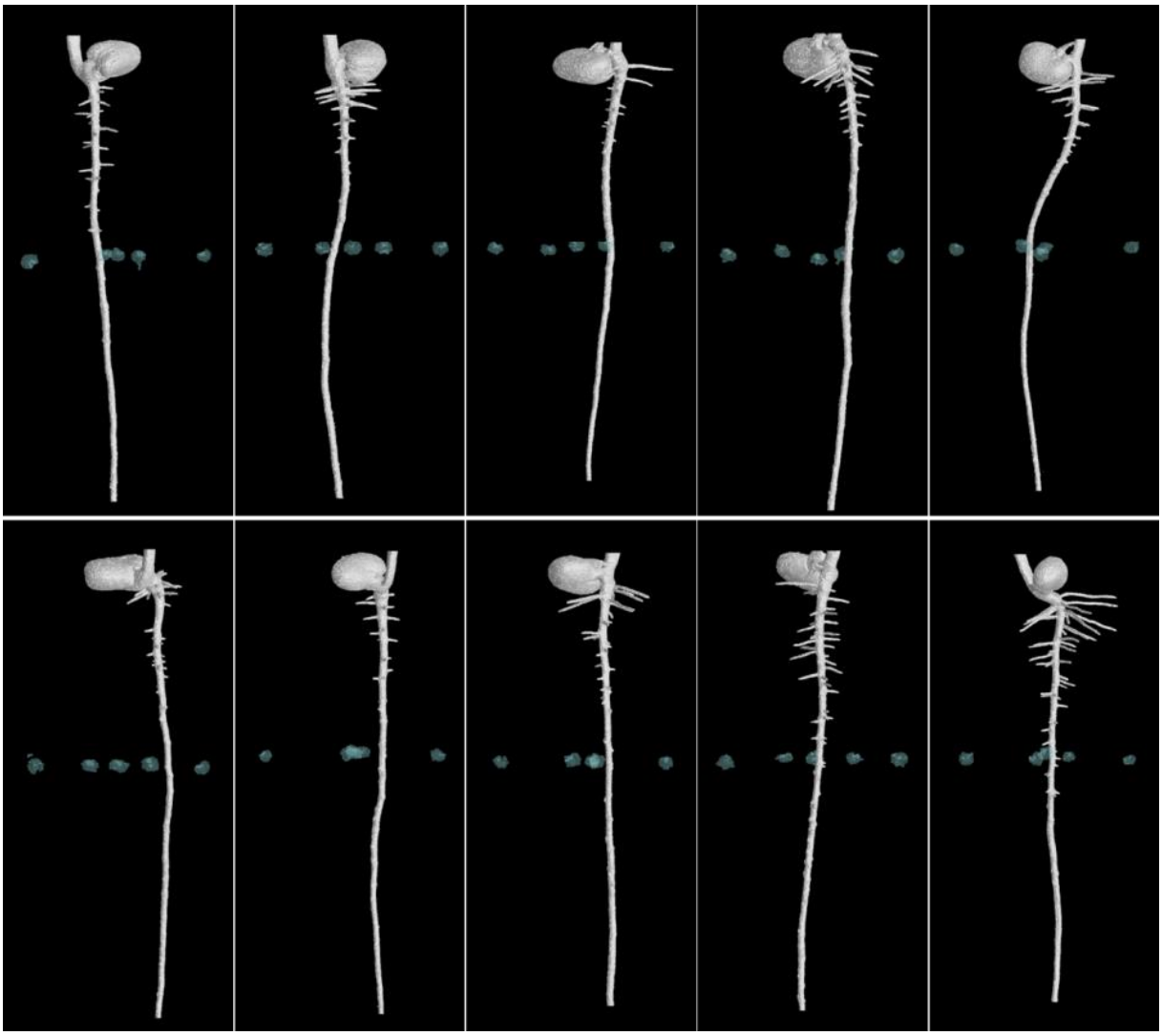

Figure A1.2: All root systems of *Vicia faba*, acquired by X-ray CT (representative 2D projections) at 8 DAP. Top row = frequent scanning (FS); bottom row = moderate scanning (MS). All tap roots have grown below the region of interest and first order lateral roots have emerged.

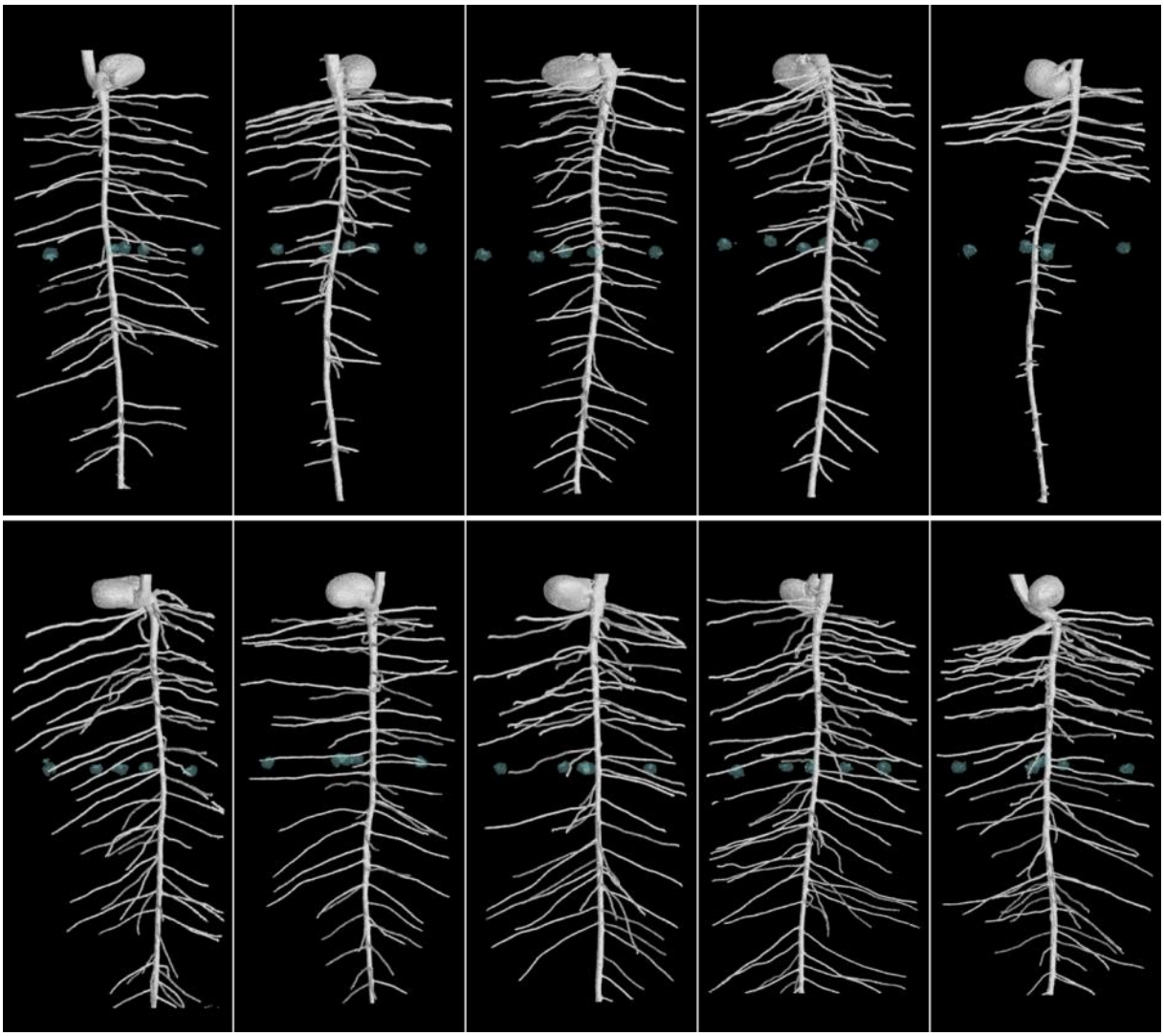

Figure A1.3: All root systems of *Vicia faba*, acquired by X-ray CT (representative 2D projections) at 12 DAP. Top row = frequent scanning (FS); bottom row = moderate scanning (MS). First order lateral roots have elongated differently for both treatments.

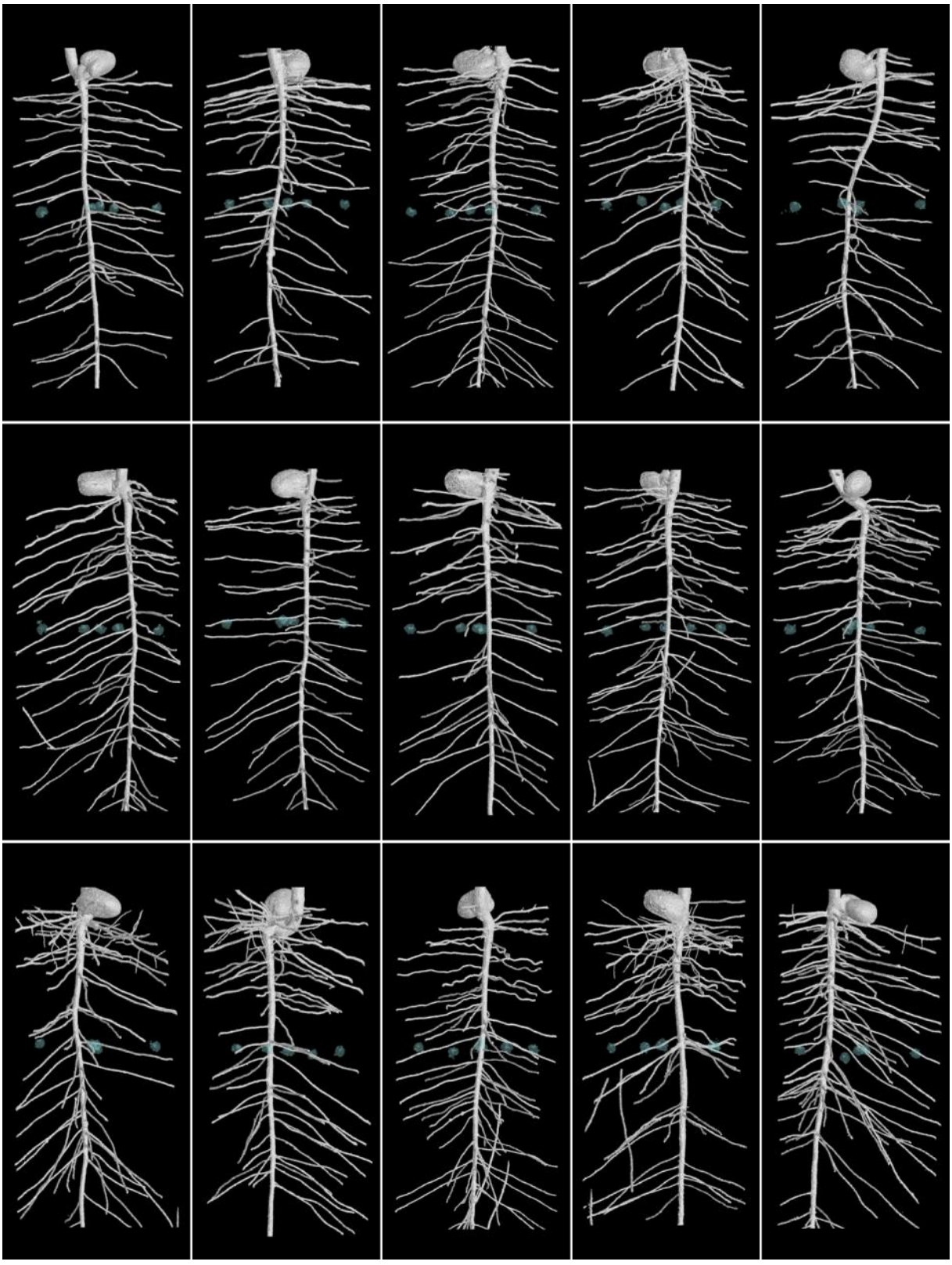

Figure A1.4: All root systems of *Vicia faba*, acquired by X-ray CT (representative 2D projections) at 16 DAP. Top row = frequent scanning (FS); middle row = moderate scanning (MS); bottom row = control (only this one scan was performed). Second order lateral roots are much more pronounced at the control treatment.

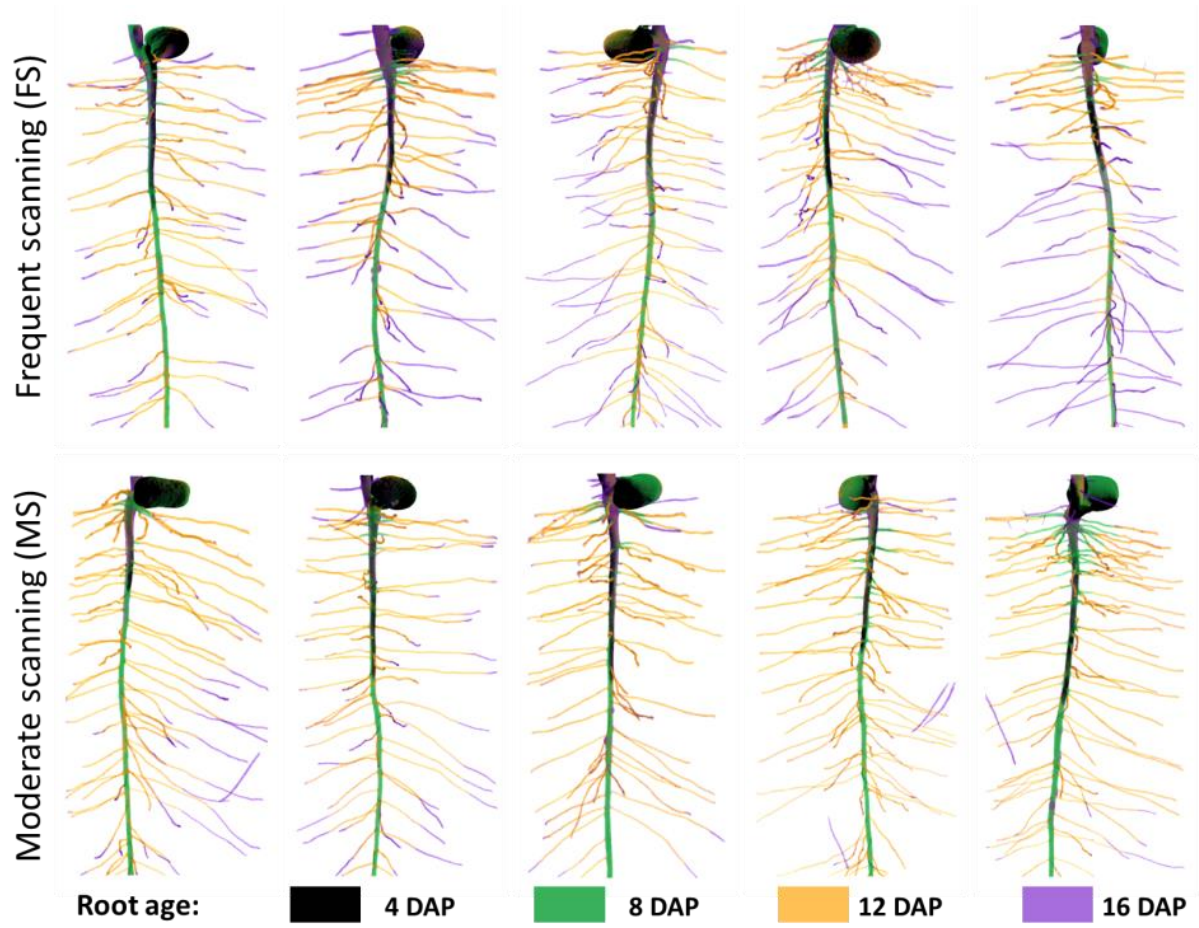

Figure A1.5: Time series of root system development of all *Vicia faba* replicates, acquired by X-ray CT. Top row = frequent scanning (FS); bottom row = moderate scanning (MS). Root age is colour coded for 4 (black), 8 (green), 12 (orange) and 16 (purple) days after planting (DAP). Changes in position are also recorded; this is the reason for the green shade at the seed in b). Secondary thickening can also be seen by the purple shade around the upper part of both tap roots.

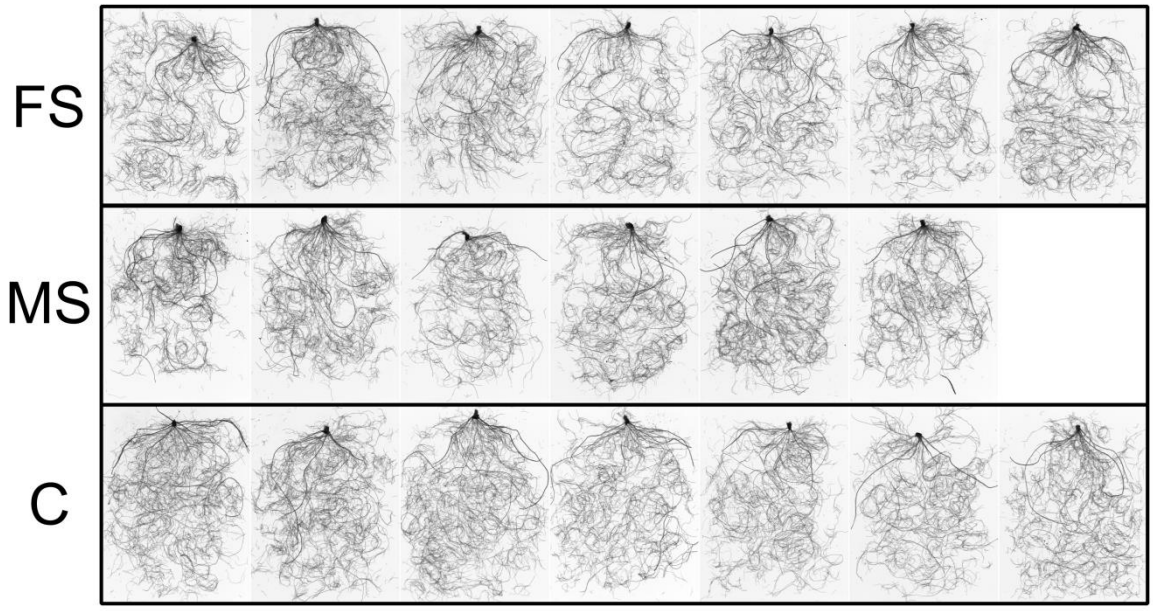

Figure A1.6: Washed-out root systems from *Hordeum vulgare* of all treatments at the end of the experiment (17 DAP). Top row = frequent scanning (FS); middle row = moderate scanning (MS); bottom row = control (C).

Additional material:

Video 1: Video of root grow of *Vicia faba* for one sample from the treatment frequent scanning (FS), acquired by X-ray CT. Root age is colour coded for 4 (black), 8 (green), 12 (orange) and 16 (purple) days after planting (DAP). Changes in position are also recorded; this is the reason for the green shade at the seed in b). Secondary thickening can also be seen by the purple shade around the upper part of both tap roots.

Link to video 1:

https://journals.plos.org/plosone/article/file?id=info%3Adoi/10.1371/journal.pone.0193669.s008&type=supplementary

Video 2: Video of root grow of *Vicia faba* for one sample from the treatment moderate scanning (MS), acquired by X-ray CT. Root age is colour coded for 4 (black), 8 (green), 12 (orange) and 16 (purple) days after planting (DAP). Changes in position are also recorded; this is the reason for the green shade at the seed in b). Secondary thickening can also be seen by the purple shade around the upper part of both tap roots.

Link to video 2:

https://journals.plos.org/plosone/article/file?id=info%3Adoi/10.1371/journal.pone.0193669.s008&type=supplementary

Appendix 2

Supporting information corresponding to the article

„When Drew meets soil - Dynamics of localised supply of nitrogen-species in soil and their relevance for root system morphology – A comparison between faba bean (*Vicia faba*) and barley (*Hordeum vulgare*)"

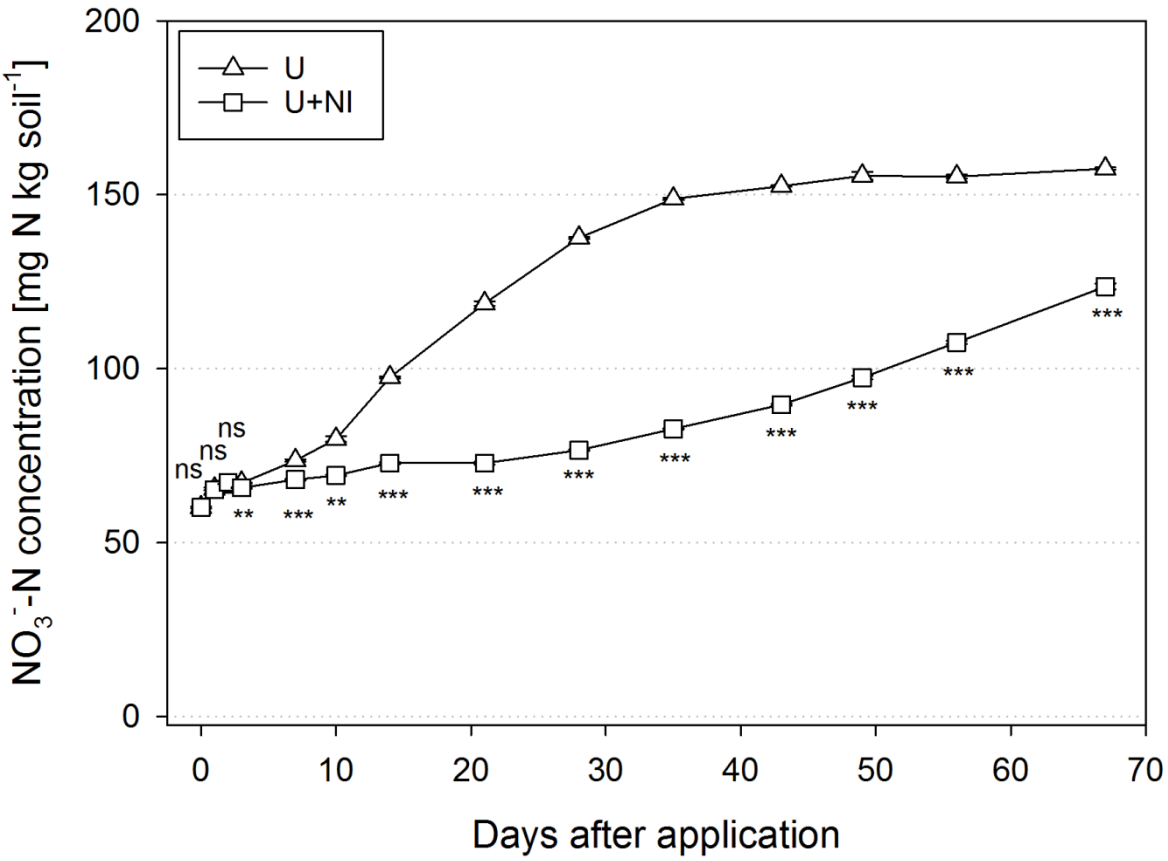

Figure A2.1: Temporal change of NO_3^--N concentration under standardised conditions in experiment 1 for treatments urea ("U", open triangle) and urea with nitrification inhibitor ("U+NI", open square) in soil extract (1 M KCl). Asterisk indicates significant difference between treatments at p<0.05 (*), p<0.01 (**) and p<0.001 (***), ns = not significant. Error bars are masked by treatment symbols in most cases.

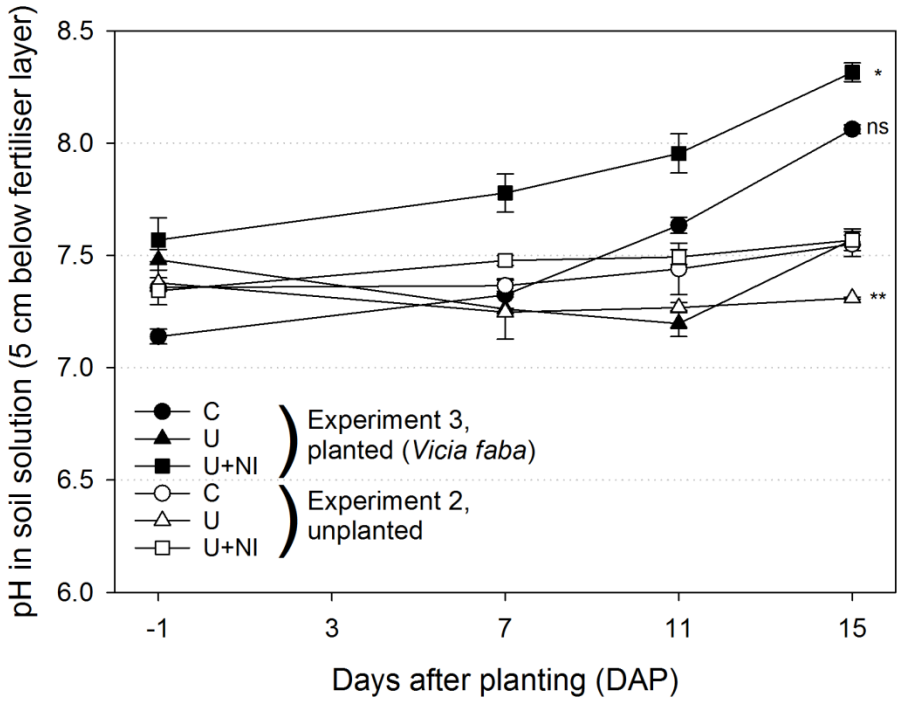

Figure A2.2: Change of soil solution pH with time 5 cm below the fertiliser layer in experiments 2 (open symbols) and 3 (closed symbols) with *Vicia faba*. First sampling point (-1 DAP) defines starting conditions for root growth in experiment 3. Asterisk indicates significant difference between planted and unplanted treatments at p<0.01 (**), ns = not significant. Error bars indicate standard error.

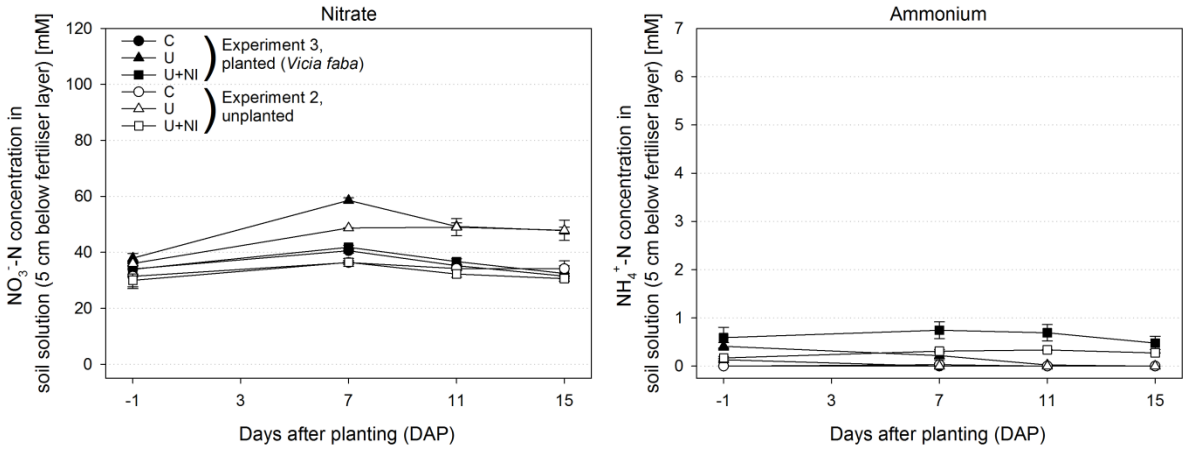

Figure A2.3: Temporal change of NO_3^- (left) and NH_4^+ (right) concentrations in soil solution 5 cm below the fertiliser layer from experiments 2 and 3 with *Vicia faba* after three weeks of incubation for treatments C (circles), U (triangles) and U+NI (squares). Filled symbols indicate plant presence (experiment 3), open symbols indicate plant absence (experiment 2). Starting point is one day before plants were introduced in experiment 3.

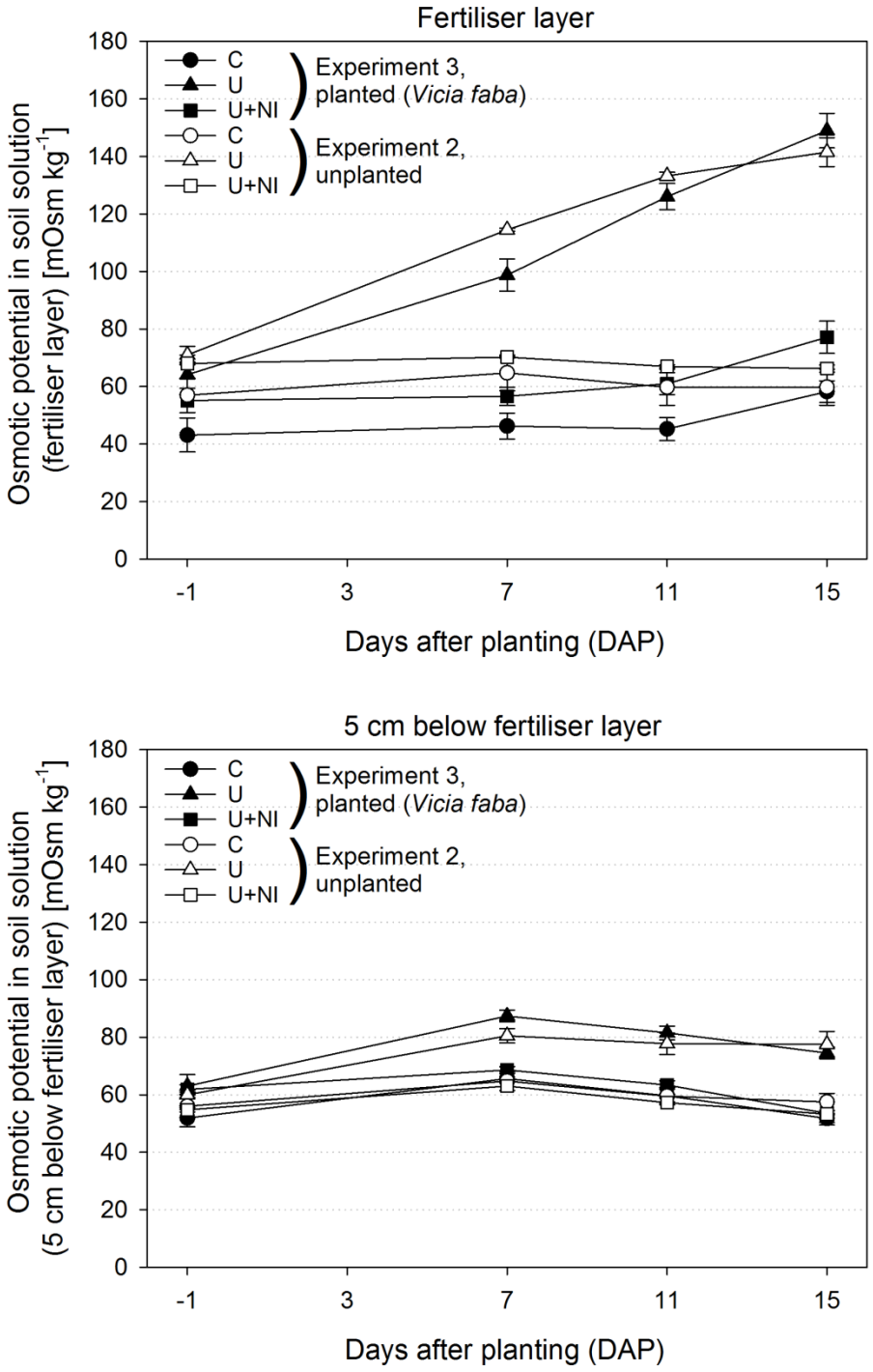

Figure A2.4: Temporal change of osmotic potential in soil solution in both layers from experiments 2 and 3 with *Vicia faba* after three weeks of incubation for treatments C (circles), U (triangles) and U+NI (squares). Filled symbols indicate plant presence (experiment 3), open symbols indicate plant absence (experiment 2). Starting point is one day before plants were introduced in experiment 3.

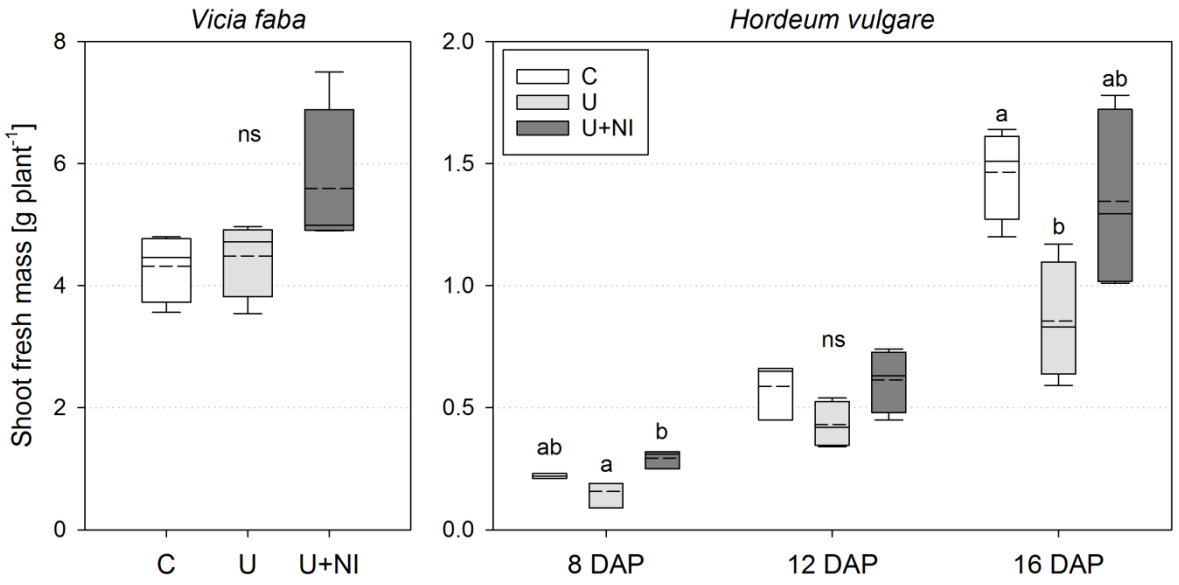

Figure A2.5: Shoot fresh mass for *Vicia faba* (left) after 16 days and for *Hordeum vulgare* (right) over time. Significant differences (p<0.05) between treatments are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

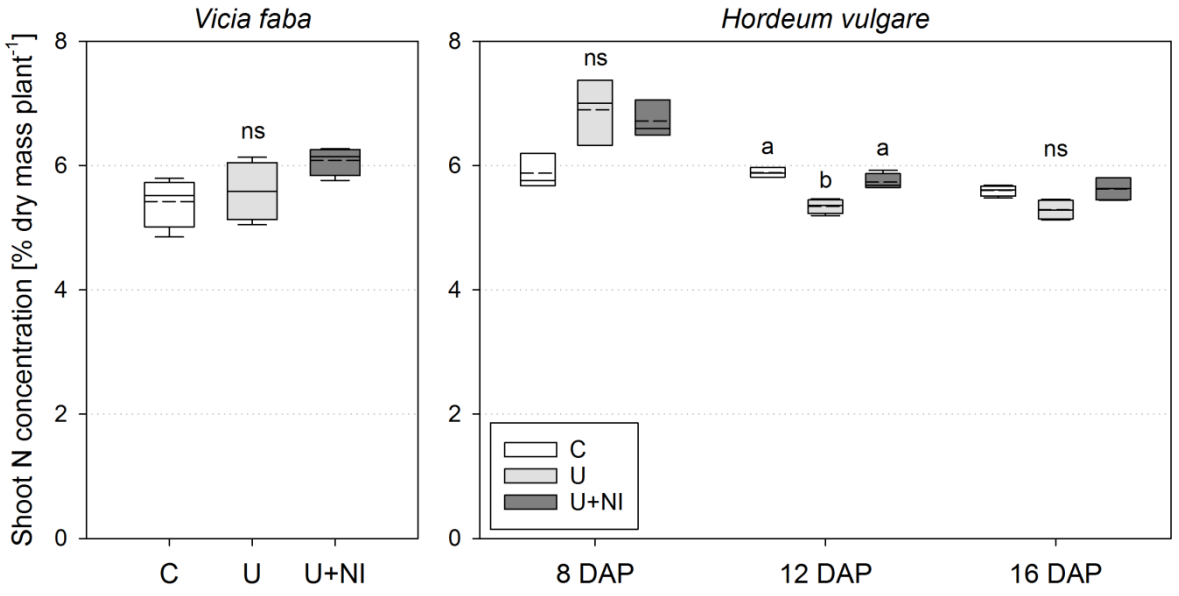

Figure A2.6: Shoot N concentration for *Vicia faba* (left) and *Hordeum vulgare* (right) in percent per dry mass per plant. Significant differences between treatments (p<0.05) are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

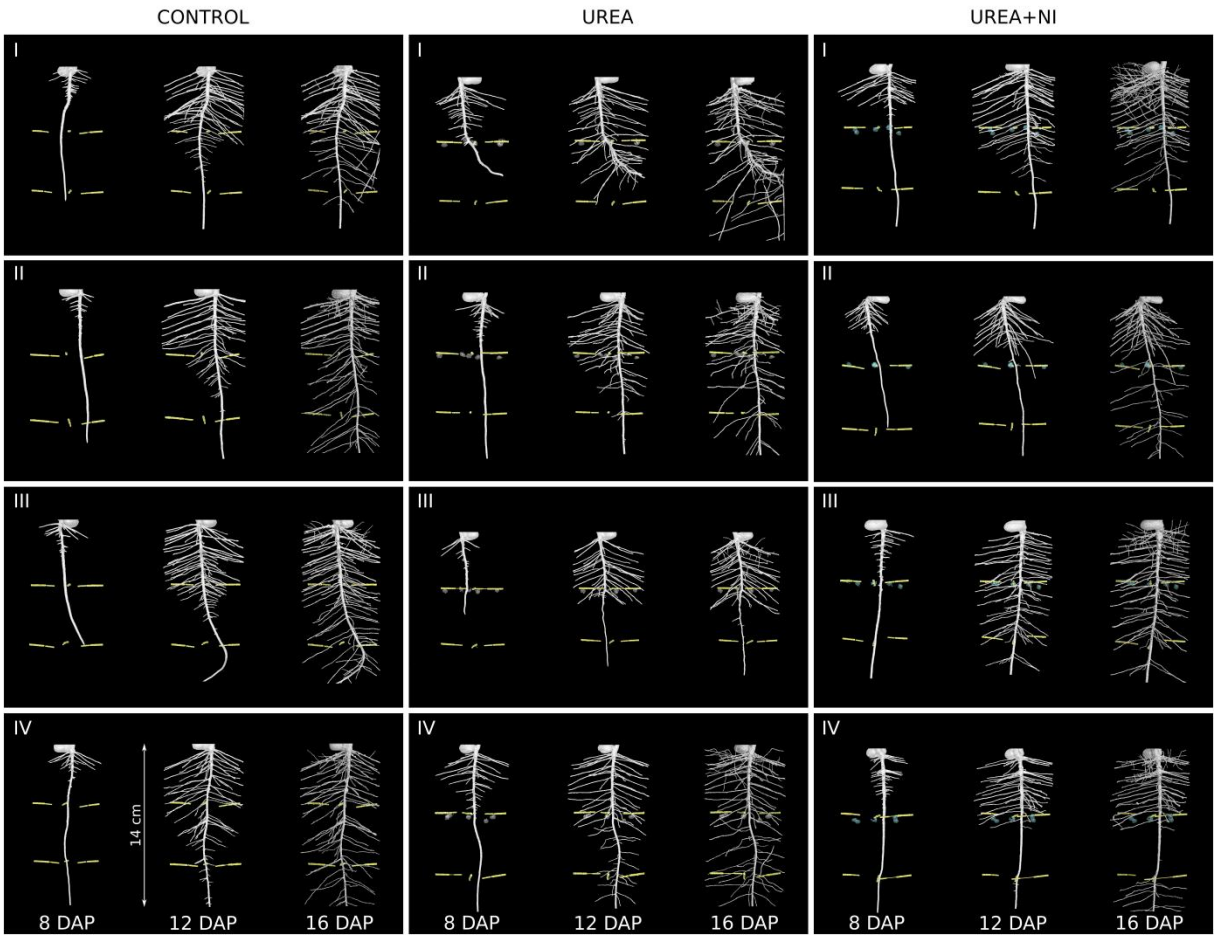

Figure A2.7: All steps of root growth development of *Vicia faba*, acquired by X-ray CT. Shown are all replicates (I-IV) for all treatments over time (8, 12 and 16 DAP). Micro suction cups are shown in yellow, fertilizer granules in grey (U) or turquoise (U+NI).

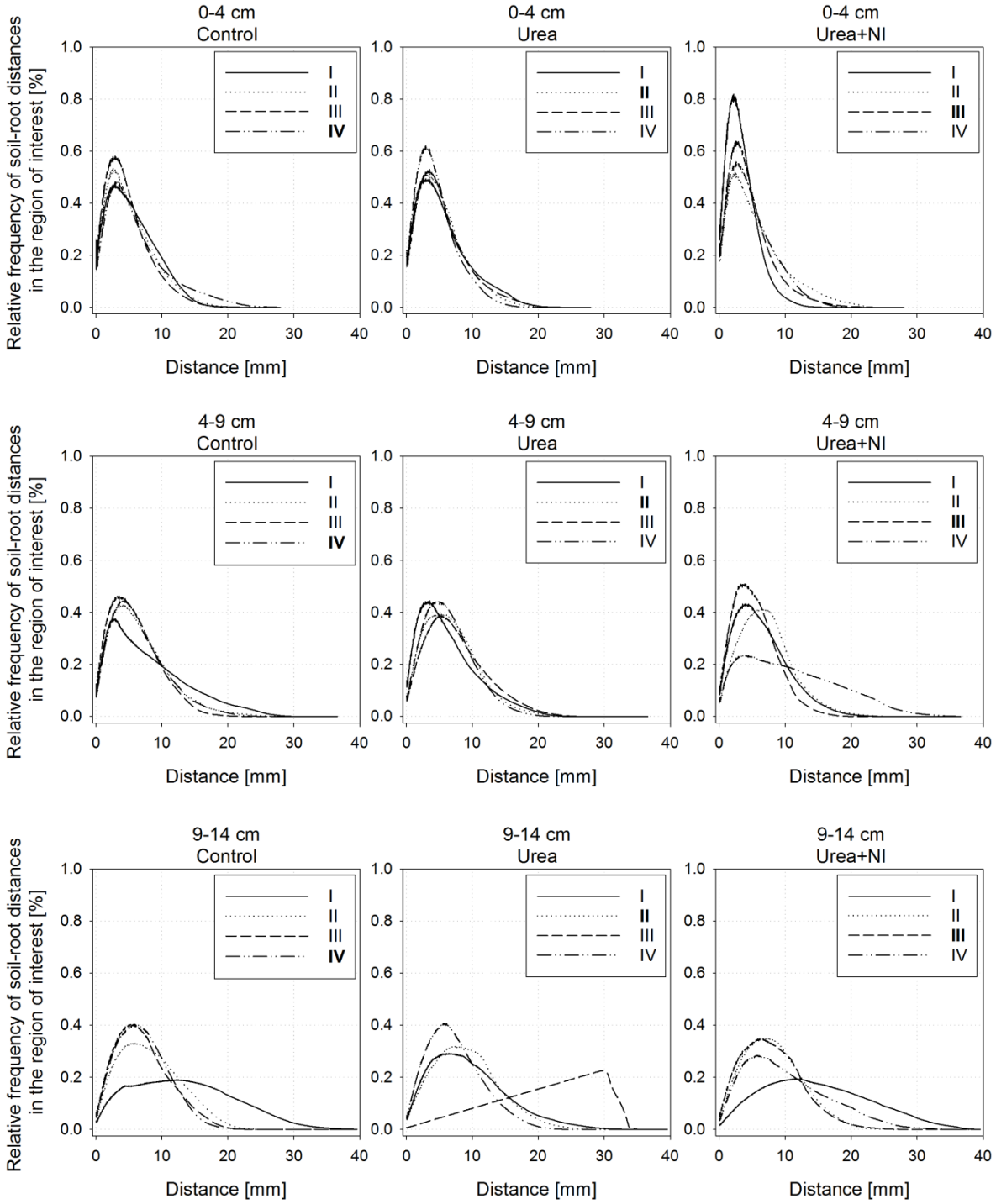

Figure A2.8: All relative frequencies [% of all soil voxels in the respective layer] of soil-root distances at 16 DAP for *Vicia faba*, separated in 3 layers, representing the fertiliser layer and both parts above and below the fertiliser layer. Replicate labelling refers to replicate numbering in Figure S6. Bold printed labels refer to the replicates closest to the treatment mean for root parameters selected for the presentation in Fig. 7.

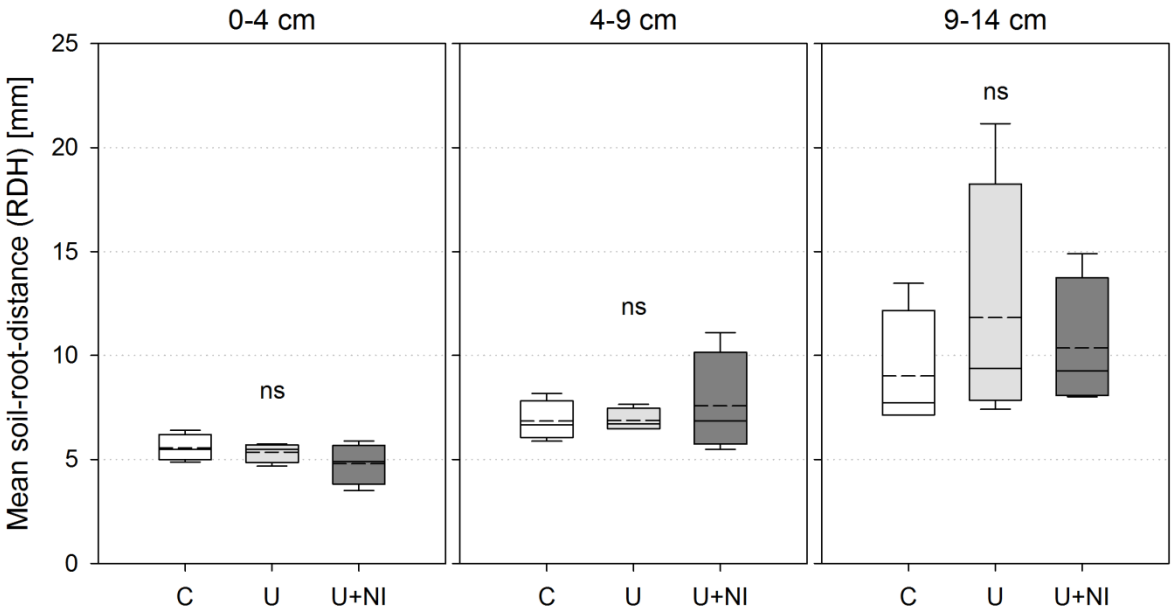

Figure A2.9: Mean soil-root-distances (RDH) at 16 DAP for *Vicia faba*, separated in 3 layers, representing the fertiliser layer and both parts above and below the fertiliser layer. Significant differences ($p<0.05$) between treatments are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

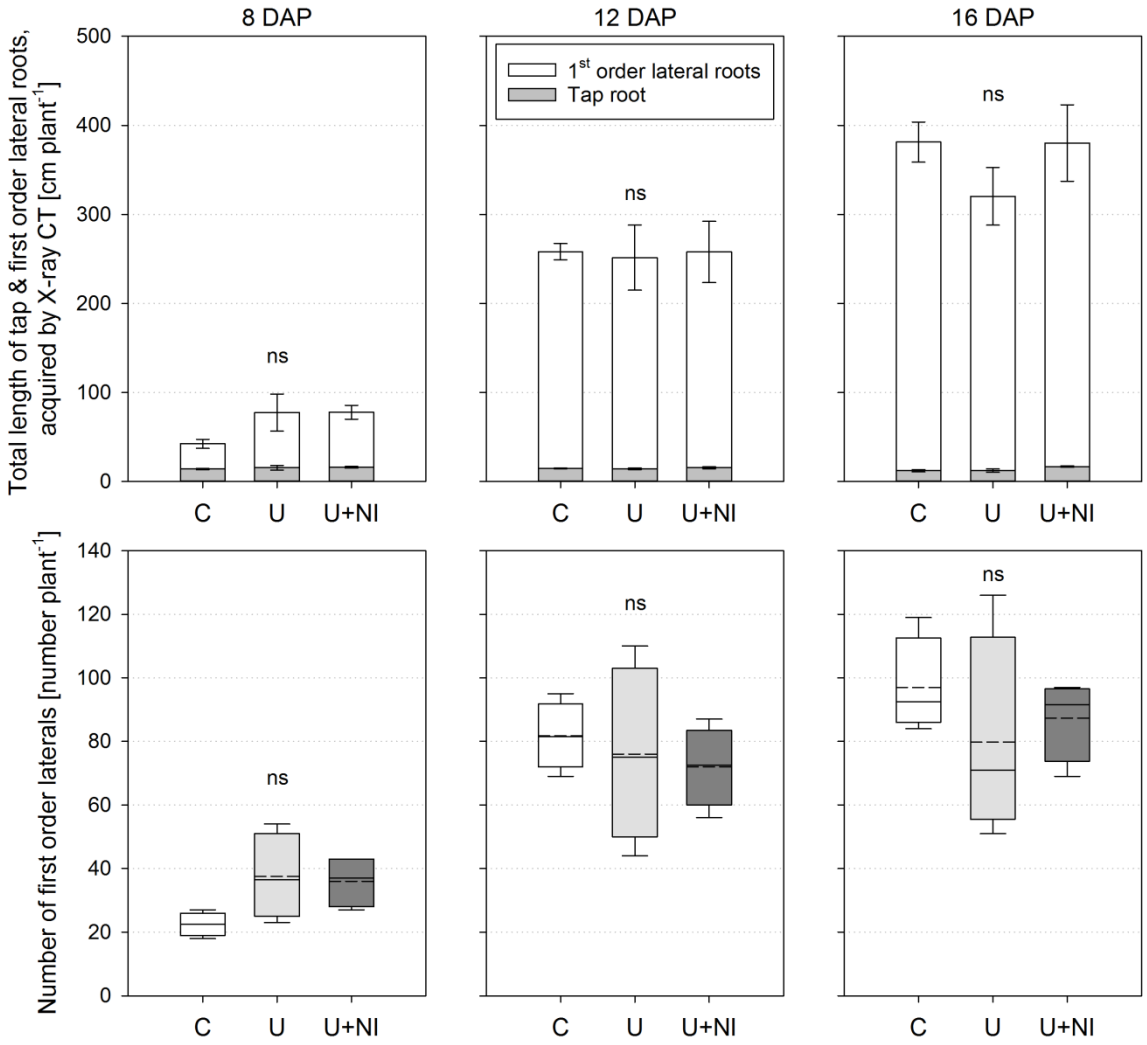

Figure A2.10: Root growth development of *Vicia faba* plants over time, acquired by X-ray CT. Upper row: white bars represent length of first order lateral roots per plant and grey bars represent length of tap root per plant. Bottom row: number of first order laterals per plant. Significant differences ($p<0.05$) between treatments are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

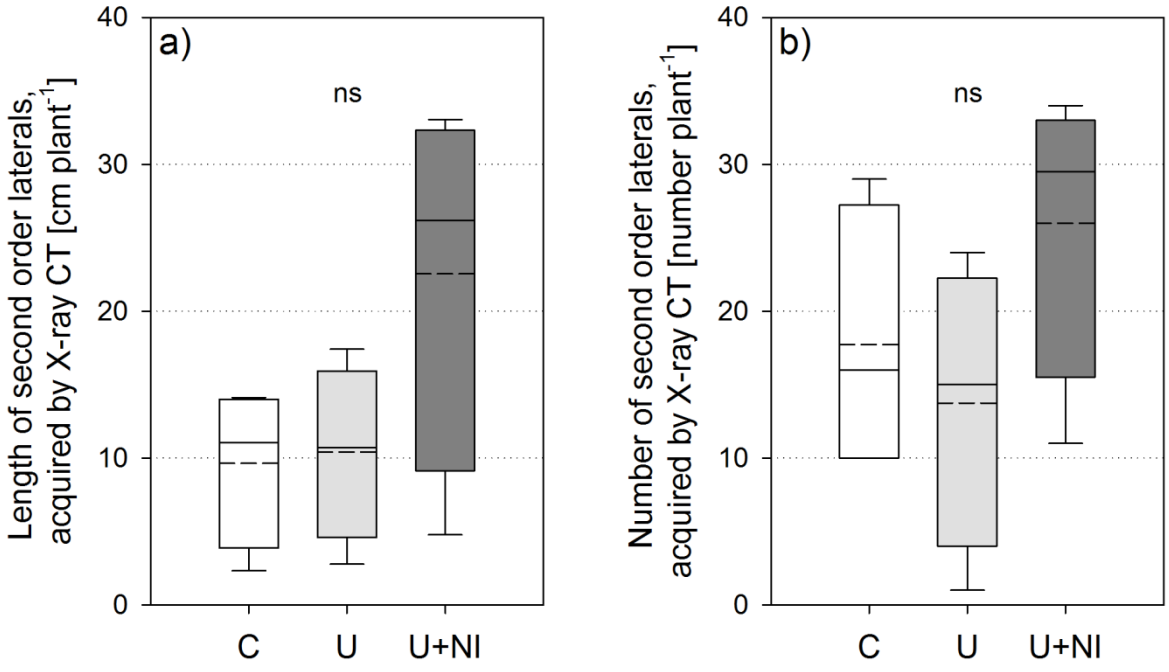

Figure A2.11: a) Length and b) number of second order lateral roots per plant of *Vicia faba* after 16 days of growth, acquired by X-ray CT. Significant differences ($p<0.05$) between treatments are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

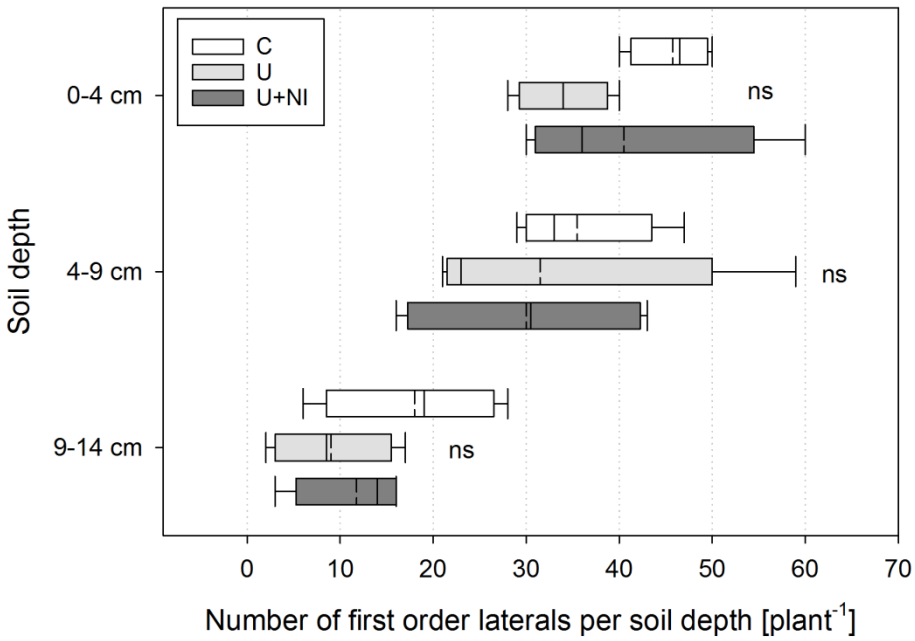

Figure A2.12: Depth distribution of number of first order laterals from *Vicia faba* after 16 days, acquired from X-ray CT data. Significant differences ($p<0.05$) between treatments are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

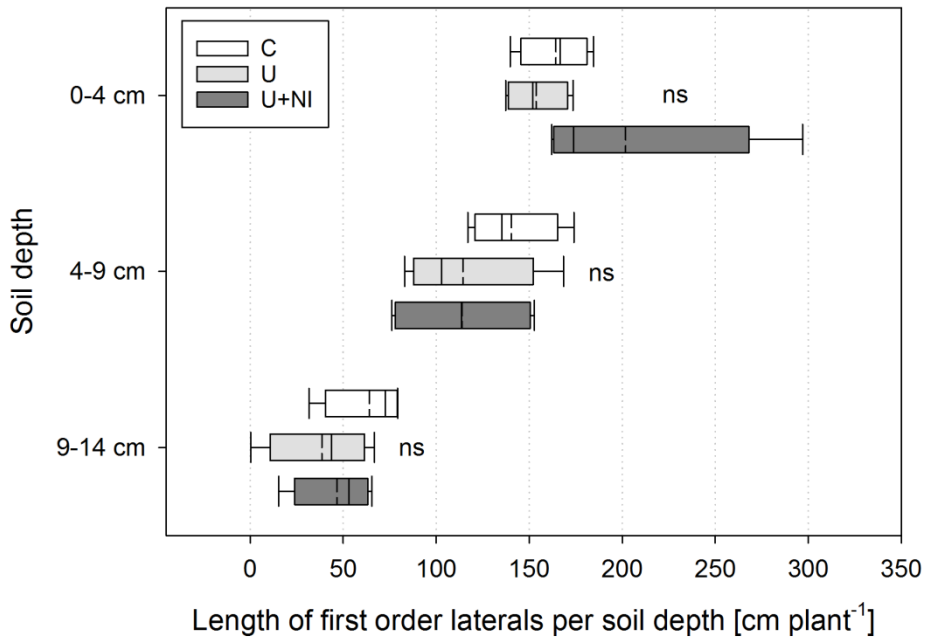

Figure A2.13: Depth distribution of first order lateral root length from *Vicia faba* after 16 days, acquired from X-ray CT data. Significant differences (p<0.05) between treatments are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

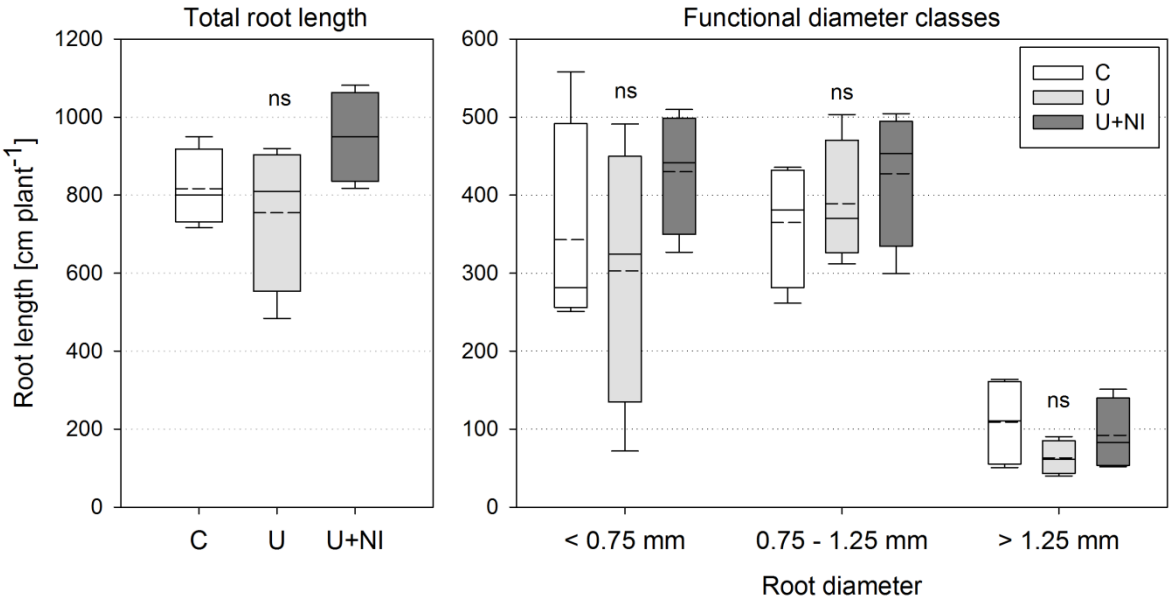

Figure A2.14: Total root length and root length per functional diameter classes (referring to root orders: second order laterals, first order laterals and tap root) for *Vicia faba* after 16 days acquired by WinRHIZO. Significant differences (p<0.05) between treatments are indicated by different letters, ns = not significant; dashed lines in the boxplots represent mean values while solid lines represent the median.

Figure A2.15: Example of root growth inhibition by high NO_3^- in barley. Scanned roots in the fertiliser layer in the treatment U, 16 days after planting.

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Eidesstattliche Erklärung / Declaration under oath

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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Ich widme diese Arbeit
Agnes, Anton, Marianne und Georg

In nomine Patris, et Filii, et Spiritus Sancti. Amen.

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