Improving agricultural nutrient use efficiencies: effects of crop rotation, high carbon amendments, and fertilizer application timing on barley

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Summary

Excessive fertilizer use leads to nutrient imbalances and losses of these to the environment through leaching, runoff and gaseous emissions. Nutrient use efficiency (NUE) in agriculture is often low and improving it could increase the sustainability of agricultural systems. The main aims of this thesis were to gain a better understanding of plant-soil-microbe interactions in order to improve agricultural NUEs. The studies included experimentally tested how crops respond to addition of high carbon amendments, fertilizer application rates and timing, and crop rotations. Furthermore, methods for measurement of roots were compared and a protocol for measurement of roots was developed.

The first experiment simulated an agricultural field using mesocosms. In this setting, we tested the effect of 4 previous crops (precrops), which either had or did not have a symbiosis with arbuscular mycorrhizal fungi (AMF)/rhizobia, on the focal crop (winter barley). We also tested the addition of high carbon amendments (wheat straw/sawdust) for immobilization of residual soil nitrogen (N) at harvest of the previous crop. Overall, the findings were that non-AMF precrops had a positive effect on winter barley yield compared to AMF precrops. Wheat straw reduced N leaching, whereas sawdust addition had a negative effect on the yield of winter barley.

Root traits are often measured in static environments, whereas agricultural fertilizer is applied once or multiple times at different crops growth stages. The second experiment tested the effect of different fertilizer (N/phosphorus (P)) application timings on plant traits grown in rhizoboxes. Overall, delaying N application had a more detrimental effect on plant biomass than delaying P application. The root system increased its root length initially due to N-deficiency, but was quickly thus N-limited that root length was relatively lower than the control group. This study emphasizes the need to dynamically measure roots for a mechanistic understanding of root responses to nutrients.

Because of the many root related measurements in the second experiment, a step-by-step method for measuring root traits under controlled and field conditions was developed and included in this thesis. This method paper describes precisely how root traits of interest can be measured, and helps with deciding which approach should be taken depending on the experimental design. Additionally, we compared the bias and accuracy of several popular root measurement methods. Although methods well correlated with a reference method, most methods tended to underestimate the total root length.

Overall, these results highlight the importance of crop choice in crop rotations and the plasticity of root systems in relation to nutrient application. Our results show high carbon amendments could reduce nitrate leaching after the harvest of crops, especially those with high risk of nitrate leaching, although they had only small impacts on yield. Future research should investigate the applicability in a farm setting, also taking into account financial and practical aspects. Non-AMF precrops could possibly increase yield of the next crop due to a shift to parasitism in agricultural fields, but whether this plays a large role in crop yields should be further investigated for specific soil, crop, and climate conditions. Our results also show the plasticity of root systems in response to nutrients. Understanding and using this plasticity can be useful for improving NUEs by optimizing fertilizer application and selecting root traits that are beneficial for specific environmental conditions.

1. General introduction

1.1 Sustainable agriculture

The green revolution, starting roughly in the 1950s, led to a remarkable increase in global food production. It consisted of two main innovations, namely breeding for new and improved cultivars with an increased yield and better disease resistance, and cultivation practices, such as irrigation and use of pesticides and synthetic fertilizers (Evenson, 2003). This type of intensive agriculture, however, did not come without costs to the environment: The fertilizers and pesticides necessary to maintain the high productivity can be lost to the environment and into natural systems. There they may cause imbalances in ecosystem functioning by altering the abiotic and biotic components of the ecosystem, leading to eutrophication and biodiversity loss (Diaz and Rosenberg, 2008; De Schrijver et al., 2011). For instance, the use of pesticides, such as neonicotinoids, has been more contested recently, as their impact stretches far beyond the targeted pests to (natural) insect communities (Hladik et al., 2018).

Generally speaking, the more fertilizer is applied, the lower the increase in yield becomes, i.e. it tapers down due to other limiting factors (Lassaletta et al., 2014). In extreme cases, the yield might even be reduced at high fertilization due to toxicity levels. Hence, the nutrient use efficiency (NUE; although multiple definitions can be further specified, here I refer to it as the ratio of nutrient input in the form of fertilizer, and output in harvested product) of crops inherently decreases with an increase in fertilizer application. This also holds true for the global budget of nitrogen (N) application and N use efficiency over the last decades (Tilman et al., 2002). Following this reasoning, a low NUE inevitably leads to either losses out of the agricultural system, and/or accumulation within the system. In this regard, the behavior of the specific element in the soil plays a crucial role. For example, N is easily lost as nitrate in leachate or as NO_x in gaseous emissions, whereas phosphorus (P) is more easily fixed in the soil (accumulation) or lost with subsurface runoff. Improving NUE has long been spoken of to be a crucial improvement to the sustainability of current intensive agricultural systems (Van Noord wijk, 1999; Spiertz, 2010). **This thesis focuses on N and P, because they 1) represent the two most common macronutrients limiting plant production in agricultural ecosystems and therefore externally applied as fertilizers and 2) show contrasting behavior in the plant-soil environment.**

1.2 Management options for increasing nutrient use efficiencies

Overall, the NUE in agricultural systems is low. Worldwide, more than half of the N applied is not recovered in the harvested product (Lassaletta et al., 2014). The efficiency of P fertilization is strongly determined by the soil properties. Fertilization of agricultural soils poor in P content and moderately to highly P-sorbing is often inefficient due to P accumulation in the soils (Simpson et al., 2011). On low P-sorbing, P-saturated soils, surface run-off and leaching is the main reason for low P use efficiency (Ulén et al., 2007). However, how to improve NUEs is a complex, multifaceted question. Improvements can be made at different levels, for example at the crop level with more efficient crop varieties adapted to low nutrient conditions (Hirel et al., 2011; Veneklaas et al., 2012), but also at a system wide level, for example better synchrony in N supply and crop demand (Cassman et al., 2002) and the use of catch crops to capture N with high risk of leaching (Abdalla et al., 2019)**.** Knowledge of these systems can result in novel agricultural management options that can be transferred into policies and subsequently be implemented on a large scale. For example, policy changes in allowed fertilizer application time and amount reduced nitrate leaching significantly in Denmark (Kronvang et al., 2008). In the Netherlands it is now mandatory to grow a catch crop after maize cultivation on sand and loess soils, as this crop/soil combination has been shown to be most vulnerable to leaching of nitrate surpluses to the groundwater (Fraters et al., 2015).

Management options can also potentially affect soil fertility and the nutrient buffer capacity of the soil (Ogle et al., 2005). Soils subject to intensive agriculture are often low in (organic) carbon, because of regular soil disturbance, crop harvests, and lack of organic inputs (Loveland and Webb, 2003). Therefore, the microbial mediated processes which are central in nutrient (im-)mobilization are often hampered (Kumar et al., 2017). The use of (organic) amendments to the soil to counter on one hand low organic carbon levels, and low microbial biomass on the other hand, has been of increasing interest (Kallenbach and Grandy, 2011). Biochar has especially been the subject of much research due to its promising capacities to improve soil quality, stimulation of microbial life and improve carbon sequestrations (Lehmann et al., 2011; Singh et al., 2015; Kuppusamy et al., 2016). However, the unknown long-term effects and cost-effectivity, and availability for commercial use, are hampering widespread use so far (Maroušek et al., 2017). Other more easily available amendments could also prove useful in improving soil quality and reducing agricultural environmental pressures. **This thesis tests the applicability and effectiveness of high carbon amendments to reduce nitrate leaching in agricultural ecosystems.** To this end, we used wheat straw and spruce sawdust as easily available high carbon amendments (HCAs). Wheat straw has a high C/N ratio (~70) and is already available on farm, whereas sawdust has a very high C/N

ratio (~500) but would have to be bought as an external input. Moreover, these HCAs could be used when winter crops are grown, and other nitrate-reducing measurements such as cover crops, are not possible.

1.3 Plant-soil-microbe interaction in cropping systems

Soils contain an immense amount of, and diversity in, microbial life (Roesch et al., 2007). However, an even higher number of microbes occur in the narrow area around the roots (called the 'rhizosphere'). The microbial communities in the rhizosphere differ strongly from that of bulk soil and acquire a part of their energy demand from plant rhizodeposits (including root exudates) (Bakker et al., 2012). Moreover, it is the area where tight co-evolved symbioses between plants and microbes take place. The most common one is a symbiosis with arbuscular mycorrhizal fungi (AMF). These fungi take part in bilateral exchange with plants providing nutrients and water in exchange of photoassimilates (Smith and Read, 2010). Another example of intimate symbiosis is between plant roots and rhizobia. Rhizobia infect the roots and force the plant to create structures ('nodules'), in which they fix atmospheric N for the plant to use, while receiving organic acids from the plants (Udvardi and Poole, 2013). Although this symbiosis is limited mostly to plants of the family *Fabaceae*, nearly all plants in this family have the ability to form this symbiosis with some exceptions, such as lupines.

Root AMF colonization is widely regarded as a mutualistic symbiosis of benefit to both the plant and the fungi (Smith and Read, 2010). Yet, their function in intensive agriculture remains unclear and has been the topic of recent debate (Smith and Smith, 2011; Rillig et al., 2019; Ryan et al., 2019). Several meta-analyses have attempted to disentangle the effect of AMF performance on crop yields (Lekberg and Koide, 2005; Zhang et al., 2019). Lekberg and Koide (2005) found an overall increase in yield if soils were additionally inoculated with AMF. However, this effect was less strong if overall soil P was high or indigenous AMF levels were high already. One of the difficulties in measuring the overall effect of AMF in agricultural systems is the multitude of interactions between AMF and management practices and specific crops. For example, high soil P availability (due to fertilizer addition) or deep tillage, both reduce AMF performance (Mäder et al., 2000; Jansa et al., 2002). Because AMF are obligate symbionts, they are dependent on the crop that is currently cultivated for their energy, survival and reproduction (Harrison, 2005). However, not all crops have an AMF symbiosis, most notably plants in the family *Brassicaceae*, and also to a degree in the family *Amaranthaceae*. Interestingly, the effect of non-mycorrhizal crops on the next mycorrhizal crop in crop rotations has received little attention so far. For example, only 3 out of 34 articles in the meta-analysis of Lekberg and Koide (2005) tested this exact effect.

Legumes play an important role in agriculture, because their symbiosis with rhizobia allow them to fix atmospheric N, thus requiring less to no N fertilization while simultaneously bringing N into the system (Herridge et al. (2008) estimated that around 20-22 Tg N is fixed annually by legume crops). However, the role of legumes in cropping systems varies widely, which makes estimates difficulties. Legumes are used as main crops (dry seed production), pulses (green seed production), and forage crops, but also as catch crops (to 'catch' leftover N and improve soil conditions) and cover crops (to prevent soil erosion, weed growth, and improve soil quality (Stagnari et al., 2017; Abdalla et al., 2019). The next crop often profits from the relatively high N contents in plant residues that remain in the field (Chalk, 1998). But the benefit of the next crop is not exclusive to the extra N in the system, as multiple studies show other potential effect of (species-specific) legume benefits to the next crop (Stevenson and Van Kessel, 1996; Danga et al., 2009; Golding and Dong, 2010). Despite all these benefits, legume use in Europe declined over the past decades (Zander et al., 2016). An increase of legume use in crop rotations could thus contribute to a more sustainable agriculture.

Although the effect of legumes and AMF crops has been subject to study in many agricultural field studies, disentangling cause and effect remains difficult. In the case of AMF, it can be difficult to find a direct link between certain AMF parameters, such as root colonization or spore count, and yield parameters (Ryan and Graham, 2018; Zhang et al., 2019). For one, this might be due to the context of which these are measured. High soil P, for example, can reduce or obscure any beneficial effect AMF colonization might give, especially in domesticated plants (Smith and Smith, 2011; Martín-Robles et al., 2018). Legumes can improve the soil fertility, and especially do so in a low N context. Similar to the benefit of AMF colonization, the N benefit of legumes can be obscured by certain management practices such as high N fertilizer rates or excessive tillage (Peoples et al., 2009). Moreover, the use of legumes can also come with potential environmental costs; High N legume residues, if left on the field, can result in increased N leaching after harvest (Plaza-Bonilla et al., 2015). **This thesis tests the effect of these symbiotic plant-microbe interactions in crop rotations on crop yields and nitrate leaching.**

1.4 Plant roots

Plant roots are essential for their role in providing anchorage and taking up water and nutrients from the soil. Yet, they have been overlooked long in agricultural use, for the focus of breeding efforts was on aboveground yield traits (Aziz et al., 2017). Also in the field of ecology, roots have not received as much attention as aboveground plant parts as trait-based approaches became popular (Bardgett et al., 2014).

However, there has been an increased interest in the scientific community, both ecological and agriculturally related fields, in the root system and its diverse functional components (Lynch and Brown, 2012; Bardgett et al., 2014; Paez-Garcia et al., 2015). Although the aim of each discipline might differ, there is quite some overlap in interest in the response of roots to their external environment. One of these interests is how plant roots respond to differences in external nutrient conditions. These are required both to better understand ecosystem functioning, nutrient cycling, drought resistance and plant competition in both natural and agricultural systems but also how to optimize plant production and plant adaptations to low nutrient conditions in agricultural systems specifically.

The response of roots to external nutrient concentrations was first described in detail by Drew (1975). In an experimental setup where only part of the root system was supplied with high concentrations of nutrients, compared to low concentrations elsewhere, the roots proliferated tremendously in these high concentration sections. This also led to the finding that root responses are not the same for all nutrients. Gruber et al. (2013) recently quantified responses for a multitude of nutrients for the model plant *Arabidopsis thaliana*. However, these experiments were performed in fairly artificial setups, whereas roots usually grow in heterogeneous soils and need to forage for these nutrients. Experiments similar to those of Drew (1975) have been conducted in more complex, realistic settings since then. For example, the influence of micro-organism competition in organic nutrient patches (Hodge et al., 2000), the effect of local P and ammonia application on maize root growth and yield (Ma et al., 2013), or the result of local digestate depot placement on the root system and yield of *Sida hermaphrodita* (Nabel et al., 2018), as well as root responses to nutrient stoichiometry (Kumar et al., 2020).

The aforementioned proliferation responses are root responses to high, localized nutrient concentrations, whereas reverse responses can also be true. Those are an increase in root investments when faced with overall low nutrient conditions. Hence, this is rather a plant level response as opposed to local proliferation. For N and P, generally two contrasting response are found. N, most commonly found as nitrate in temperate cropping systems, is more soluble in water and thus has a higher depletion zone around the root and leaches more easily to deeper soil layers (Di and Cameron, 2002). Hence, it is favorable for a plant to explore deeper soil layers to 'catch' nitrogen (Trachsel et al., 2013). This in contrast to P, that has low diffusion rates, is more susceptible to adsorption to soil minerals, and is thus mostly found in upper soil layers (Shen et al., 2011). Because of this immobility, adaptations have evolved to access this P fraction, such as root hairs, cluster roots, mycorrhizal association, or release of exudates to mobilize P (Lambers et al., 2006). From a plant breeding perspective, these insights can prove very

valuable for improving crop resource acquisition. For example, Lynch (2013) describes a maize root ideotype, which would be efficient at taking up N and water by foraging in deep soil layers. **This thesis tests the response of roots to N and P fertilizer application, with emphasis laid on the timing of fertilizer application. .**

A great challenge still lies in the quantification of root systems and their responses to environmental stimuli. Different from aboveground parts, where non-invasive or destructive measurements are relatively easily performed, belowground parts require extensive labor and highly destructive methods for analysis. If grown in soil, extracting of the roots is difficult and has to be done carefully not to damage the roots or lose parts thereof. Non-invasive measurements to track the root system over time are even more difficult, but there are a range of options available nowadays, such as mini-rhizotrons, growing paper, rhizoboxes and x-ray computed tomography, yet they remain labor-intensive or time and plant size limited (Atkinson et al., 2019)**.** Artificial experimental setups, such as hydroponics or an agar gel, provide easy quantification but are either limited in space and growing duration or have little relevance to realistic (a)biotic conditions. Depending on which experimental setup is chosen, there are a plenty of options available to analyze the extracted root data (for example see https://www.quantitative-plant.org/). In the end, large trade-offs need to be made between the amount of replicates, plant size or age, and the amount and detail of root system measurements.

Secondly, attempts to make generalizations of root traits to function have proven difficult. For example, the leaf economic spectrum relates the cost of leaves to their function (Wright et al., 2004), yet a similar framework for root traits is not established. Valverde-Barrantes and Blackwood (2016) propose that this difficulty is because of the higher diversity in root traits that can be used and maximized for function. Moreover, the direct comparison between leaf traits and root traits can obscure root-specific processes. This has impact on the species-specific interpretation of root traits. For example, roots with a relatively large diameter, low root hair density and short root hairs, can be favorable for nutrient uptake if a plant is dependent on a mycorrhizal symbiosis for the uptake of nutrients (mainly N or P) limiting plant growth (Brundrett, 2002; Bardgett et al., 2014)**.** If a plant is less reliant on a mycorrhizal symbiosis then thinner roots with long root hairs and root hair density, which explore a larger soil volume would be superior. However, using these traits as a predictor for plant growth to AMF inoculation might prove difficult, as shown by a meta-analysis of (Maherali, 2014). **This thesis describes a protocol for root trait measurements and tests several root measurement methods on their accuracy and bias.**

1.5 Scope and aims of the thesis

The overall objective of this thesis is to gain a better understanding of plant-soil-microbe interactions (based on both experimental approaches and methodological research) in order to improve agricultural nutrient use efficiencies. I carried out experimental work testing hypotheses of specific mechanisms which occur within these interactions. The main experimental work consisted of an outdoor, 2-year mesocosm crop rotation experiment (**paper 1)** and a controlled rhizobox experiment (**paper 2)**. Furthermore, because the measurements on roots involves a lot of methodological work, I included two studies, one describing a step-by-step method of analyzing root traits **(paper 3)** and one comparing several common root measurement techniques **(paper 4)**.

The thesis focusses on the following aims:

1) To assess the effect of different plant functional group precrops, based on arbuscular mycorrhizal and/or rhizobial symbioses, on the following crop in a crop rotation (Paper 1).

2) To test the use of high carbon amendments (HCAs) to prevent nitrate leaching in the fall/winter after crop harvest (Paper 1).

3) To test the effect of fertilizer (N/P) application timing on root system architecture and below- and aboveground plant traits, taking *Hordeum vulgare* as a model crop (Paper 2).

4) To develop a step-by-step method for analyzing root traits in the field and in rhizoboxes (Paper 3).

5) To investigate the accuracy and precision of common root measurement techniques (Paper 4).

These aims are expanded on in the papers of this thesis. The overlapping aims are described in Figure 1. In short, **paper 1** describes the effect of crop rotation, high carbon amendments, and N fertilizer addition on the yield of the focal crop, barley (*Hordeum vulgare*) in a mesocosm experiment. In **paper 2**, we also use barley as a model crop species, but instead investigate root traits at greater detail in a controlled rhizobox experiment testing the effect of fertilizer application timing. The methods used to measure root traits non-invasively in rhizoboxes are described in the protocol of **paper 3**. Lastly, this protocol builds upon the results of **paper 4**, in which we compare and discuss different root length measurements techniques.

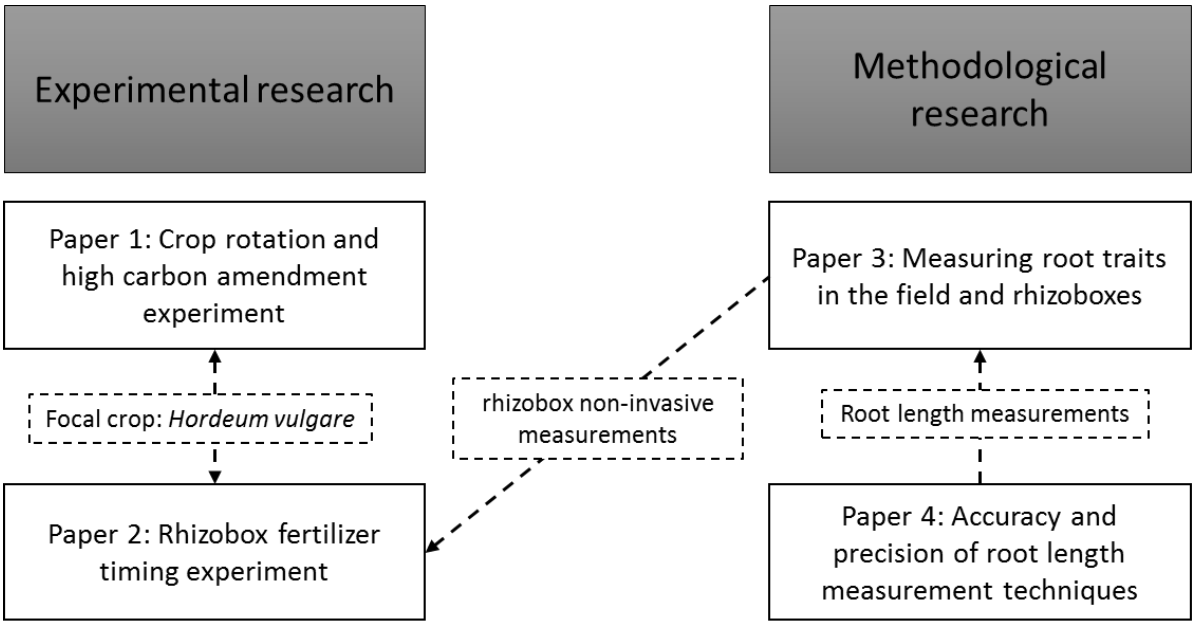

Fig. 1. Conceptual diagram of the relationships between the (experimental and methodological) studies included in this thesis.

Box 1: The INPLAMINT project

The work in this thesis was embedded within the INPLAMINT (Increasing agricultural nutrient-use efficiency by optimizing plant-soil-microorganism interactions) project funded by the Bundesministerium für Bildung und Forschung (BMBF). The INPLAMINT projects consists of several work packages involving multiple universities and research institutes (see www.inplamint.de). The central hypothesis of INPLAMINT is that "plant-soil-microbe interactions are a key driver of agricultural nutrient use efficiency". Because of the nature of these interactions, and the aim to produce viable management techniques from the mechanistic understanding of the interactions, the INPLAMINT team consists of researchers of a wide variety of disciplines. Although the work in this thesis constitutes an independent scientific work, it is also embedded in the INPLAMINT project with the aim to produce synergies between the research activities of the partners involved. Especially because of the scope (the soil is an enormously complex system, and the transition of fundamental research to applied management techniques require a multidisciplinary approach), it was essential to communicate clearly and collaborate on ideas and execution of plans. In short, the following synergies were established between the papers in this thesis and other INPLAMINT partners.

The effectivity of HCAs is dependent on multiple factors, for example specific composition of the HCA, water content and nitrogen in the soil. In order to better understand which HCAs could pose an effective management technique, we set up multiple experiments within the INPLAMINT project, ranging from controlled experiments to field experiments. The controlled lab experiment was performed in

Forschungszentrum Juelich, in which HCAs were added to soil columns with different levels of N (Reichel et al., 2018). The main advantage of this study was the possibility to accurately measure parameters such as HCA decomposition, N_2O emissions and N immobilization, but also changes in microbial biomass and the microbial community composition. Reichel et al. (2018) also found, similar to results in **paper 1**, that wheat straw was the most effective, at least short-term, immobilizer of mineral N. The field study is being done at the Kiel University by at their experimental farm site. In this setup, HCAs are added to a common German crop rotation and certain parameters are measured (soil mineral N, N_2O emissions and yield) and incorporated into models. Because of the nature of field experiments (larger variation due to year and weather effect and less homogeneous conditions overall), this experiment continues to run and collect data to verify long-term effects. Our study (**paper 1**) fits in between these two experiments. The duration of our experiment was longer than the controlled lab experiment, (2 years) and kept outside to have semi-realistic environmental conditions. However, it was performed in mesocosms which do not completely mimic realistic field conditions and agricultural management, such as in the experimental field at the Kiel University.

Another important aspect of the INPLAMINT project consists of plant-microbe interactions. Within **paper 1** we applied a functional group approach based on symbioses with microbes, namely arbuscular mycorrhizal and/or rhizobial symbioses. This approach is ideal to better understand what impact the preceding crop has on the performance of these symbiotic microbes and the overall microbiome. Thus, we sampled the soil and roots in the mesocosms multiple times. Project partners at the Freie Universität Berlin (focusing on AMF) and Helmholtz Zentrum München (focusing on bacteria) are analyzing these samples. Together with the yield and soil parameters of **paper 1** we hope to elucidate the impact of specific crops on the microbiome.

Lastly, fertilizer application and stoichiometric constraints were investigated in collaboration with the University of Cologne. At the University of Cologne, our project partners investigated a gradient of N/P fertilizer application and absence/presence of AMF on plant biomass. In order to have as much synergy between our results, we used a similar soil, plant species (*Hordeum vulgare* L. cv. Barke) and fertilizer method (Hoagland solution), as described in **paper 2**. Whereas the experiments at the University of Cologne explored a wide range of N/P stoichiometries and its effects, we investigated the effect of N/P fertilizer application timing on root traits.

2. Experimental design and methodology

The main experimental work in this thesis consists of two studies; an outdoor two-year mesocosm experiment (**paper 1**) and a greenhouse rhizobox experiment (**paper 2**). In the following chapter I provide an overview of the experimental designs and methods and reasoning behind them. The method-orientated work consists of a book chapter on root trait measuring methods (**paper 3**) and a comparative study on methods to study root length (**paper 4**). For a more detailed description of the methods see the material and methods section of the respective full-texts of the papers.

2.1 Mesocosm experiment (paper 1)

We set up an outdoor two-year mesocosm experiment starting in May 2016. The aim was to simulate a crop rotation from an agricultural field, including standard agricultural practice for applying fertilizer. For this purpose, we collected soil from the top 30 cm from the experimental field site of the University of Kiel (54°19’05.6”N 9°58’38.8’’E). The soil had a total C content of 1.26% and total N of 0.14%**.** More importantly, the soil had a history of agricultural use and was thus relatively low in carbon but had a high N and P content. This was also very relevant for the microbial parameters, because the microbial community is strongly shaped by agricultural practices. The soils were placed in square mesocosms with a volume of 38L. The experiment was set up in a factorial design, i.e. all possible combinations of factors were included with 5 replicates each. The factors we tested were the effect of precrops (4 levels; canola, spring barley, faba bean, white lupine; see Fig. 2), high carbon amendment (3 levels; control, wheat straw and sawdust) and nitrogen fertilization on the focal crop winter barley (2 levels; 40 and 160 kg N/ha). This factorial setup allowed to statistically test for interactions between either of the three factors.

	Precrops				Focal crop
Symbiosis	Spring canola	Spring barley	White lupine	Faba bean	Winter barley
AMF	☒	☑	☒	☑	☑
Rhizobia	☒	☒	☑	☑	☒

Fig. 2 Symbiotic relationships with AMF and rhizobia of the crops in the mesocosm experiment (paper 1). The first four crops (precrops) spring canola, spring barley, white lupine and faba bean were grown the season before winter barley (focal crop).

In this experiment we applied a functional group approach in an agricultural experimental setup. The concept of plant functional groups originated within the discipline of ecology. Although multiple interpretations of this concept have been used, the main idea is to separate species into different groups based on their function in certain (ecosystem) processes, rather than taxonomic or genetic relatedness (Blondel, 2003). Hence, by sorting species this way, a trait-based approach can aid to better characterize and quantify related functions. We opted to sort by symbiotic relationship with microbes, namely rhizobia and arbuscular mycorrhizal fungi. Moreover, with the factorial setup explained previously, it would be possible, to a certain degree, to relate performance outside of characteristics of the species themselves, but rather based on the functional traits they possess.

Lastly, we constructed a lysimeter setup to measure a subset of mesocosms for nitrate leaching over winter to test the effectivity of HCAs in reducing nitrate leaching (Fig. 2). This setup allowed us to directly measure the amount of nitrate in the leachate and to quantify the effectivity of HCAs. We opted, due to time and space constraints, to only compare nitrate leaching after faba bean and spring barley as a

precrop in order to be able to compare a legume and a non-legume. Legume residues are characterized by high N contents due to the species' symbiosis with nitrogen fixing bacteria.

During and at the end of the experiment we measured multiple parameters at the soil and plant level. The factors were incremental and build upon each other: First the precrops were sown, after its harvest the HCAs were added and lastly N fertilization for winter barley was added in low and high levels. We took soil cores during the experiment at the precrop harvest, before N fertilization of winter barley, and at the winter barley harvest. This made it possible to link soil and plant C/N levels to the experimental factors. Standard plant yield characteristics were also measured, such as seed and straw weight, and N uptake.

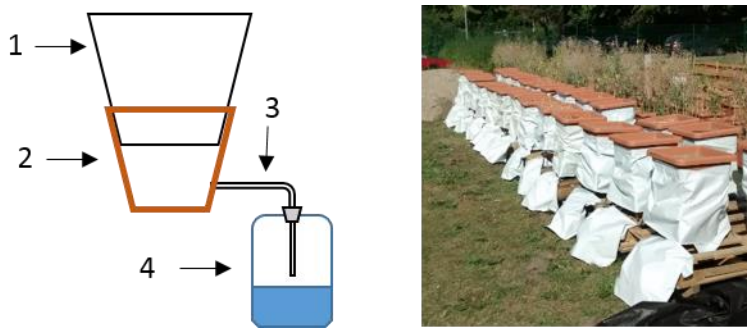

Fig. 3 Lysimeter setup for nitrate leaching measurements. Left: Schematic drawing of the lysimeter setup. The mesocosm (1) was placed into a smaller mesocosm (2), which was connected with a drain (3) into a canister (4) to collect the leachate. Right: Picture of the lysimeter setup at the experimental garden of the Leuphana University Lüneburg. The pots were covered with white plastic to avoid contamination, and heating by sunlight.

2.2 Rhizobox experiment (Paper 2)

We set up a rhizobox experiment lasting from June 12th to july 19th 2017 (38 days) to test the effects of timing of fertilizer application on spring barley (*Hordeum vulgare* L., cv. Barke) above- and belowground traits. Since we found very little previous studies explicitly testing the timing of application, we opted for a 2/3/4 week interval, to ensure that most nutrients in the seeds themselves were exhausted. The nutrients were applied using a modified Hoagland solution, a well-known nutrient solution often used in experimental plant biology (Hoagland et al., 1950). The main advantage of using a solution like this is the ability to easily manipulate specific nutrients while keeping others equal. This removes possible interference due to a lack of nutrients outside of the ones of interest**.** The timeframe was chosen to measure the roots response over the following weeks after fertilizer application, as the experimented ended after 5.5 weeks. Experimental time is limited in rhizoboxes, because plants reach the bottom and the side walls of the rhizobox rather quickly. Continuing the experiment too long may lead to artefacts in the results (Poorter et al., 2012).

In the present experimental setup it was essential to have a very nutrient poor soil to ensure that 1) plants were N/P-limited at an early stage, 2) responded to N/P fertilizer application. After some pilot experiments we found a suitable combination of soil, which was nutrient poor but still mimicked a natural soil (more than hydroponics, agar or pure sand culture would). Eight parts sand (v/v) were mixed with two parts of loess soil from a mine near Jackerath (Jackerath, Germany, 51°05'04.8"N 6°27'38.4"E) and one part peat potting soil (Nullerde, Einheitserde Werkverband e.V., Germany).

In this rhizobox setup it was possible to measure the root length over time, without interfering with the plants growth. Thus, this setup is ideal if one wants to look explicitly at time related shifts in root growth. Yet, only part of the root system grows along the glass plate of the rhizobox (depending on the species). We took the pictures using a photobox setup to avoid reflections of the glass plate**.** With these pictures I was able to extract the visible root length (VRL) using the SmartRoot plugin (Lobet et al., 2011) for ImageJ (Schneider et al., 2012). SmartRoot enables the user to semi automatically draw the visible roots and provides an overlay option for succeeding days and exports this as a Root System Markup Language (RSML) file. This file was read using archiDART (Delory et al., 2016) to facilitate data analysis (Fig. 4). The final root system of a subset of replicates were washed, scanned and analyzed with WinRHIZO and correlated with the VRL measurements.

Lastly, we measured several aboveground and belowground traits related to biomass (allocation) and nutrient uptake. The rhizobox was cut at 10 cm depth intervals and the roots were washed and dry weight was measured to quantify biomass allocation by depth. Aboveground biomass was also dried and weighed, and subsequently C, N, and P contents were measured with a C/N analyzer (Vario EL, Elementar, Germany) and P content with an ICP-OES (Optima 3300 RL, Perkin Elmer Inc., USA).

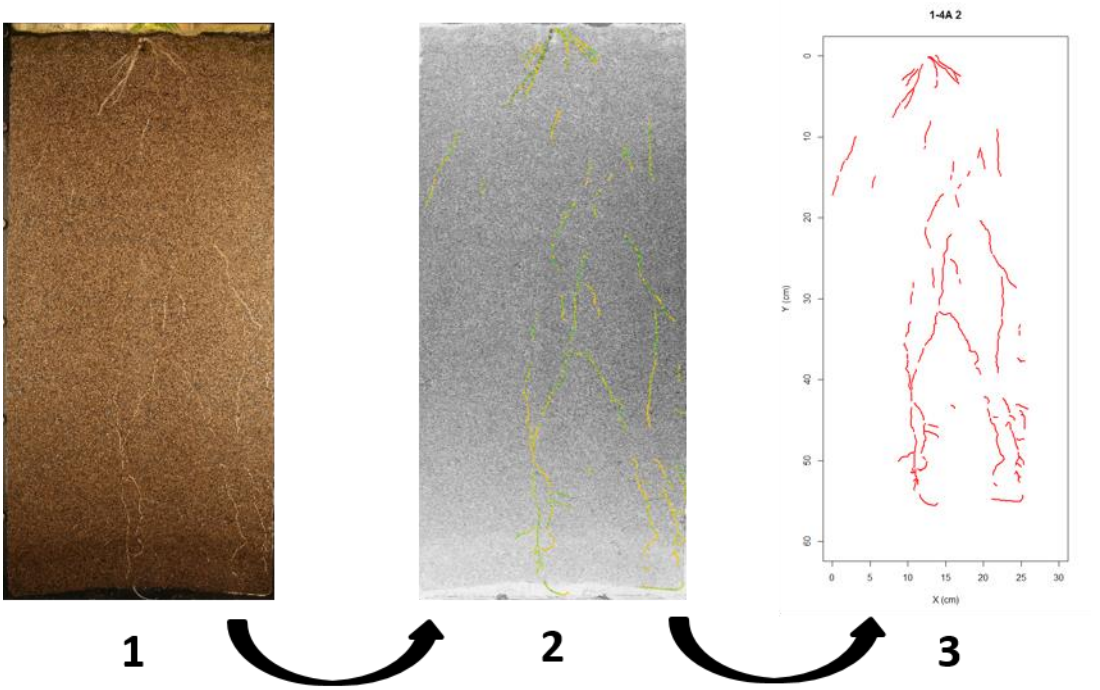

Fig 4. Workflow of acquiring root trait data in rhizoboxes. 1) High resolution photograph of rhizobox in photobox setup, 2) semi-automatic drawing of roots using Smartroot and 3) root trait extraction using archiDART.

2.3 Method papers (paper 3 and 4)

In the first method paper (**paper 3),** we give step-by-step procedures for measuring root traits in the field and in rhizoboxes. These are methods we used in our lab group at the Leuphana University. We distinguish three main sampling approaches: root cores taken in the field (standing root biomass), the ingrowth core method (to measure fine root productivity), and (non-)invasive quantification of root traits in rhizoboxes. The acquisition of roots depends on the sampling approach, but thereafter many procedural steps are similar, depending on the root traits to be measured. After collection of the roots, they need to be separated from organic material and soil particles by washing. This can be done in multiple stages of washing the roots, depending on how difficult it is to separate these. Furthermore, we describe suitable settings for acquiring images of washed roots using a high resolution scanner. These settings are dependent on the required measurements. For example, staining the roots with methyl red greatly increases the contrast and the ability of root analyzing software to distinguish roots from the background, but can interfere with subsequent chemical analyses.

In the second method paper (**paper 4**), we further investigated the methods available to measure root length from (scanned) images. We compared several automated software packages, such as WinRHIZO and IJRhizo with manual methods such as the Tennant method (Tennant, 1975) and a manually drawn reference method. These methods differ in their cost, time, and accuracy. We compared the strength of the correlation between these methods with a Pearson's product moment correlation coefficient and subsequently fitted Model II linear regression. With this regression we could test whether there was a proportional bias (95% confidence interval of slope did not include from 1) or fixed bias (95% confidence interval of the intercept did not include 0). We also evaluated the agreement of the different methods, including comparisons with the reference method, with a Bland-Altman analysis.

3. Results and discussion

3.1 Precrop functional identity and high carbon amendment effects on winter barley yield

Nitrate leaching is an urgent environmental problem associated with intensive agriculture (Di and Cameron, 2002). The amount of inorganic soil N and potentially mineralizable soil N left over after the harvest, which in turn would be prone to leaching over winter, depends on the soil conditions and the crops grown that season. In **paper 1,** we tested the effect of different precrops, HCAs, and low or high N fertilization on yield of the focal crop (winter barley) in a full-factorial experiment. The N fertilization allowed us to test for interactions with the precrops and HCAs. We expected legume precrops to have a bigger positive effect on winter barley yield under low N conditions because of their high N residues and lower use of soil N.

As to be expected, N fertilization had the biggest effect on winter barley performance. However, we were mostly interested in the interaction between N fertilization and precrops and high carbon amendments, of which we found strong effects. The strongest effect was due to precrops under high N fertilizer, with the non-AMF precrops lupine and canola resulting in higher winter barley yield (23% increase). However, there was only a slight precrop effect under low N, indicating mostly a lower yield due to N-limited conditions. Contrary to our expectations, HCAs only had a small effect yield irrespective of the amount of N fertilizer applied. Sawdust might lead to an undesirably high (from a crop yield perspective) increase in N immobilization due to its high C/N ratio, although this was not entirely supported by the data. One would then expect a larger negative yield effect of sawdust under low N fertilizer conditions, which was not the case. More of interest was the reduction in nitrate leaching. Faba bean as a precrop (a legume with low C/N ratio crop residues) resulted in significantly higher nitrate leaching than spring barley. Also, HCAs, especially wheat straw, were effective in countering the increased nitrate leaching related to faba bean.

The results in **paper 1** indicate that non-AMF precrops might have a positive effect on the next crop, a result which has been found previously, yet the mechanisms behind this effect are not well understood (Lekberg and Koide, 2005; Koide and Peoples, 2012). The positive effect could be because of the history of the agricultural soil: The AMF community tends to be parasitic over mutualistic in intensive agricultural settings due to no added benefits to plants in terms of their C investment to AMF (Verbruggen and Kiers, 2010). Hence, reduced AMF performance could have been the cause for the positive effect on winter

barley yield. More research is needed to disentangle the crop rotation effect on AMF performance, and ultimately the focal crops yield. It would also be intriguing to investigate if such negative effects of precrops disappear if the precrops are mixed cultured (intercropping), having both AMF and non-AMF crops as compared to crop monocultures (in our case).

The legume precrops (faba bean and white lupine) did not show a clear positive or negative effect on winter barley yield. Surprisingly, legumes also did not have a more positive effect under low N fertilizer. We expected a positive effect, because legumes commonly increase the yield of the next crop by leaving high N crop residues and not using up the soil N stock (Chalk, 1998; Angus et al., 2015). This could partly be the result of the mesocosm setup, which were only 37 cm deep. Thus, a large part of the residual N could be lost as leachate out of the mesocosm, which would be still within the root zone of the next crop under natural circumstances, depending on the precipitation surplus and soil characteristics. However, our N leaching results also show that it is essential to hold the N in the system after the harvest of crops with a high residual N, such as faba bean (Jensen et al., 2010).

Wheat straw showed potential to reduce nitrate leaching, especially under crops with high N residues (faba bean). Annual additions of wheat straw to the soil are most likely too expensive, but additions after crops with high risk of N leaching could be a viable management technique to improve N use efficiency. Reichel et al. (2018) used wheat straw and sawdust, as well as lignin, as HCAs in an incubation study and found an initial immobilization of 42 kg N/ha with two thirds released in the following four months. However, they also emphasize that controlled release of the immobilized N might be difficult to achieve under field conditions, as also stated by Chaves et al. (2007). Thus, for the successful application of HCAs clear aims should be set, such as: minimizing nitrate losses to the environment without major yield loss. Furthermore, future research is needed to optimize the addition of HCAs (substrate, particle size, depth, and timing) and the following crop fertilization due to potential net immobilization at the start of the growing season.

3.2 Effects of nitrogen and phosphorus fertilizer application timing on the root system architecture of barley

Achieving a mechanistic understanding of how roots respond to fertilization is essential for improving nutrient use efficiencies. The experimental setups that attempt this are often related to the concentration of the nutrient added (e.g. low concentration of nutrients result in root foraging; Linkohr et al., 2002;

Gruber et al., 2013) or the spatial distribution (e.g. a nutrient patch in which roots proliferate, see Hodge, 2004). Although the temporal aspect of fertilizer application is one that could result in new management practices, it is rarely explicitly tested. In **paper 2**, we combined N and P fertilization application timing in one experimental setup, in order to investigate the difference in plant response to these nutrients. We used spring barley as a model plant and used rhizoboxes to track the roots over time.

We found that applying N late had the biggest impact on spring barley overall performance (measured as biomass production). No matter the time that elapsed between N applications (2/3/4 weeks) the total biomass was severely lower (52-61% compared to the control treatment receiving N from the start of the experiment). Late P application only reduced total biomass by 25-37% if applied later than two weeks; before that date, no reduction in biomass occurred, despite a lower shoot P uptake compared to a control group. Root mass fractions (i.e. root biomass relative to shoot biomass) did not change between treatments. However, the biomass allocation to different depths of the rhizobox changed depending whether N or P was added later, with a relative increase of root biomass in deeper layers if N application was delayed. This could be explained by the fact that nitrate is more mobile in the soil and more easily leached to deeper soil layers. Trachsel et al. (2013) found similar root responses to N-deficiency (in maize) in two field experiments in the USA and South Africa.

One of the main aims of **paper 2** was to address how roots (in terms of root length allocation) respond to fertilizer application timing. With the use of photographs of the rhizoboxes, we were able to trace shifts in visible root length (VRL) over time. These observations are especially useful because they do not require any destructive measurements. We found two main patterns regarding N and P application timing. First, an increase in VRL compared to the control treatment early on if N was limiting (root foraging), but a lower overall root length 12 days after sowing onwards. Then, if N was still not applied after 4 weeks, the VRL increased a second time compared to plants that had received N at this time point. Although withholding P application for more than two weeks resulted in a decrease in biomass, there was no increase in VRL compared to the control group. Instead, VRL was initially similar, but lower at the end of the experiment. There are, however, very few experiments explicitly testing the effect of fertilization application timing on root systems. One field experiment by Peng et al. (2012) showed that maize roots also responded similarly, with an initial increase in root length at N-deficiency, but overall lower root lengths during later growth stages.

These results provide insight into the response of the early root system to fertilizer application. Translating this information to agronomic practice can improve nutrient uptake efficiencies. Initial N-deficiency might

lead to a more extensive root system (Peng et al., 2012), preventing N-losses if fertilizer application is paired with extensive rainfall and leaching of N to deeper root layers. Delaying N fertilization might not lead to yield losses under field conditions, as shown by a study by Scharf et al. (2002). The response of roots to N might also interact with the response to drought, which is expected to become more frequent in certain areas due to climate change. Years of breeding for high yielding crops led to efficient, but small, root systems (Aziz et al., 2017). With sufficient fertilizer, the need for the plant to exploit deeper layers for N also decreases. Hence, plants with a deeper root system might be more drought tolerant and N-efficient (Lynch, 2013). Additionally, droughts can lead to increased losses of N (and thus lower NUE) if crops are fertilized at normal levels but suffer a yield loss and reduced N uptake.

3.3 Methods for measuring root traits

Measuring roots is not an easy endeavor. The fact that roots are hidden in the soil often requires one to extract the roots and perform destructive measurements. Alternatively, non-destructive method do exist, such as the use rhizoboxes and minirhizotrons, but are limited in other forms (size and time). In **paper 3**, we described step-by-step procedures for measuring roots in the field and in rhizoboxes and explain which root traits can be measured best in which setup. The steps provide a concrete starting point for anyone who wants to measure root traits, and tackle common pitfalls (such as sample treatment and storage). In addition, important references are provided for more detailed studies on specific facets of root sampling.

An important root trait for soil exploration and nutrient uptake is root length. Simultaneously, root length is used in other composed root traits such as root length density (length of roots per unit area) and specific root length (length per dry weight) (Bardgett et al., 2014). To acquire the root length, most commonly roots are removed from their substrate (e.g. soil or some growth medium such as agar or nutrient solution), cleaned, and scanned. In **paper 4**, we compared different methods for measuring root length of washed, scanned root samples. Pairwise comparisons between the investigated methods showed that 11 out of 12 methods deviated significantly from a 1:1 relationship. Although all methods were highly correlated with a reference method, most tended to underestimate the total root length. These results show that comparisons of raw root length values between studies can lead to wrong conclusions. Ultimately, the most appropriate method depends on the available equipment, software, and time for analysis.

Both papers contribute to the standardization of root measurement methods. Methods sections often do not describe root measurements in great detail. However, it can be easy to lose fine roots during washing or to lose root biomass in sampling handling (Van Noordwijk and Floris, 1979). Methods on root analyses are becoming increasingly advanced (e.g. minirhizotrons and X-ray computed tomography). Yet, as the results in **paper 4** show for root length, these methods need to be carefully compared to a reference method, and for the foreseeable future classical approaches will most likely still play an important role. However, technological advances, such as high throughput root phenotyping (Nagel et al., 2012), can considerably speed up the classical approaches. Furthermore, the increased interest of the scientific community in open and shared data also lead to databases with large amount of plant trait data, for example in the Fine Root Ecology Database (Iversen et al., 2017). All this progress taken together will improve our understanding of plant roots for both functioning of ecosystems and plant communities, as well as help solve agronomical and environmental problems.

3.4 Conclusions and outlook

The research in this thesis contributes to an improved understanding of plant-soil-microbial interactions and NUEs in agricultural systems. The main results show that: 1) non-AMF precrops lead to yield increases of the following crop, possibly due to a shift of the AMF community to parasitism, 2) HCAs can reduce nitrate leaching, especially in crops with high residual N, but have a relatively small effect on yield, 3) N-deficiency in barley initially leads to an increased root length, but overall poor plant performance later on, whereas initial P-deficiency is less detrimental to overall barley performance, and 4) common methods to measure root length produce different results and can underestimate the total root length.

The next steps are two-sided. First, a further mechanistic understanding of the plant-microbe interactions and the role of the microbial communities in crop rotations are necessary to harness this knowledge for improving NUEs. Secondly, this knowledge needs to be translated into management practices for farmers. This same concept can also be applied to the use of HCAs. A better understanding of remineralization of immobilized N under field conditions is important. Moreover, the knowledge has to be translated for farmers, e.g. by calculating for which crops it is worthwhile, from both a financial as well as an environmental standpoint, to invest in HCA application. These steps are partly addressed by other subprojects within the INPLAMINT consortium. Lastly, crops that are not only selected for high yields, but also have root traits that are adapted to their environment, e.g. deep rooting in dry environments or shallow rooting in soils with low P status, will be pivotal in improving NUEs.

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5. Full texts of papers

5.1 Paper 1: Precrop Functional Group Identity Affects Yield of Winter Barley but Less so High Carbon Amendments in a Mesocosm Experiment

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Precrop Functional Group Identity Affects Yield of Winter Barley but Less so High Carbon Amendments in a Mesocosm Experiment

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Nitrate leaching is a pressing environmental problem in intensive agriculture. Especially after the crop harvest, leaching risk is greatest due to decomposing plant residues, and low plant nutrient uptake and evapotranspiration. The specific crop also matters: grain legumes and canola commonly result in more leftover N than the following winter crop can take up before spring. Addition of a high carbon amendment (HCA) could potentially immobilize N after harvest. We set up a 2-year mesocosm experiment to test the effects of N fertilization (40 or 160 kg N/ha), HCA addition (no HCA, wheat straw, or sawdust), and precrop plant functional group identity on winter barley yield and soil C/N ratio. Four spring precrops were sown before winter barley (white lupine, faba bean, spring canola, spring barley), which were selected based on a functional group approach (colonization by arbuscular mycorrhizal fungi [AMF] and/or N_2-fixing bacteria). We also measured a subset of faba bean and spring barley for leaching over winter after harvest. As expected, N fertilization had the largest effect on winter barley yield, but precrop functional identity also significantly affected the outcome. The non-AMF precrops white lupine and canola had on average a positive effect on yield compared to the AMF precrops spring barley and faba bean under high N (23% increase). Under low N, we found only a small precrop effect. Sawdust significantly reduced the yield compared to the control or wheat straw under either N level. HCAs reduced nitrate leaching over winter, but only when faba bean was sown as a precrop. In our setup, short-term immobilization of N by HCA addition after harvest seems difficult to achieve. However, other effects such as an increase in SOM or nutrient retention could play a positive role in the long term. Contrary to the commonly found positive effect of AMF colonization, winter barley showed a greater yield when it followed a non-AMF precrop under high fertilization. This could be due to shifts of the agricultural AMF community toward parasitism.

Keywords: crop rotation, arbuscular mycorrhizal fungi, rhizobia, barley, high carbon amendment, immobilization, plant functional group, nitrate leaching

INTRODUCTION

An ever-increasing yield is necessary to feed the growing world population, but this is coupled with high fertilizer use and associated environmental problems (Matson, 1997). Nitrate leaching is one of these problems, especially in intensive agriculture (Nixon and European Environment Agency, 2003), leading to multiple negative effects such as eutrophication of surface waters, or pollution of drinking water with consequences to human health (Di and Cameron, 2002). In temperate agroecosystems, the most crucial time point for leaching to occur is in the fall and winter, when crop residues decompose, and plant nitrogen uptake and evapotranspiration is low (Di and Cameron, 2002). Certain management practices to avoid nitrate leaching at this time point have been tested, such as addition of a substrate with a high C/N ratio (high carbon amendment; HCA) in an attempt to immobilize the nitrate microbially. The rationale behind this is that microbes in soils are commonly C-limited (Kallenbach and Grandy, 2011; Farrell et al., 2014), and by adding easily available carbon the microbes will take up the excess carbon and simultaneously immobilize excess mineral nitrogen. The advantage of this concept has been tested various times already with mixed success in mechanistic incubation studies (Zavalloni et al., 2011; Congreves et al., 2013a) and field studies (Thomsen and Christensen, 1998; Vidal and López, 2005; Burke et al., 2013; Congreves et al., 2013b; Török et al., 2014), for both agricultural and restoration purposes. However, due to the complexity of soil nitrogen dynamics, it is not clear whether remineralization of the immobilized N takes place the following spring, thus bridging the high leaching risk period in fall/winter and providing nitrogen when plant uptake is high (Chaves et al., 2007).

The amount of nitrogen susceptible to leaching in fall also depends on the previous crop (from now on referred to as 'precrop'). This can largely be affected by the crop type. Cereals like wheat have relatively low leftover N and risk of N leaching (Maidl et al., 1991; Francis et al., 1994), whereas N-intensive crops with a deep rooting system, such as canola (*Brassica napus*), or vegetable crop residues typically have very high leftover N (Henke et al., 2008; Agneessens et al., 2014). Similarly, grain legumes can increase leaching risk due to easily decomposable high-N plant residues (Chalk, 1998; Plaza-Bonilla et al., 2015). However, in the case of legumes as a precrop, of which benefits commonly have been attributed to a more positive N balance due to atmospheric N fixation (Maidl et al., 1996; Herridge et al., 2008), the overall effects of legumes in crop rotations cannot solely be attributed to increased N benefits. Reduced soil-borne pathogens, reduced soil water usage, and deep tap root systems loosening the soil can also positively affect the next crop (Peoples et al., 2009). This generally results in a yield increase compared to non-legumes in cereal cropping systems (Chalk, 1998; Angus et al., 2015).

Besides the positive effect of legumes in crop rotation, precrops that form a symbiosis with arbuscular mycorrhizal fungi (AMF), thus providing a host, typically increase the AMF spores in the soil and colonization of the next (mycorrhizal) crop. Although the benefits of AMF are usually linked to increased phosphorus and water uptake, there might also be benefits to N uptake, although this matter is still open to question (Smith and Smith, 2011; Thirkell et al., 2016), and disease resistance (Cameron et al., 2013). However, most studies neglect the possible role of AMF in affecting yields, in contrast to the well-studied effects of management practices, such as fertilizer addition and tillage, on subsequent crop yields. One reason for this might be that both fertilizer levels (especially P), tilling depth and the extent of fallow periods generally negatively affect AMF performance (Lekberg and Koide, 2005; Fester and Sawers, 2011). Moreover, these intensive agricultural practices could indirectly select for AMF strains which do not provide the benefits to the host species, but instead are closer to the parasitic end of the spectrum, investing more in their own reproduction and maximizing carbon acquisition from the host plants (Ryan and Graham, 2002; Verbruggen and Kiers, 2010). However, it is not clear what effect non-mycorrhizal crop species (the most common ones belong to the *Brassicaceae)* in crop rotations have on AMF community and structure both during the cropping with the non-mycorrhizal plant species and for the subsequent mycorrhizal crop (Kirkegaard et al., 2008; Verbruggen and Kiers, 2010). We know little about the extent to which having a mycorrhizal vs. a non-mycorrhizal precrop affects the yield and performance of the subsequent crop.

The effect and applicability of HCAs to counter nitrate leaching might depend on the specific precrop. To this end, we combine precrops with an ecologically based plant functional group approach based on two common plant-microbe symbioses: colonization by AMF and/or N_2-fixing bacteria. We explore the role of the symbiotic status of the precrop by combining all possible combinations of these two symbioses in the precrop, e.g., from rhizobial and mycorrhizal to non-rhizobial and non-mycorrhizal species (see **Table 1**). We include high and low N fertilization to disentangle effects of precrop functional groups and HCAs and their interactions. To our knowledge, this is the first study that explicitly tests the role of such plant functional groups (based on symbiosis) with a full factorial design within an agricultural experiment. Therefore, our study incorporates an ecological concept within a mainly agricultural experimental setup.

We experimentally investigated the role of HCA, precrop functional group and nitrogen fertilization on winter barley yield in an outside mesocosm experiment. We measured a subset, consisting of faba bean and spring barley as precrops, for the effect of these precrops and HCA on nitrate leaching over winter. We asked the following questions:

(1) Does the previous crop identity affect winter barley yield and do precrops forming root symbioses (rhizobia/AMF) show a bigger positive effect under low N?
(2) Is the effect of HCA on winter barley yield modulated by N fertilization level?
(3) Is the effect of HCA affected by the precrop identity? More specifically, does nitrate leaching increase after harvest of a legume precrop compared to a non-legume precrop and is this reduced by HCAs?

Overall, we hypothesized that an AMF-colonizing legume precrop amended with wheat straw under high N conditions

TABLE 1 | Sowing and harvest dates, fertilizer amount and symbioses with AMF and/or rhizobia of the crops used in this study.

	Crop	Sowing date	Harvest date	Fertilizer addition (kg/ha)					AMF	Rhizobial
				N	K_2O	P_2O_5	MgO	S		
Precrops	Spring barley	26/05/16	26/08/16	75	130	40	35	98	X	
	Spring canola	26/05/16	22/09/16	100	140	70	50	90		
	Faba bean	26/05/16	07/09/16	0	50	115	35	60	X	X
	White lupine	26/05/16	28/09/16	0	50	60	35	65		X
Focal crop	Winter barley	07/10/16	10/07/17	160/40	100	70	50	86	X	

Precrop complete fertilizer was added at precrop sowing date, while complete fertilizer addition to winter barley occurred at 23/03/17, except for two more N additions at 01/05/17 and 15/05/17 in the high N treatment.

results in the highest winter barley yield. Specifically, we hypothesized that:

(1a) Legume precrops have a positive effect on winter barley yield (especially under low N fertilization), since they introduce extra N into the system.
(1b) AMF crops have a positive effect compared to non-AMF crops on winter barley yield; we expect the highest yield increase with faba bean (both rhizobial and AMF).
(2) HCA has no effect under optimal N conditions, but could either decrease or increase winter barley yield under low N conditions by continuous N-immobilization or N-immobilization followed by remineralization in the spring, respectively.
(3a) Precrop identity modifies HCA effects on winter barley yield, since we expect more leftover mineral N after harvest of the legumes and canola. This would lead to potentially higher N immobilization over winter.
(3b) Nitrate leaching after precrop harvest is higher for a legume precrop compared to a non-legume precrop (faba bean vs. spring barley) and decreases for both with HCA addition.

MATERIALS AND METHODS

Experimental Site and Conditions

Our mesocosm experiment was conducted outside in an experimental garden of the University of Lüneburg (Lüneburg, Germany, 53°14′23.8″N 10°24′45.5″E). Mean annual temperature and rainfall is 9.2°C and 718 mm respectively. For detailed meteorological measurements during the experiment see Supplementary Figure S1, data was taken from the nearby weather station of the Deutsche Wetterdienst, Wendisch Evern (53°12′49.0″N 10°28′13.1″E).

Experimental Design

We applied a mesocosm experiment to quantify treatment effects on winter barley yield. We used relatively square mesocosms with an edge length of 37.5 and 26.5 cm at the top and the bottom, respectively, and a height of 37 cm; the resulting volume was 38 L. We used a surface area of 0.16 m^2 when converting to g m^{-2} and calculating fertilizer and HCA rates from kg ha^{-1}. Mesocosms were subject to three experimental factors (**Figure 1**): HCA (three levels; no HCA, wheat straw, sawdust), precrop identity (four levels; spring barley, spring canola, faba bean, white lupine), and N fertilization (2 levels; high: 160 kg/ha, low: 40 kg/ha). We applied a full factorial design with 5 replicates (n = 5) for each treatment combination, resulting in 120 mesocosms. Mesocosms were placed randomly (with 25 cm distance between mesocosms) in the experimental garden. Mesocosms were filled to a bulk soil density of ∼1.1 g cm^{-3} in the top 10 cm with soil passed through a 1 cm sieve. The soil originated from the top 0–30 cm of the experimental farm Hohenschulen of the Christian-Albrechts-University in Kiel (54°19′05.6″N 9°58′38.8″E). The soil is a sandy loam (Cambic Luvisol) and has a history of agricultural practice. In the growing season before the experimental start, a mixture of catch crops (such as clover and lupine) had been grown without fertilization, while the season before that maize had been cultivated and fertilized with 40 m^3 slurry (∼3% N, ∼1.8% P) and 100 kg/ha triple superphosphate (20% P). The soil had a total of 1.26% C, 0.14% N, a C/N ratio of 9.2 and a pH of 6.0 at the start of the experiment. We constructed a setup to measure nitrate concentrations in the leachate after the precrop harvest until N fertilization of winter barley at 23/03/2017 (see subsection leachate measurements). After the precrop harvest, mesocosms were reorganized and six out of ten replicates of the precrops faba bean and barley, and all HCAs were randomly selected for leachate measurements. At this time point, mesocosms were also isolated with air cushion foil (Luftpolsterfolie 3S, Hermann Meyer KG) and covered with white plastic sheets to avoid extreme temperature fluctuations within the mesocosm.

Study Species and Crop Husbandry

In May 2016, all mesocosms were sown with the precrops, which were chosen according to their ability to either be colonized by AMF or rhizobia (i.e., *Fabaceae*). The chosen precrops were spring barley (*Hordeum vulgare* cv. Barke, Saatzucht Breun), spring canola (*Brassica napus* cv. Medicus, NPZ), faba bean (*Vicia faba* cv. Tiffany, NPZ) and white lupine (*Lupinus albus* cv. Energy, Feldsaaten Freudenberger). Winter barley (*Hordeum vulgare*, cv. Antonella, Nordsaat Saatzucht), the focal crop, was sown the season after the precrops. The planting density (seeds/m^2) and row distance (cm, if applicable) was the following: spring barley 300, 9 cm; canola 120, 19 cm; faba bean 45; white lupine; 70, winter barley; 240, 13 cm. Mesocosms were fertilized according to their crop and standard agricultural practice in Germany (for exact values

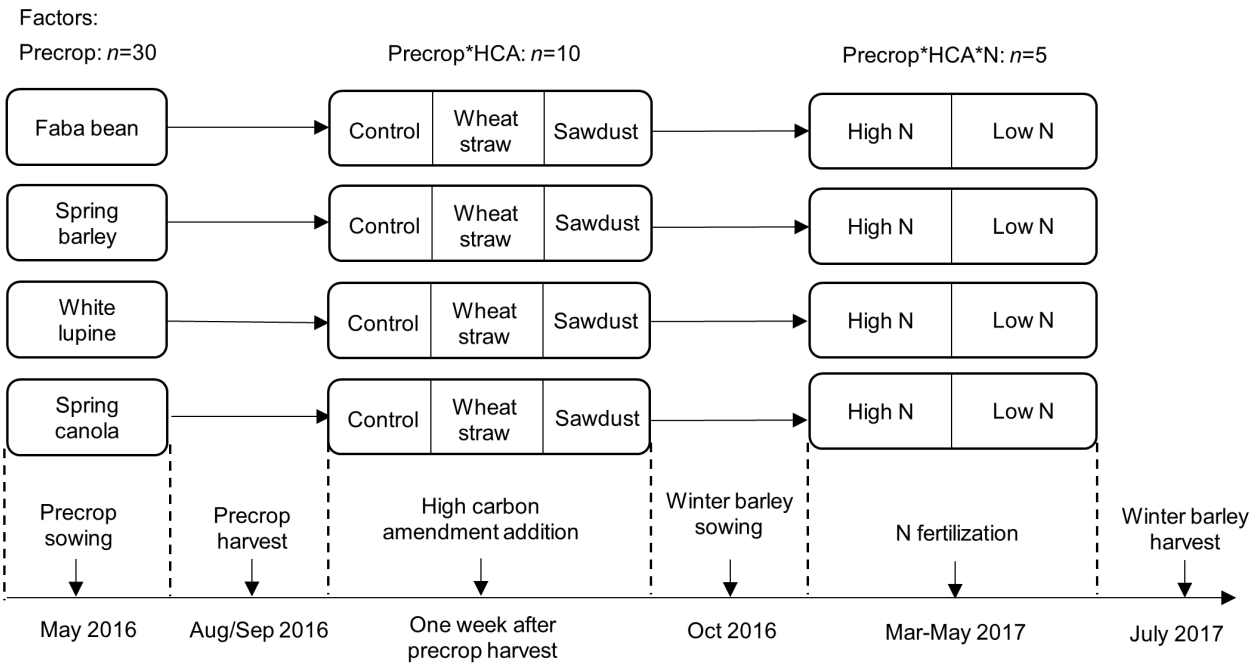

FIGURE 1 | Experimental design of the study with the main factors, precrops, HCA and winter barley N fertilization, added at different time points. For exact sowing and harvest time points see **Table 1**.

see **Table 1**) at either the sowing date (all precrops) or on 23/03/17 (winter barley). N Fertilization of winter barley for the high N treatment was spread over three time points, 60 kg N/ha at 23/03/2017 and 01/05/2017, and 40 kg N/ha at 15/05/2017. Nitrogen was added in the form of calcium ammonium nitrate, phosphate as superphosphate, potassium oxide as Korn-Kali, magnesium oxide as Korn-Kali and Epsom salt, and sulfur was contained in superphosphate, Epsom salt and Korn-Kali.

All mesocosms received 0.8 g Schneckenkorn (9.9 g/kg iron(III)-phosphate; Neudorff GmbH) on 14/06/2016 to counter plant damage by slugs. Furthermore, all mesocosms were sprayed with roughly 200 ml diluted Spruzit Schädlingsfrei per pot (45.9 mg/L pyrethrin; Neudorff GmbH) on 01/07/2016 due to an aphid infestation. No pesticides or herbicides were necessary during winter barley cultivation. Weeding was done by hand when necessary on multiple occasions. Mesocosms were watered during dry and warm spring/summer days when deemed necessary, but never during fall/winter, as to not affect the leachate amount.

High Carbon Amendments

High carbon amendments were added within 1 week after the precrop harvest. HCAs were air-dry wheat straw or spruce sawdust at a rate of 8.6 t/ha (137.6 g/mesocosm). The C/N ratios were 71 and 539, total C 46 and 51%, and total N 0.7 and 0.1%, respectively. The wheat straw had a particle size of 5–10 cm, whereas the sawdust contained particles of 1–2 cm. The HCAs were mixed in the top 10 cm of the soil and afterwards watered slightly to promote incorporation. The top 10 cm soil in the control treatment was also mixed, but without any amendment added.

Leachate Measurements

A leachate setup was built after the precrop harvest to collect water flowing through the mesocosms, which was subsequently analyzed for nitrate concentration. Mesocosm pots were put into slightly smaller mesocosms (30 × 30 × 32.5 cm) on top of two stacks of pallets. The drainage holes of the smaller mesocosms were sealed with cement and coated with a nitrogen-free resin at a slight angle so all water would flow to an attached drain. The drain was connected to a 5 L canister stored under the pallets and covered with white plastic sheet. Drainage holes of the large mesocosm were covered with nitrogen-free drainage fleece (Drainage-Geotextilvlies, Haga-Welt), to prevent contamination by soil particles or root growth into the smaller mesocosms. From 01/09/16 until 23/03/17, every 3–4 weeks (when enough water for analysis was leached through) the total leachate volume was recorded and a subsample of 50 ml was taken. The subsample was stored at −20°C before analysis for nitrate content. Samples were filtered before analysis using 0.45 μm filters (CHROMAFIL Xtra RC 45/25 membrane, Macherey-Nagel, Germany). The first two time points were analyzed with a direct UV measurement (VWR UV-3100PC, Denmark) at 220 nm and subtraction of interference at 275 nm according to (Goldman and Jacobs, 1961). However, at the third time point (28/11/16) the interference at 275 nm was too high (>10%) and we measured this and subsequent time points with an ion chromatograph (Dionex DX-120, AS14 column, United States). We correlated the UV and ion chromatograph for the third time point and the measured values

showed good agreement (R^2 = 0.961; Supplementary Figure S2), but the UV method underestimated nitrate content due to the high interference at 275 nm. Three samples were excluded from the analysis due to broken tubes.

Plant and Soil Analyses

All precrops were harvested at maturity in September/August 2016 (for exact dates see **Table 1**), winter barley at 10/07/17, and separated into seeds and other aboveground biomass tissues, i.e., stems and leaves. Stems were cut off at 3 cm above the ground and leftover stubble and roots remained in the mesocosm. Furthermore, the seeds, depending on the crop, were manually separated from the spike (spring and winter barley) or the pods (lupine, canola and faba bean) to get the final cleaned seed mass. Dry mass of each plant component was measured after drying for at least 48 h at 70°C to constant weight. Winter barley seeds free from spikes were milled (MM 400, Retsch, Germany), dried at 105°C overnight and analyzed for C and N content (Vario EL, Elementar, Germany). Soils were sampled for roots at precrop harvest for screening for AMF colonization to see if the potential for symbiosis actually resulted in a symbiotic interaction in the experiment. Pooled composite samples of 6 soil cores (0–10 cm depth, 1 cm diameter) per mesocosm were sieved at 0.5 mm and precrop root fragments were sampled and stored at −80°C until analyses. A subset of precrop root fragments were then stained with Trypan blue and screened for AMF structures.

Soils were sampled for C/N analysis on two separate occasions. From 13 till 15 March 2017, before nitrogen fertilization, a composite sample of 6 cores (0–10 cm depth, 1 cm diameter) per mesocosm was taken. Afterward, autoclaved soil of the start of the experiment was used to fill the holes, as to not interfere with the leachate setup. Sampled areas were marked with a wooden toothpick to avoid resampling the same position later on. After the winter barley harvest on 10 July 2017, a composite sample of again 6 cores (0–10 cm depth, 2 cm diameter) was taken. Samples were air-dried before sieving (2 mm), milling (MM 400, Retsch, Germany), drying 24 h at 105°C and subsequent C/N analysis (Vario EL, Elementar, Germany).

Statistical Analysis

We first fitted three-way ANOVA models testing the effect of HCA, precrop and N fertilization as fixed factors on grain yield, straw biomass, C/N ratio and total N uptake of the seeds. The factor levels were as following; Precrop: spring barley, faba bean, white lupine, canola; HCA: control, wheat straw, sawdust, N: high, low. We included all interactions, because we were mainly interested in the two-way interactions between N fertilizer and either precrop species or HCA, i.e., whether the response to these factors differs between low and high N conditions. Moreover, we tested for the interaction between precrop and HCA, in case the HCA response was dependent on the precrop. We started with the full model and also checked for significance of the three-way interaction. If this was not significant and if dropping this improved the model, which was so in all cases, the three-way interaction was dropped. In case of no interaction, we averaged over the other factors for the factor of interest when plotting means or describing effect sizes.

In most cases heteroscedasticity was observed due to the choice of extremely contrasting N levels (i.e., high and low). Thus, for all data involving N as a factor, we used a generalized least square model with the "weights" function to allow different error terms for high and low N and correct for this heteroscedasticity [varIdent (form = ∼1|N)] using the nlme package (Pinheiro et al., 2017). The same approach was applied to soil C/N analysis, but replacing N levels with HCA levels within the weights function. Multiple comparisons between groups were tested for significance by using generalized linear hypotheses with Tukey's HSD adjusted *p*-values using the lsmeans (Lenth, 2016) and multcomp (Hothorn et al., 2008) packages. The leachate data was analyzed with a two-way ANOVA testing the effect of HCA (control, wheat straw, sawdust) and precrop (faba bean, spring barley) on total nitrate leached from the precrop harvest in August/September 2016 until N fertilization in March 2017. Multiple comparisons were tested for significance using Tukey's HSD adjusted *p*-values using the multcomp package (Hothorn et al., 2008). All statistical analyses were performed using R 3.4.2 (R Core Team, 2017).

RESULTS

N Fertilization

N fertilization was the main factor affecting the yield of winter barley with an average increase of 75% at high N compared with the low N level. This main factor effect was expected and we were mainly interested in the interactions. We found strong interaction effects with N levels and precrop species for the yield parameters grain yield, straw biomass, seed C/N ratio and total seed N uptake (**Table 2**).

Precrop Species Affect Yield Under High N

The precrop had a pronounced effect on the winter barley yield, but mostly under high N conditions only (**Figure 2A**, precrop*N: $F_{3,102} = 7.56$, $p < 0.001$). Non-AMF precrops lupine and canola resulted in an on average 23% increase in yield compared to the AMF precrops spring barley and faba bean under high N. Although under low N we found significantly higher yields in winter barley when grown after lupine compared to spring barley, this effect was not as pronounced compared to the stimulating effect of having a non-mycorrhizal precrop found in the high N treatment (**Figure 2A**). For the winter barley straw yield, although roughly half the biomass of the yield in all treatments, the same pattern was found for high N but no significant difference found under low N (**Figure 2B**). Interestingly, the C/N ratio of the seeds showed the reverse pattern: Non-AMF precrops resulted in a lower seed C/N ratio than AMF precrops, but only under low N conditions (precrop*N: $F_{3,102} = 12.36$, $p < 0.001$, **Figure 2C**). Total grain N uptake, however, which is the N concentration of the seeds multiplied by grain yield,

TABLE 2 | Results of the GLS ANOVA of N fertilizer, precrop and HCA on different yield parameters.

Factor	df	Grain yield		Straw biomass		C/N seeds		Total N yield	
		F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
N	1, 102	490.58	**<0.0001**	935.99	**<0.0001**	1017.73	**<0.0001**	1749.64	**<0.0001**
Precrop	3, 102	8.12	**0.0001**	9.37	**<0.0001**	0.31	0.8179	16.96	**<0.0001**
HCA	2, 102	11.05	**<0.0001**	10.69	**0.0001**	2.18	0.1186	8.55	**0.0004**
N*precrop	3, 102	7.58	**0.0001**	6.60	**0.0004**	12.36	**<0.0001**	7.07	**0.0002**
N*HCA	2, 102	1.43	0.2433	2.13	0.1239	0.37	0.6919	0.84	0.4355
Precrop*HCA	6, 102	2.11	0.0589	2.70	**0.0179**	1.56	0.1655	1.33	0.2503

ANOVA p-values are in bold when $p < 0.05$.

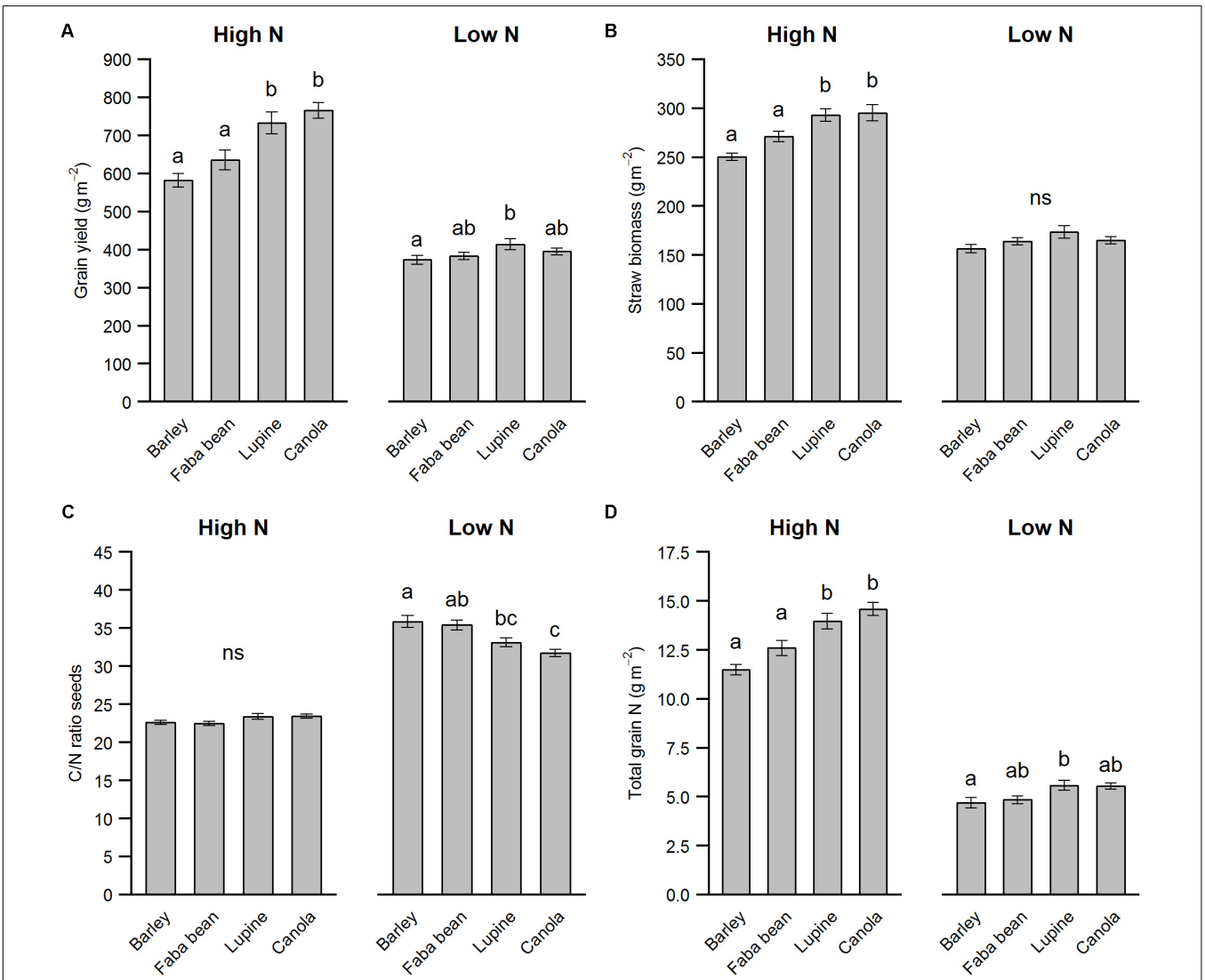

FIGURE 2 | Grain yield parameters of winter barley as affected by nitrogen fertilization and precrop species. The parameters are grain yield **(A)**, straw biomass **(B)**, C/N ratio of the seeds **(C)** total grain N **(D)**. The values are means per factor combination averaged over HCA ($n = 15$) ± SE. Different letters indicate significant differences between groups within N levels ($p < 0.05$).

showed the same pattern as the grain yield, indicating that the C/N ratio of the seeds is a less important indicator for total N uptake (**Figure 2D**). Although we had two legumes in the crop rotation, we did not see a clear legume effect on the yield, but instead observed a consistent effect of non-AMF vs. AMF precrops.

Sawdust Decreases Yield, but Increases Soil C/N Ratio

Overall, HCA application did not result in a winter barley yield increase compared to the control treatment, irrespective of N fertilization (HCA: $F_{2,102} = 11.05$, $p < 0.001$; N*HCA: $F_{2,102} = 1.43$, $p = 0.243$). However, sawdust application consistently decreased grain yield (sawdust: −6.3%, wheat straw: +3.8% compared to control, **Figure 3A**). Although seed C/N ratios were not affected by HCA (**Table 2**), total N uptake was lower in the sawdust treatment due to decreased yield (**Figure 3B**). Furthermore, we found a marginally significant HCA and precrop interaction on grain yield (precrop*HCA: $F_{6,102} = 2.11$, $P = 0.059$). This was mostly seen in spring barley and faba bean causing a slightly increased yield and white lupine and canola slightly decreased yield when wheat straw was applied.

Top soil (0–10 cm) C/N ratios increased with HCA application, more so with sawdust than wheat straw (**Table 3**). Total C and corresponding C/N ratios increased with higher addition of carbon, but no change in total N was observed before N fertilization in March 2017. However, while wheat straw resulted in an increase of soil C/N ratios in March, no lasting effect compared to the control was found by the time of the winter barley harvest in July 2017. There is a trend that C/N and total C due to sawdust addition remained high even over the main growing season of winter barley (**Table 3**), although in our experimental setup this was statistically not testable due to addition of N over the sampling period. Lastly, we did not find an effect of any precrops on the measured soil parameters.

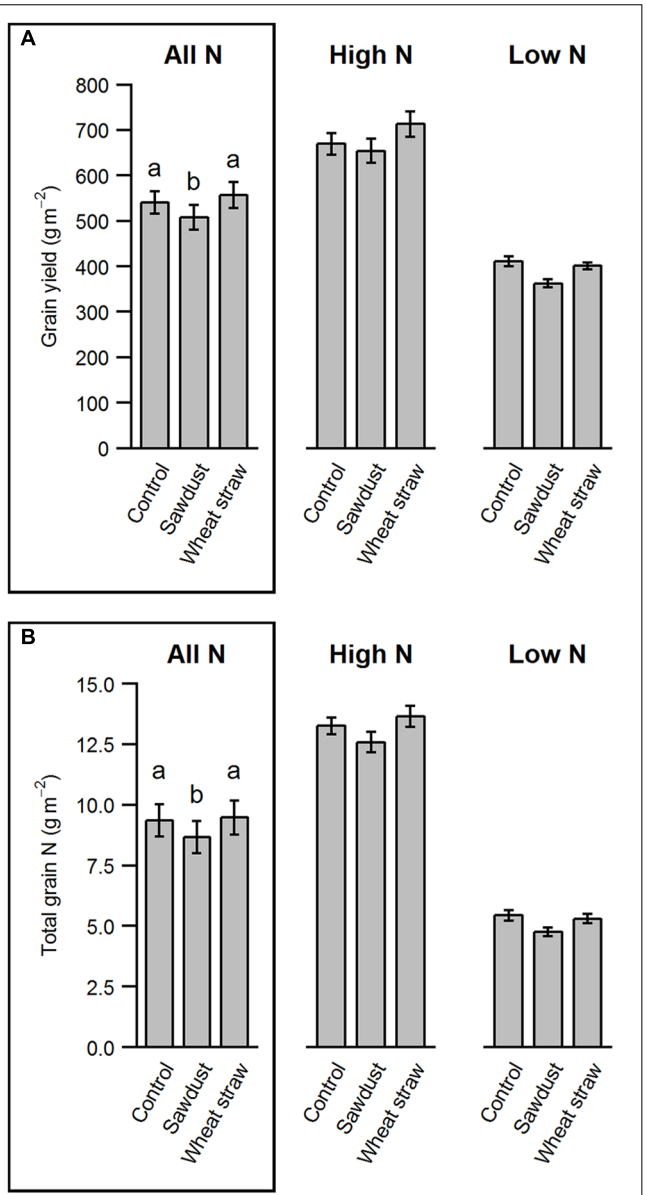

FIGURE 3 | Grain yield parameters of barley as affected by HCA. The parameters are grain yield **(A)** and total grain N **(B)**. The values are means ± SE per factor combination averaged over N and precrop ($n = 40$) in the boxed panel and separately plotted for high and low N to show variability between N levels ($n = 20$). Different letters indicate significant differences between groups ($p < 0.05$).

Leachate

Measurement of nitrate leaching showed a clear trend of increased leaching when faba bean was grown as a precrop compared to barley (**Figures 4**, **5**). The first sampling time point was 25/10/16, although we had the leachate sample setup ready 01/09/16, due to a very dry and warm September month (Supplementary Figure S1). Wheat straw showed a pattern of decreasing nitrate leaching for both precrops (**Figure 4**). For the cumulative nitrate leaching over fall and winter, faba bean, being a legume, had significant higher nitrate leaching in the control group (precrop*HCA: $F_{2,27} = 10.22$, $P < 0.001$; **Figure 5**), but wheat straw addition reduced nitrate leaching by 43% compared to the control. Although wheat straw addition lowered faba bean leaching to values similar to barley as a precrop, there was no significant overall decrease in barley due to HCAs.

AMF Colonization of Precrops

We did a screening of the roots of all four precrops to see if they showed signs of AMF or other fungal structures. Some fungal structures in these roots are shown in Supplementary Figure S4. We found that faba bean had vesicles, hyphae and spores resembling AMF structures, whereas we did not find clear signs of AMF colonization in spring barley. As expected, the non-AMF precrops canola and lupine showed no signs of AMF colonization.

DISCUSSION

We determined the response of winter barley yield to the previous crop and HCA under low or higher N conditions. We used a plant functional group approach based on two important plant–microbe symbioses (AMF and rhizobia) to disentangle their temporal effect on crop yield. Contrary to our hypotheses, we did not see a large effect of either plant functional group under N-limiting conditions. However, under high N fertilizer conditions non-AMF precrops significantly increased the yield

TABLE 3 | Effect of HCA and N fertilization on soil C/N ratio, total C (%) and total N (%).

Sampling date	HCA	N	*n*	C/N	Total C (%)	Total N (%)
13/03/17	C		20	9.68 (0.0640)a	1.29 (0.009)a	0.134 (0.0005)ns
	W		20	10.08 (0.0628)b	1.36 (0.009)b	0.135 (0.0008)ns
	S		20	11.87 (0.2445)c	1.59 (0.033)c	0.134 (0.0007)ns
10/07/17	C	High	20	9.52 (0.071)a	1.29 (0.017)a	0.135 (0.0018)a
		Low	20	9.51 (0.046)a	1.26 (0.014)a	0.132 (0.0014)a
	W	High	20	9.70 (0.057)a	1.36 (0.015)b	0.141 (0.0018)b
		Low	20	9.60 (0.062)a	1.33 (0.014)b	0.139 (0.0011)b
	S	High	20	11.48 (0.164)b	1.60 (0.022)c	0.141 (0.0018)b
		Low	20	11.27 (0.203)b	1.53 (0.035)c	0.138 (0.0015)b
N effect				ns	*	*

C: Control, W: Wheat straw, S: Sawdust. Sampling of March 2017 did not have the N treatment yet, thus a random subsample of low and high N factors was taken. Values are means ± SE averaged over precrops. Different letters indicate significant main effects of HCA (Tukey HSD; $p < 0.05$). Asterisks indicate a significant N effect (only applicable for sampling date 10/07/17).

compared to AMF precrops. Whereas HCA did not have a strong effect on the yield, it resulted in an increase in total soil C and N, indicating possible longer term positive effects on nutrient retention. HCA also directly reduced nitrate leaching in the top soil, but only for faba bean compared to spring barley as a precrop.

Effect of Precrop and Its Type of Symbiosis on Winter Barley Yield

We hypothesized that precrops that are leguminous and/or have a symbiosis with AMF would positively affect winter barley yield, especially under low N conditions. Instead, we hardly found an effect under low N conditions and a positive effect of non-AMF precrops on barley yield under high N (**Figure 2A**). The legumes were clearly fixing atmospheric nitrogen, since (a) we found large numbers of nodules on the main tap roots when mixing in the HCA after precrop harvest, and (b) we found no signs of N stress, and legume yield comparable to canola and spring barley without any N fertilization (**Table 1** and Supplementary Figure S3). Nevertheless, our results suggest that the legume precrop effect was not the dominant driver for winter barley yield, but more that the AMF-symbiosis of the precrop played a key role, since winter barley yield after canola (non-AMF and non-rhizobial) was just as high as that after lupine (non-AMF, rhizobial). This result is surprising because of the many studies showing a positive effect of legumes on the subsequent crop in crop rotations (Chalk, 1998; Angus et al., 2015).

This lack of strong legume facilitation on the subsequent crop might be explained by our crop husbandry and experimental setup. First of all, we grew grain legumes until maturity and removed all of the aboveground biomass, (both stems and seeds) which may complicate a direct comparison to typical leguminous cover crops where the goal is to increase nutrient retention and add biologically fixed nitrogen in the system (Tonitto et al., 2006). However, just the legume grain alone can contribute to 45–75% of the total aboveground biomass N (Van Kessel and Hartley, 2000), thus normally the majority of N is taken off in grain legumes. In our study, any carry over N facilitation effect would have to be mediated via decomposition of roots or direct exudation of compounds. However, belowground N contributions to the N budget are often ignored or vary widely in their estimates (Herridge et al., 2008), especially in the case of rhizodeposition (Wichern et al., 2008).

Secondly, our mesocosms were only 37 cm deep, which limits the extrapolation to field conditions, since the roots of our species could not grow as deep as in field conditions. Canola and barley, and grain legumes similar to the species in our study such as narrow leaf lupine (*Lupinus angustifolius)* and soybean (*Glycine max)* are known to have roots as deep as 1.6, 1.7, 2.5, and 1.8 m, respectively (Canadell et al., 1996; Fan et al., 2016). Thus, one would expect such roots under field conditions to be able to take up more of the excess N before being lost out of the system as leachate. After harvest in winter, nitrate will leach down to lower soil depths (Pedersen et al., 2009), some of which may then be taken up by the next crop in spring, some of which will be lost as leachate. In our study, however, we measured leachate derived from a 37 cm deep mesocosm, such that one could not know whether the N would be lost in the same way as leachate under field conditions. Field experiments and models show large amounts of nitrate leaching into deeper soil layers (Pedersen et al., 2009). Leachate measurement in our study nevertheless allowed us to compare differences between a legume and non-legume precrop. We know that cropping systems with legume species tend to have a larger leachate problem than non-leguminous crops since legumes tend to not rely on soil N as much as other crops, and leave low residues with low C/N ratios (Francis et al., 1994; Hauggaard-Nielsen et al., 2009). Our direct finding that faba bean had higher nitrate leaching compared to spring barley as a precrop (**Figure 5**) confirms this.

In addition to a positive legume precrop effect, we expected a positive effect of AMF precrops on the winter barley yield. This was not the case in the high N treatment (**Figure 2A**) and a surprising finding, because, assuming a positive effect on mycorrhizal colonization when the previous crop is a host to AMF compared to a non-host, a higher AMF colonization is associated with a higher yield (Lekberg and Koide, 2005). However, due to inclusion of low and high N, we can rule out a significant N carry-over of the previous crop, because

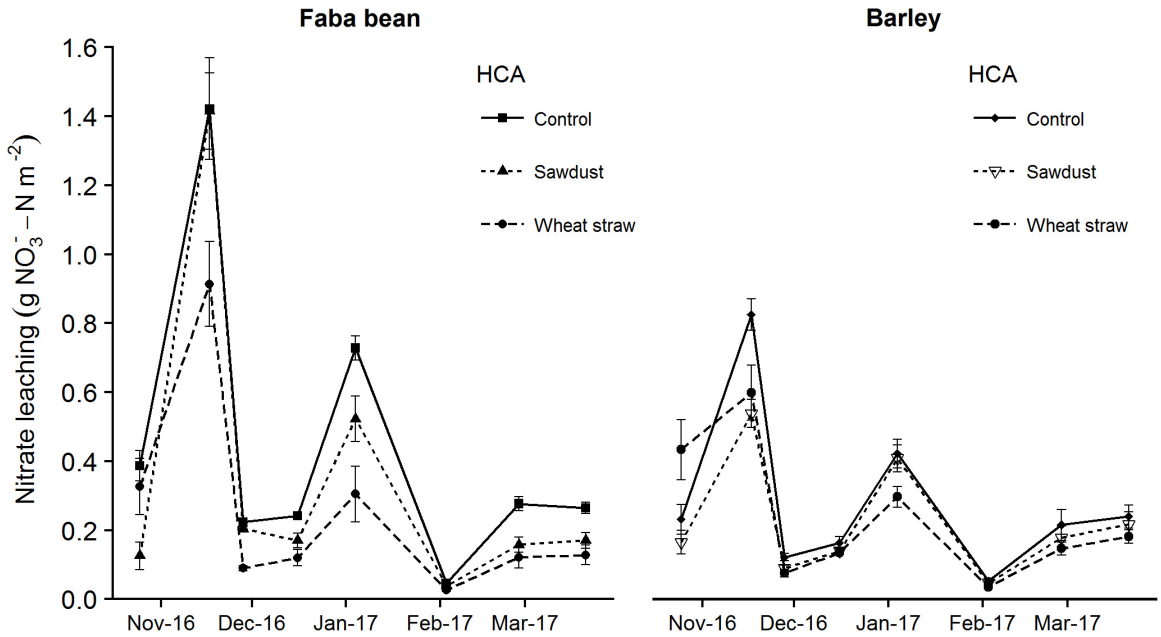

FIGURE 4 | Nitrate leaching after precrop harvest until first N fertilizer application as affected for the precrops faba bean and spring barley, and HCA. Nitrate leaching is calculated as the concentration of a subsample times the volume of water leached through the mesocosm. The values are means ± SE (n = 4–6).

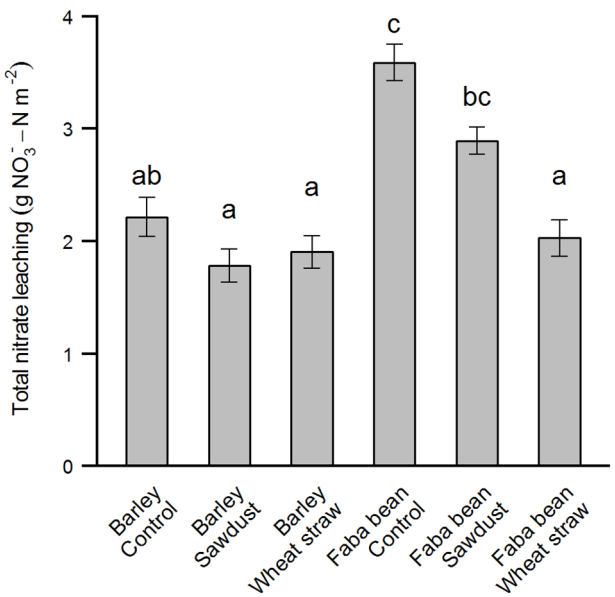

FIGURE 5 | Total amount of nitrate leached between 01/09/16 and 21/03/17 as affected by the precrops faba bean and barley and HCA. Total nitrate leaching is calculated as the concentration of a subsample times the volume of water leached through the mesocosm summed over all time points between 25/10/16 and 21/03/17. The values are means ± SE (n = 4–6). Different letters indicate significant differences between groups ($p < 0.05$).

we found no legume-exclusive effect compared to non-legumes (spring barley or canola; **Figure 2A**). Thus, the non-AMF precrop effect under high N might be attributed to other factors, such as reduced AMF colonization or a reduction in soil-borne pathogens by bio-fumigation of canola (Matthiessen and Kirkegaard, 2006). However, a bio-fumigation effect would not explain the similar positive effect of lupine. Therefore, a decrease in winter barley yield due to AMF precrops might be the most plausible explanation. Root staining showed AMF colonization in faba bean roots, but no clear colonization in spring barley (in comparison, in canola or lupine we found other fungal structures, but no colonization by AMF; Supplementary Figure S4). We can therefore not say with certainty whether the negative precrop effect of spring barley on winter barley yield compared to canola or lupine is directly related to AMF performance.

Explicit comparisons, other factors being equal, between non-mycorrhizal and mycorrhizal crops in crop rotations are limited (Ryan and Graham, 2002; Lekberg and Koide, 2005). Although rather controversial, Ryan and Graham (2002) and Ryan and Kirkegaard (2012) question the function of AMF and their contribution to crop yields in intensive agriculture. Similarly in our experiment, nutrient conditions were standard for German agriculture, which is generally regarded as very high (de Vries et al., 2011; MacDonald et al., 2011). High fertilizer rate/soil nutrients, especially soluble P, is known to negatively affect AMF colonization (Mäder et al., 2000; Treseder, 2004), but could also change the functioning of the AMF community toward more parasitism (Verbruggen and Kiers, 2010). If the AMF community represented a typical agricultural community (due to the history of our soil), this could explain the positive effect of non-AMF precrops, with the AMF precrops possibly introducing rather parasitic AMF to the system that may have contributed to the lower yield in winter barley after these crops in our study.

HCA Effects on Winter Barley and Soil Parameters

Addition of HCAs to reduce nitrogen leaching specifically after harvest has been attempted multiple times, with mixed results (Thomsen and Christensen, 1998; Vidal and López, 2005; Chaves et al., 2007; Congreves et al., 2013b). HCAs can have effects on a number of parameters. Some studies found effects on the soil chemistry (which is often the main focus), whereas effects on the subsequent crop performance are much rarer (Congreves et al., 2013b). This is surprising, since the preferred outcome of HCA N immobilization over winter would be to retain more N in the topsoil, thus making it more available to the next crop and reducing the N fertilizer needs of the subsequent crop.

In our study, we did not find strong evidence of remineralization of immobilized N due to HCA (**Table 3**). On the contrary, sawdust application had a negative effect on winter barley yield, potentially caused by strong N immobilization under either N fertilizer levels. Wheat straw application resulted in a positive trend of winter barley yield under higher N conditions (**Figure 3A**), which could be either caused by remineralization of immobilized N during the growing season, but also due to decomposition and subsequent N release contained in the wheat straw itself (Di and Cameron, 2002). We found no difference in total N content in soils in March 2017, which indicates a lack of N transfer over fall/winter, although at winter barley harvest we did find a significant increase of total N in the wheat straw and sawdust treatment compared to the control (**Table 3**). This increase might be mainly due to fertilizer added during the winter barley growing season being immobilized in the soil rather than an N carry-over effect from the precrop. It is worthwhile noting that because of the small particle size of sawdust some particles were not sieved out with a 2 mm sieve before milling, while pieces of wheat straw were, which could inflate the soil C measurement. However, the results were consistent and wheat straw also showed a higher total C (%) than the control (**Table 3**).

We found a strong reduction in nitrate leaching when wheat straw was applied to faba bean as a precrop (**Figure 5**). Other studies on HCAs and nitrate leaching show mixed results. Chaves et al. (2007) found a reduction in N leaching of 56–68% due to wheat straw or sawdust after high N vegetable crop residues, which might be comparable to the increased leftover N in legumes. On the other hand, little to no reduction in nitrate leaching due to straw incorporation was found by Thomsen and Christensen (1998) when cereal crops or sugar beet were grown beforehand, similar to our findings for spring barley as a precrop. A common finding in both these studies is that remineralization in the next spring does not seem to occur in considerable amounts. Paradoxically, HCAs could increase N leaching when immobilized N is being mineralized next fall instead. Finally, we did not find a precrop species effect on soil C/N ratios or total soil C or N contents in either March before N fertilization or at winter barley harvest, despite the clear reduction in nitrate leaching after faba bean amended with wheat straw. This could be because the mineral N pool is relatively small compared to the total N pool, and, coupled with the hypothesis of long-term immobilization due to HCAs, might explain the lack of a positive HCA effect, especially under low N conditions.

CONCLUSION

Using a semi-natural setup our experiment bridged the gap between short-term artificial greenhouse experiments and the heterogeneity of field studies, allowing for relatively realistic weather conditions and temporal scale whilst reducing spatial heterogeneity, in order to improve our understanding of carry-over effects of precrops. We found evidence that AMF precrops had possibly parasitic effects on the subsequent winter barley when large amounts of fertilizer were added to the system, whereas there was no clear legume precrop effect. In our setup, short-term immobilization of N by HCA addition after harvest was not generally achieved, despite a slight positive effect of wheat straw on winter barley yield. HCAs do show potential to counter nitrate leaching of high-risk leaching crops such as grain legumes. Furthermore, other effects such as an increase in SOM or nutrient retention could play a positive role in the long term, since we found higher soil total C and total N nearly a year after application of HCAs.

AUTHOR CONTRIBUTIONS

VT and RvD designed the experiments. RvD and JR collected the data. RvD analyzed the data. RvD and VT led the writing. WH reviewed the manuscript. All authors contributed to critical revisions of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00912/full#supplementary-material

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5.2 Paper 2: Timing Matters: Distinct Effects of Nitrogen and Phosphorus Fertilizer Application Timing on the Root System Architecture of *Hordeum vulgare*

van Duijnen, R., Uther, H., Härdtle, W., Kumar A., and Temperton, V. M. Timing Matters: Distinct Effects of Nitrogen and Phosphorus Fertilizer Application Timing on the Root System Architecture of *Hordeum vulgare*.

Timing matters: Distinct effects of nitrogen and phosphorus fertilizer application timing on the root system architecture of *Hordeum vulgare*

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Abstract

Background While different plant foraging responses to the two macronutrients nitrogen (N) and phosphorus (P) are well researched, the effect of timing of fertilizer application on root system architecture (RSA) remains largely unknown.

Aims We therefore aimed to understand how RSA of *Hordeum vulgare* L. responds to timing of N and P application.

Methods Plants were grown in rhizoboxes for 38 days in nutrient poor soil and watered with nutrient solution, lacking either N or P, with the absent nutrient applied at either 2/3/4 weeks after sowing. Controls were continuous N and P and a negative control receiving both N and P once after 3 weeks. We tracked root growth over time, measured plant biomass and nutrient uptake.

Results Late N application strongly reduced total biomass and visible root length compared to continuous NP and late P application. Root mass fractions remained similar over all treatments, but relative allocation (% of total root biomass) was higher in lower depth with late N application. Shoot P concentrations remained relatively stable, but late P plants accumulated more N. Late N application had overall more negative effects on early plant growth compared to late P.

Conclusions Hence, sustainable fertilization practices need to consider nutrient specific timings for optimal fertilizer use.

1. Introduction

The increase in food production over the last century was coupled with a steep increase in inorganic fertilizer production and use (Tilman et al., 2002). Although yields increased significantly, excessive fertilizer use also lead to multiple negative effects on the environment, such as eutrophication of terrestrial and aquatic ecosystems (Bennett et al., 2001; de Vries et al., 2013). Nitrogen (N) and phosphorus (P) are, next to potassium (K), the major macronutrients essential for plant growth, and thus make up the bulk of the fertilizer mass. There are differences in terms of the relative importance of these two elements for plant growth, depending on background soil fertility of these elements in soils. P stocks in European agricultural soils are generally high due to a surplus of P imports and accumulation of P in fixed form (van Dijk et al., 2016). This residual soil P can provide an important steady source of P to crops over multiple years, and is often ignored (Sattari et al., 2012). Hence, for European farming settings, P is often not as limiting to plants as N. Although it is seemingly common knowledge amongst (European) farmers, that adding N too late to a plant crop is more detrimental to plant performance than adding P too late, this has so far received barely any attention in agronomic research. Understanding the relative effects of adding N or P fertilizer later than the other on crop performance could lead to strategies for improving nutrient uptake efficiency, efficient fertilizer use, and hence sustainability outcomes.

Most studies so far have looked at either timing of application of single N or P fertilizer elements (Scharf et al., 2002; Van Es et al., 2004; Efretuei et al., 2016; Finnan et al., 2019) or specifically at effects of *spatial* differences in nutrient availability using nutrient patches on plant performance (Drew, 1975; Hodge, 2004; Ma et al., 2013; Li et al., 2014). Recently, Nabel et al. (2018) used both a spatial and temporal approach, adding maize biogas digestate in patches to the prairie plants *Sida hermaphrodita*, and found that roots initially avoided the nutrient patch until ammonium had been converted to nitrite and nitrate, after which massive root proliferation into the patch occurred. To our knowledge, no-one has yet studied how relative temporal availability of N and P affect plant performance either via aboveground or belowground plant

trait plasticity. A multitude of crop root systems have been investigated for their response to N or P deficiency (Liao et al., 2001; Niu et al., 2013; Trachsel et al., 2013). Generally speaking, these responses are linked to the spatial availability of the specific nutrient element. For example, P-deficient plants allocate more resources into top soil foraging where most of the P is located (Lynch and Brown, 2001). Furthermore, morphological and root system architecture (RSA) responses, such as an increase in lateral root length over amount of laterals, increase in lateral root elongation steeper angle of laterals, and an increase in root hair length/density are all common plant responses as a means to increase P uptake when it is limiting (Linkohr et al., 2002; Haling et al., 2013). In contrast to P-limited plants, N-limited plants tend to invest more roots in deeper layers, since N (especially in the form of NO_3^- ions) is more mobile in soil than P, and they achieve this by changing root growth angles – a root architectural response (Trachsel et al., 2013). Consequently, breeding for these specific adaptations in crops could prove advantageous for productivity in N and P deficient soils (Lynch, 2011, 2013).

What about the timing, and the relative timing of N and P application however? Fertilizers are often applied to crops at the time of sowing, although this is likely to be suboptimal regarding nutrient-use efficiency due to low initial uptake by seedlings, risk of leaching and gaseous losses (Wuest and Cassman, 1992; de Oliveira et al., 2018). Split N application, whereby N fertilizer is applied successively over time to adapt to the ontology and phenology of the crop to enhance uptake and reduce losses, has alleviated the environmental problems significantly (López-Bellido et al., 2005; Peng et al., 2012). However, the risk remains that crop establishment will be poor and that yield losses occur if plants are nutrient-deficient early in their growth (Binder et al., 2000; Grant et al., 2001). Besides the nutrients stored in the seed, the root system is responsible for providing N and P to the whole plant shortly after germination. Understanding how young roots respond to N and P availability relative to one another could improve nutrient use efficiency and sustainability in cropping systems.

Plants are known for their phenotypic plasticity in response to nutrient deficiencies and heterogeneous nutrient supplies (Hodge, 2004). Since nutrients are taken up by plants directly from the soil, nutrient-deficient plants often invest relatively more resources belowground for nutrient acquisition via altered RSA and interactions with the rhizospheric microbiome. A frequently measured response to nutrient deficiencies, as predicted by resource optimization hypothesis (Bloom et al., 1985), is an increase in the root mass fraction (RMF) in relation to the total biomass ((Agren, 2003; Hermans et al., 2006). These adaptations are species-specific and range widely from physiological responses (changes in quality and quantity of root exudates) and morphological responses (root diameter, root hair length and density) to overall RSA responses (spatial distribution of roots, rooting depth, root orders and length, see (Koevoets et al., 2016). RSA responses to nutrient deficiencies have been especially well quantified in the model plant *Arabidopsis thaliana* (Gruber et al., 2013). Heterogeneous nutrient conditions often evoke a root proliferation response in the concentrated nutrient patch (Drew, 1975; Hodge, 2004; Li et al., 2014). In this way, the root system can adapt to environmental conditions and this proliferation response might also confer a competitive advantage (Robinson et al., 1999).

RSA responses to nutrient deficiencies in model plants are mostly measured in non-dynamic experimental setups however, that do not allow tracking of responses over time (but see Nabel et al., 2018) often with one time fertilizer application mixed in the starting soil, continuous application using a solution or in a solid growth medium (Xu et al., 2016; Hanlon et al., 2018). Hydroponics approaches provide an easy method to control the abiotic root environment by replacing the growing solution with a different combination of nutrients, but fail to mimic soil conditions where roots face mechanical impedance and heterogeneity in water and nutrient availability, meaning plants have to place their roots selectively. Very few studies explicitly tested timing of N and P fertilization on the root system (but see (Peng et al., 2012; de Boer et al., 2016), despite the common knowledge in agriculture of the importance of fertilizer timing and split fertilizer application. In addition, spatially explicit studies (finding root proliferation in and around

nutrient patches) far outnumber temporally explicit studies, potentially because of the difficulty of observing roots non-invasively over time. Given the key role of N and P for plant performance, it remains curious that so little attention has been paid to the effects of the timing of their application in relation to plant ontology and phenology. Because of the dynamics between nutrient mobility in the soil and nutrient uptake and depletion rates it is not clear how roots would respond over time. For example, under constant nutrient conditions in *Arabidopsis thaliana* seedlings, Gruber et al. (2013) found that slight N deficiency leads to root foraging (increase in total root length), but severe N deficiency to a decrease in total root length, whereas total root length always decreased with increasing P deficiency. Knowledge on interactions between root foraging and nutrient deficiencies over time would provide useful mechanistic information for innovative and more sustainable fertilization practices.

The objective of this study was to dynamically quantify root responses of spring barley (*Hordeum vulgare*) to relative timing of N and P fertilization, whilst also assessing overall shoot response and nutrient uptake. To do this we set up a 5.5 week rhizobox experiment and applied N or P at 2/3/4 weeks after sowing, giving a factorial design with three levels of N applied late, whilst P was added in straight away and continuously, and vice versa (P applied late and N applied straight away). The experiment involved one classical control with both N and P supplied continuously, as well as a negative control with both N and P applied only after 3 weeks. We measured the visible root length (VRL) non-destructively at the transparent front plate of the rhizobox over time, and root biomass allocation by depth, total shoot/root biomass, and C/N/P uptake at harvest at the end of the experiment.

We asked the following questions:

1a) How does the RSA of spring barley respond to N being applied later than P and vice versa?

1b) How does the length of time elapsed between fertilizing with one element and the next affect the RSA?

2) How do biomass allocation and plant nutrient (N, P) uptake respond to late N/P fertilization treatments?

We hypothesized that:

1a) Late N or P application will result in an increase in VRL compared to the control up to a point that N/P deficiency limits overall plant growth. Root elongation rate will decrease after the missing nutrient is applied due to sudden availability of this missing nutrient.

1b) The response described in 1a will be more pronounced the later the missing nutrient is applied (root foraging).

2a) Plants will increase biomass allocation to the root system when either N or P fertilization occurs later. These responses will differ between late N and late P however, with an increased investment of root biomass in deeper layers (N) or to top layers (P).

2b) Plants will exhibit a decrease in total shoot nutrient uptake and nutrient concentrations in shoot tissue for the nutrient which was applied later.

2. Material and Methods

2.1 Experimental setup

The experiment was conducted in the greenhouse of the Leuphana University Lüneburg (Lüneburg, Germany, 53°14'23.8"N 10°24'45.5"E) from June 12th 2017 to July 19th 2017 for a total of 38 days (five and a half weeks). The average day and night temperature and relative humidity were 21.2°C, 17.0°C and 63% and 78%, respectively. Rhizoboxes (58.0 × 26.6 × 2.0 cm^3, volume approximately 3L) were filled with a ~5 kg mixture of sand (dried at 60 °C, sieved at 2 mm), a nutrient poor, calcium carbonate rich, loess soil from a lignite mine near Jackerath, (Jackerath, Germany, 51°05'04.8"N 6°27'38.4"E; dried at 60 °C, sieved at 2 mm) and a peat potting soil (Nullerde, Einheitserde Werkverband e.V., Germany) in the ratio of 8:2:1 respectively. We chose this very low nutrient soil so as to more easily control relative nutrient applications with fertilizer as well as for a complementary study with and without mycorrhizae (data not shown). Soil was compacted by dropping the rhizobox twice from a set distance of 30 cm after each liter of soil was added. The soil mixture resulted in a nutrient poor soil with a total N content of 170 mg/kg, total P content of 135 mg/kg, available P content of 10.47 mg/kg (Olsen P), and a pH of 6.69 (0.01 M $CaCl_2$). At the start of the experiment, rhizoboxes were saturated with deionized water and left to drain for 24h, and watered with Hoagland solution for the rest of the experiment.

We sowed spring barley (*Hordeum vulgare* L. cv. Barke, Saatzucht Breun, Germany) at a rate of one plant per rhizobox. Seeds were pre-germinated for 24h on wet tissue paper and seeds with a radicle of 1-2 cm were transplanted at 1.5 cm depth. Rhizoboxes were placed at an angle of 45 ° to promote root growth along the front plate due to gravity. Five rhizoboxes were placed adjacently in a container to prevent light infiltration into the rhizobox and the front rhizobox was covered with a black plate. The last rhizobox was covered with a white polystyrene plate to prevent high temperatures during the day. The positions of the rhizoboxes were randomized twice weekly.

We used 8 different nutrient treatment levels to test the effect of timing of application and nutrient identity on root system architecture, biomass accumulation and nutrient uptake of spring barley. We applied all nutrients, except N or P, continuously with the total amount of lacking N or P applied at once after 2, 3, or 4 weeks. We included a control group (C; continuous N and P application) and a negative control (F; both N and P applied after 3 weeks) for a total of 8 treatments with 5 replicates (*n*=5) resulting in 40 rhizoboxes (see Table 1). Rhizoboxes were watered 3 times per week with modified Hoagland solutions. 120 ml per week was added for the first 4 weeks, which was increased to 180 ml per week for the last 1.5 weeks. Full Hoagland solution (macronutrients: 5 mM KNO_3, 5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 2 mM $MgSO_4$, micronutrients: 46.3 µM H_3BO_3, 9.2 µM $MnCL_2$, 0.77 µM $ZnSO_4$, 0.36 µM $CuSO_4$, 0.01 µM MoO_3, 50.12 µM FeNaEDTA, pH set to 5.8-6 with 2M NaOH) was used for the control group. For treatments lacking N or P, the Hoagland solution was modified to keep the osmotic potential and other continuous nutrients equal to the control group. For late N, 5 mM $Ca(NO_3)_2$ and 5 mM KNO_3 were replaced with 5 mM $CaCl_2$ and 5 mM KSO_4, respectively. For treatments lacking P, 1 mM KH_2PO_4 was withheld and 5 mM KNO_3 was increased to 6 mM and 5 mM $Ca(NO_3)_2$ to 4 mM to accommodate for the loss of K. This change resulted in a slight difference in total N applied in these treatments due to the different molar ratios of N in KNO_3 and $Ca(NO_3)_2$. For the negative control (F), both these changes to the Hoagland solution were made. The treatments lacking either N and/or P received the respective total amount that the control group received over the 38 days (151.3 mg N and/or 22.3 mg P) at 15, 22 or 29 days after sowing (DAS). N was applied as 5 ml of 2.16M KNO_3 and P as 5 ml of 0.144 M KH_2PO_4.

Table 1: List of treatments related to timing of N/P application and abbreviations used throughout this study. The nutrient that is not mentioned in the abbreviation is applied continuously.

Treatment	N and/or P application
C (control)	Continuous N+P
F (negative control)	N+P applied at 3 weeks
P2W	P applied at 2 weeks
P3W	P applied at 3 weeks
P4W	P applied at 4 weeks
N2W	N applied at 2 weeks
N3W	N applied at 3 weeks
N4W	N applied at 4 weeks

2.2 Measurements and harvest

A ‘photobox’ was built to quantify root length over time by acquiring images of the front plate of the rhizobox (as described in (Delory et al., 2018). The photobox consisted of metallic frame to hold the rhizobox, a camera holder at a set distance of 54 cm, and two LED lights (BB&S Pipeline FREE, 4300K, 60 cm length), incorporated within a lightproof container. The inside of the container was lined with black cloth**,** and together with the LED lights allowed us to illuminate the rhizobox uniformly without reflections of the transparent front window. Starting 5 DAS, pictures were taken three times per week with a digital camera (Canon EOS 5D Mark III) equipped with a 28 mm lens (Canon EF 28mm f/2.8) at a resolution of a 5760 x 3480 pixels in JPEG. Afterwards, pictures of each sampling day were converted to 8-bit grayscale, and visible roots along the transparent front plate were drawn manually using the SmartRoot plugin (Lobet et al., 2011) for ImageJ (Schneider et al., 2012). The corresponding Root System Markup Language (RSML) file produced by SmartRoot was converted to a table using archiDART (Delory et al., 2016) and the VRL per day was calculated and plotted using R (R Core Team, 2017).

All plants were harvested 38 DAS for shoot and root measurements. One control plant was excluded from all analyses due to poor growth and visible signs of disease at harvest. Shoots were cut off at the base of the stem and dried at 80 °C. Rhizoboxes were opened carefully and soil layers were cut at 10 cm depth intervals. These layers were washed with a hose above a 2 mm sieve. A comb was used to catch washed roots and the 2mm sieve was checked for washed through roots. Washed roots were stored at -20 °C until fine washing with a brush and tweezers to remove the last soil and organic peat material from the roots. A subset of 8 random washed samples (1 per treatment) was stained overnight with an excess of 1.7 mM neutral red, extensively rinsed after, cut into smaller pies to avoid root overlap, and scanned at 400 dpi using an Epson Perfection V800 Photo (Epson, Japan). Scanned images were analyzed with WinRHIZO (Regent Instruments, Québec, Canada), using the automated global thresholding and local adjustments were made manually to prevent over- or underestimation of the total root length (Delory et al., 2017).

The total root lengths (TRL) obtained with WinRHIZO were then used to correlate with the respective VRL at the harvest date. Afterwards, all roots were dried at 60 °C until constant weight and dry weight per depth layer was measured. Shoot material was ground with a ball mill (MM 400, Retsch, Germany) to a fine powder and total C and N was analyzed with an elemental analyzer (Vario EL, Elementar, Germany). For total P analysis, 200 mg plant sample was digested with 3 ml 37% HCl, 3 ml 65% HNO_3 and 1 ml 30% H_2O_2 in a microwave (MARS Xpress, CEM GmbH, Germany). Digested samples were analyzed with an ICP-OES (Optima 3300 RL, Perkin Elmer Inc., USA).

2.3 Statistical analysis

We used one-way analysis of variance (ANOVA) to test for the effect of the treatments (fixed factor, different combinations of N and P timing, total of 8 levels) on root and shoot biomass, root biomass by rhizobox depth, VRL at the end of the experiment, and nutrient related measurements such as N%, P%, C:N and N:P ratios, and total N and P uptake. If the ANOVA results were significant ($p< 0.05$), we followed up with a Newman-Keuls post-hoc test to determine significant differences between the treatments at $\alpha=0.05$ using the R package agricolae (De Mendiburu, 2014). Residual and QQ-plots were used to visually assess normality and homogeneity of variance. We determined the Pearson correlation coefficient between the VRL and TRL and fitted a regression line. All statistical analyses were performed using R 3.4.2 (R Core Team, 2017).

3. Results

3.1 Biomass responses to treatments at harvest

Overall, both for aboveground and belowground biomass, applying N late reduced total biomass on average by 52-61% compared to control plants supplied with continuous full Hoagland solution, and how long the plant had to wait for N did not generally matter (Fig. 1). Similarly, applying P later also reduced biomass, both for roots and shoots, but only when the time until fertilization occurred exceeded two weeks (as with the reduction in biomass when applying N later but less strongly – a reduction of on average 25-37% in biomass in the P3W and P4W treatments compared to the control). When P was applied after two weeks, we found no significant differences in above- or belowground biomass compared to the control plants. Late application of both N and P after 3 weeks (negative control; F) was most similar to, and not significantly different from, N applied after 3 weeks (N3W). However, this treatment had significantly higher shoot biomass compared to sole late N application after 4 weeks (N4W). Interestingly, relative root biomass investment at harvest (expressed by RMF) was not affected by treatments (average RMF by treatment: 0.18-0.21; one-way ANOVA, p=0.78). Despite similar RMFs, plants in late N treatments invested relatively less into root biomass in the top 10 cm of the rhizobox and more in deeper layers compared to plants of late P and control treatments (Fig. 2; Table 2).

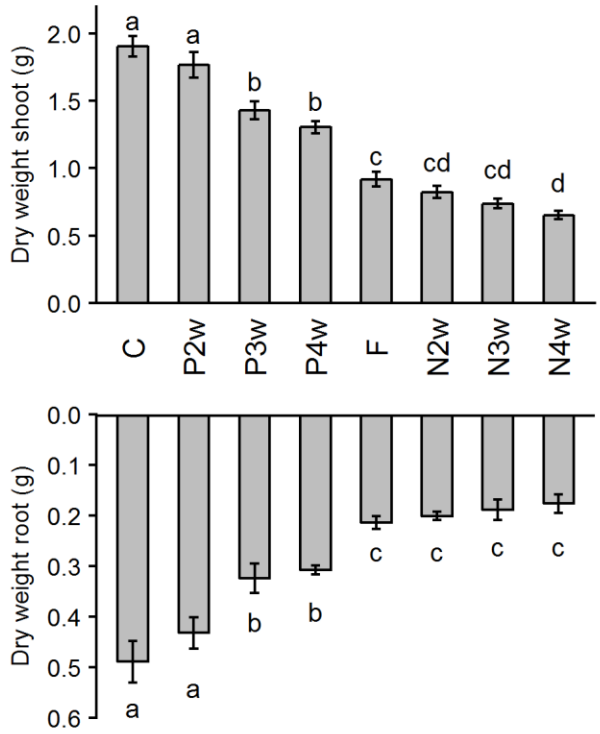

Figure 1: **S**hoot and root biomass at harvest as affected by the N and P timing treatments; Applying N later than P had a stronger effect on both above- and belowground biomass than adding P later than N, but waiting more than two weeks before adding P had a detrimental effect compared to waiting two weeks. Treatments are control (C; continuous N and P), P applied after 2/3/4 weeks (P2/3/4W), negative control (F; N and P applied after 3 weeks) and N applied after 2/3/4 weeks (N2/3/4W). Values are mean±SE (*n*=4-5). Different letters indicate significant differences between treatments (Newman and Keuls test, $P<0.05$).

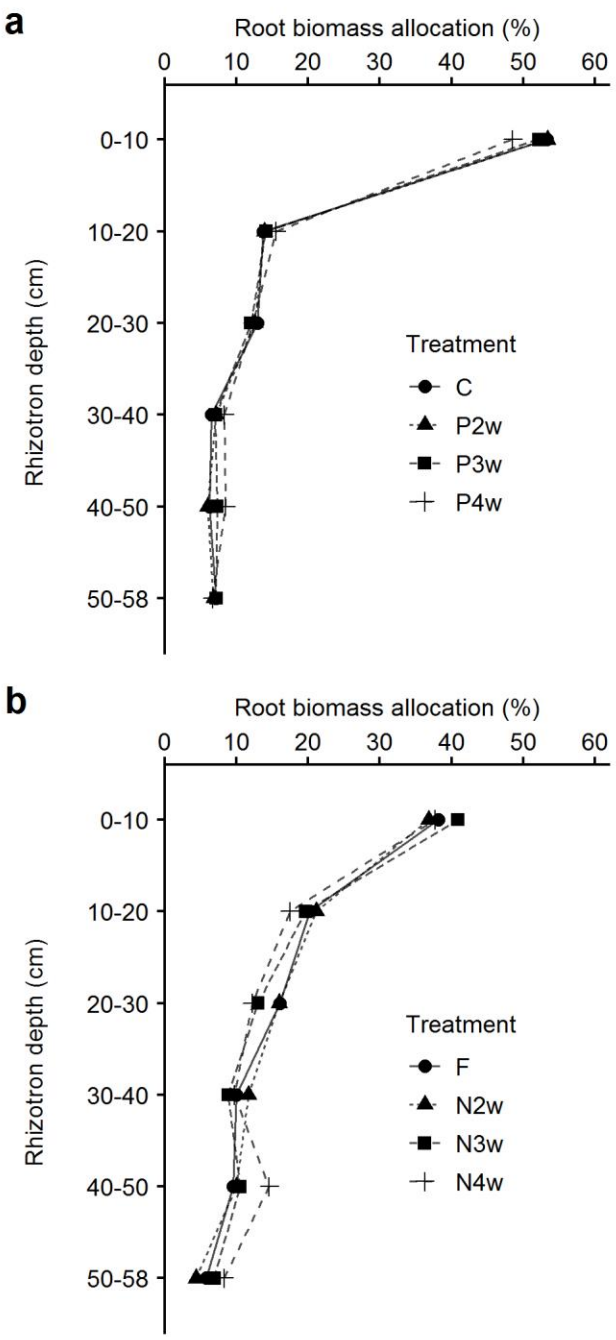

Figure 2: Relative biomass allocation of root biomass (%) for **a)** control (C; continuous N and P) and P applied after 2/3/4 weeks (P2/3/4W) treatments, **b)** negative control (F; N and P applied after 3 weeks) and N applied after 2/3/4 weeks (N2/3/4W) treatments. Values are means of the percentage of root in the respective depth layer (*n*=4-5). Standard errors and statistical tests are omitted for better visualization and can be found in Table 2.

Table 2: Relative root biomass allocation at harvest per depth layer. Values are means ± SE. Different letters indicate significant differences between treatments within the respective layers (Newman-Keuls test, $p<0.05$).

	Relative root biomass allocation (%)								
Depth (cm)	C	P2w	P3w	P4w	F	N2w	N3w	N4w	ANOVA *p*-value
0-10	53.3±1.1 [a]	52.8±3.4 [a]	53.6±1.2 [a]	48.1±2.0 [ab]	38.5±2.5 [b]	38.6±5 [b]	41.7±2.8 [ab]	37.1±3.3 [b]	**<0.001**
10-20	13.7±1 [a]	14.5±0.7 [a]	14.1±0.2 [a]	15.5±0.9 [a]	20.3±0.7 [b]	20.3±1.7 [b]	18.2±1.2 [ab]	18.0±0.5 [ab]	**<0.0001**
20-30	12.9±0.7	13.0±0.6	11.6±0.8	12.2±0.2	16.4±4.1	15.5±1.1	13.6±2.5	12.2±0.2	0.5406
30-40	6.5±0.6 [a]	6.9±0.6 [a]	6.6±0.6 [a]	8.4±0.5 [ab]	9.1±1.3 [ab]	11.5±1.2 [b]	8.5±1.7 [ab]	9.4±0.6 [ab]	**0.0225**
40-50	6.3±0.3 [a]	5.8±0.8 [a]	7.4±0.3 [a]	9.0±0.9 [a]	9.3±1.2 [a]	9.9±1.1 [a]	10.3±1.0 [ab]	14.9±1.8 [b]	**<0.0001**
50-58	7.2±1.0	7.1±1.0	6.7±0.8	6.8±0.8	6.4±1.0	4.1±0.5	7.8±3.5	8.3±0.9	0.7035

3.2 Visible root length over time

Visible root length increased similarly over time for late P application compared to the control (Fig. 3a), although VRL was slightly reduced from 26 DAS onwards when P was applied after 3 or 4 weeks. Late N application showed a different pattern, with an overall much lower VRL (Fig. 3b). The VRL increased starting from 22 DAS if N was still not applied after 4 weeks (N4W) compared to N2W and N3W. This was followed by an alignment (lower VRL increase) at harvest date when N was eventually applied after 4 weeks. Initial N-deficient plants increased their VRL compared to P-deficient or control plants up to 12 DAS, but had lower VRLs thereafter (Fig. 3c). These results show average trends (including errors) over time, but these are not, at least on a temporal scale, statistically testable mainly due to repeated measurements on the same biological replicates. We opted to statistically test the outcome of the different treatments on the VRL at the harvest time point instead.

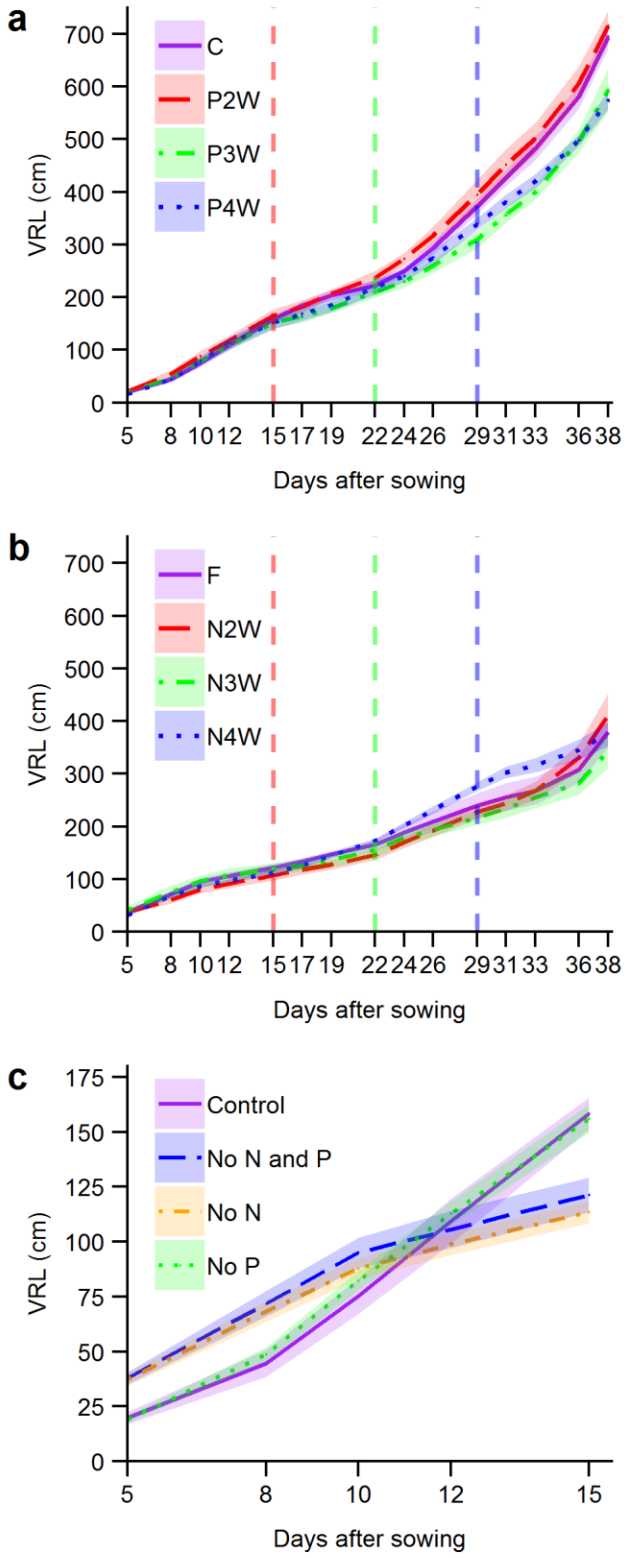

Figure 3: Visible root length (VRL) over time for **a)** control (C; continuous N and P) and P applied after 2/3/4 weeks (P2/3/4W) treatments, **b)** negative control (F; N and P applied after 3 weeks) and N applied after 2/3/4 weeks (N2/3/4W) treatments, and **c)** early response of root length until first N/P application at 15 days after sowing of the late N/P treatments. Vertical lines in panels **a)** and **b)** indicate the 2, 3 and 4 week time points at which N or P was applied. In panel **c)** plants that did not receive N or P yet until 15 days after sowing (treatments N2/3/4W for no N, P2/3/4W for no P, treatment F for no N and P) are grouped. Values are means ± se (*n*=4-5 for panels **a** and **b**, *n*=4-15 for panel **c**).

3.3 VRL at harvest date and correlation with scanned subsample

All plants, except those that received P after two weeks (P2W), had significantly reduced VRL compared to the control (15-17% reduction for P3W/P4W, up to 41-51% reduction for late N treatments; Fig. 4). Furthermore, late N application had a stronger effect compared to late P application. Although there was a timing effect regarding P application, late N and late N and P had similar visible root lengths, regardless of the week when N was applied. These patterns are similar to those found in the root biomass at harvest (see Fig.1).

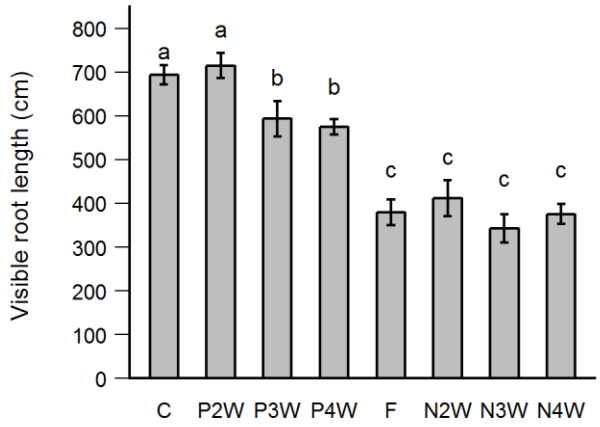

Figure 4: Visible root length at harvest (38 DAS) as affected by the treatments**.** Adding N late has stronger effect than adding P late. Treatments are control (C; continuous N and P), P applied after 2/3/4 weeks (P2/3/4W), negative control (F; N and P applied after 3 weeks) and N applied after 2/3/4 weeks (N2/3/4W). Values are mean ± SE (*n*=4-5). Different letters indicate significant differences between treatments (Newman and Keuls test, P<0.05).

The visible root length at harvest time correlated very well with a subsample of roots scanned with WinRHIZO (Fig. 3; ρ=0.959, p<0.001). Overall, 4.5-5.1% of the total root length (Fig. 5, calculated from fitted regression line: TRL=17.6*VRL+16.3) was visible along the Plexiglas side of the rhizobox at the end of the experiment.

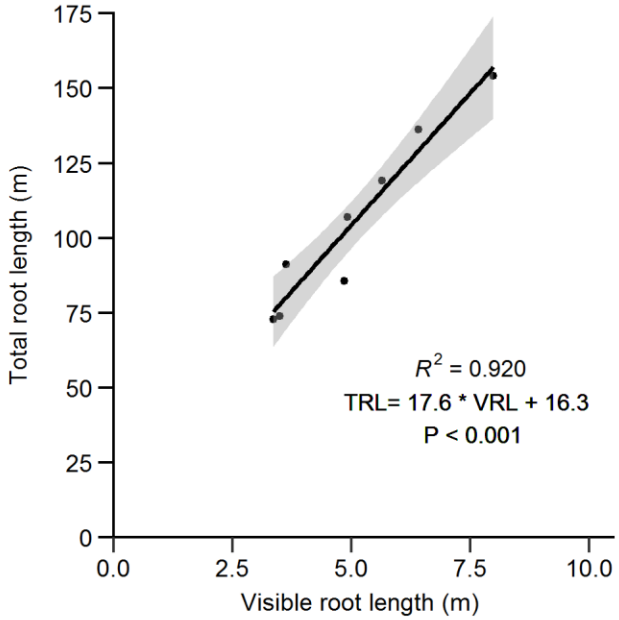

Figure 5: Correlation between visible root length and total root length of randomly selected (1 per treatment) scanned samples measured with WinRHIZO at harvest. The solid line indicates a regression line between visible root length and total root length and the grey shade the confidence interval of the regression fit.

3.4 Nitrogen and phosphorus uptake

Late P or N application resulted in two very different responses regarding shoot nutrient concentrations. Late P had a trend of increased N concentrations and corresponding decrease in C:N ratios with increased delay in P application (Fig. 6a, c). In contrast, plants within the late N treatments showed no significant differences in leaf N concentrations (%) and C:N ratios irrespective of how much time elapsed before N was applied (Fig. 6a, c). P concentrations were less variable across treatments and only the most contrasting treatments showed significant differences (Fig. 6b): Earliest P application (P2W) had the lowest leaf P concentrations whereas the latest N application (N4W) had the highest. N:P ratios indicate that P late treatments were quite similar to the control, but a trend that P2W was more P-limited (N:P ratio >18; see (Koerselman and Meuleman, 1996) and all N late and the negative control treatments were N-limited (N:P ratio <15; Fig. 6d). Total N and P uptake was severely lower for all late N treatments, mainly

due to a decrease in biomass (Fig. 6e, f, also see Fig. 1). A similar pattern as plant biomass was found for total N uptake in late P, although total P uptake was similar for all three late P treatments (Fig. 6e, f).

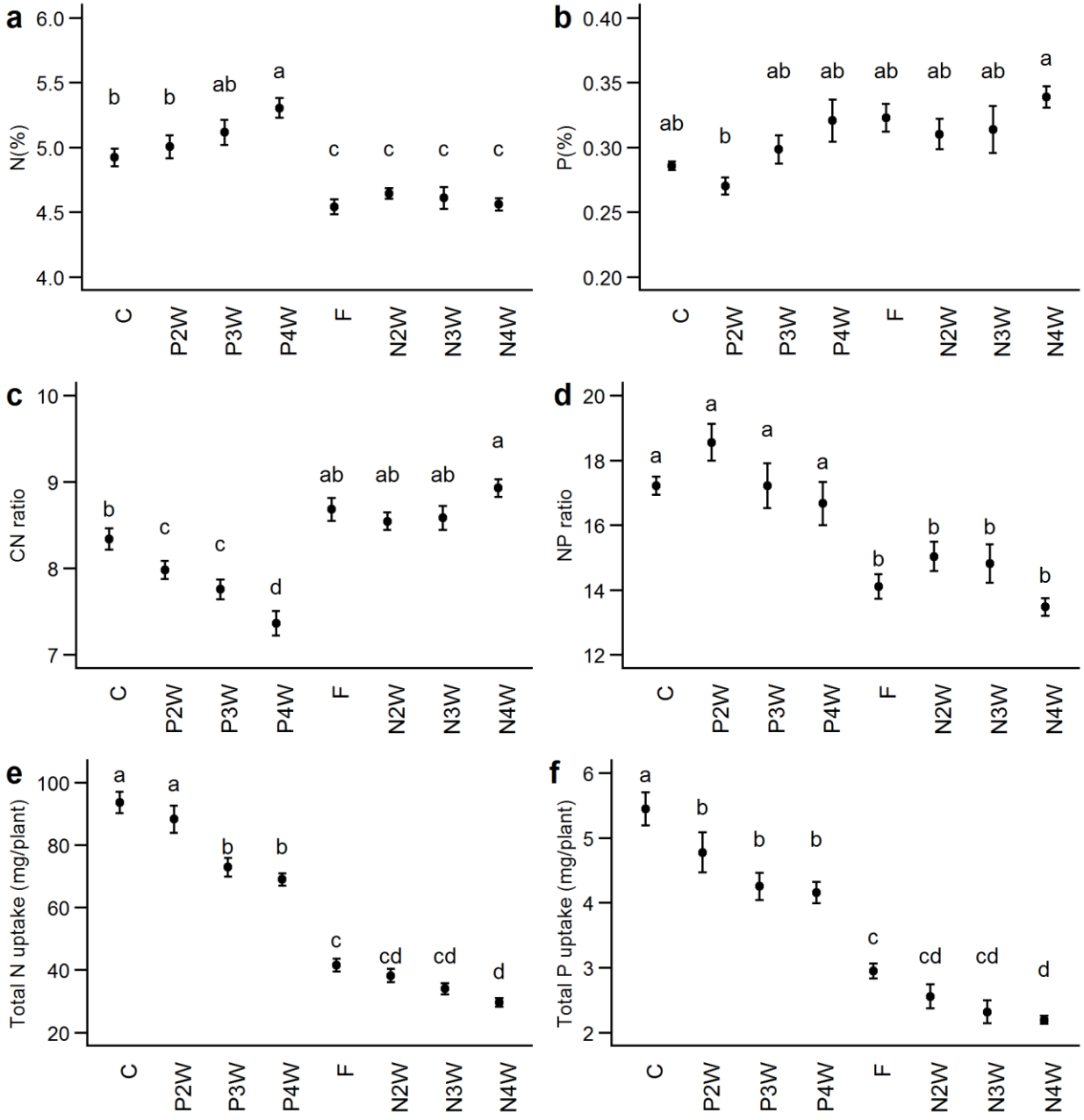

Figure 6: Effect of the treatments on shoot **a** N%, **b** P%, **c** C:N ratio, **d** N:P ratio, **e** shoot total N uptake and **f** total P uptake. Treatments are control (C; continuous N and P), P applied after 2/3/4 weeks (P2/3/4W), negative control (F; N and P applied after 3 weeks) and N applied after 2/3/4 weeks (N2/3/4W). Values are means ± SE. Different letters indicate significant differences between treatments (Newman-Keuls test, $p<0.05$).

4. Discussion

In this study we determined RSA and below- and aboveground responses of *H. vulgare* to timing of N and P fertilization. Late P fertilization, beyond two weeks after sowing, resulted in a significant decrease in root length and biomass, whereas late N application had a detrimental effect irrespective of time and differences between timing of fertilization mattered less. To our knowledge, this is the first study to explicitly test timing of fertilizer application (including both N and P) on root length over time with dynamic, non-invasive root measurements. The use of rhizoboxes allowed us to see an initial root response to N deficiency in the first ±10 DAS and an additional root foraging response when N had still not been applied after 3 weeks.

4.1 Fertilizer timing effect on visible root length

We hypothesized that VRL would, at least initially, increase in all treatments compared to the control treatment, which had a constant supply of all nutrients and thus would have to invest the least in root foraging for nutrients. Moreover, this would occur in the timeframe that the lacking nutrient was not yet applied; after the nutrient was applied there would be a temporary excess of that nutrient thus less need to invest in root exploration. We did not observe any fast root responses, i.e. increase or decrease in root elongation within treatments days after N or P was applied, compared to treatments that would have already received this nutrient (Fig. 3a, b). However, the first hypothesis was only confirmed for the initial elongation response in, mostly, the seminal root system in early N-deficient plants (until ±10 DAS, Fig. 3c) compared to the positive control treatment. The fact that this response was not observed in the early P-limited plants could be due to sufficient P reserves in the seeds complemented by soil P. (Nadeem et al., 2011) found that maize plants rely solely on seed P up to 5 DAS, a mixture of seed and soil P up to 15 DAS and exclusively on soil P thereafter. This roughly fits our timeline of a decrease in plant performance when P was applied after three or four weeks. Still, in all late N/P applications, except for P2W, VRL was

eventually lower than the control group (Fig. 4). Thus, the absolute VRL did not increase significantly due to nutrient deficiencies.

Despite an initial rather strong root response to low N (steeper slopes of the lines over first 10 DAS), we observed another increase in VRL starting around 26 DAS, if N was still a limiting factor at this point e.g. in the N4W treatment (Fig. 3b). This response was noticeably absent in plants that had already received N by then. Interestingly, in line with our hypothesis this root elongation response was followed by a decrease in root elongation rate after fertilization after 4 weeks (31 DAS, and this effects lasted until end of the experiment). These root dynamics, with an increase followed by a decrease, resulted in net similar VRL values for all late N treatments by the end of the experiment (Fig. 4). This dynamic would not have been observed if the root length was only quantified at harvest time. We are not aware of any other studies finding such a response to nitrogen deficiency and subsequent fertilization. In a slightly similar experiment, in which *Zea mays* plants were initially N-deficient and thereafter subjected to a split root low/high N treatment, in ‘t Zandt et al. (2015) found that plants increased their root elongation rates in the high N side within 4 days, but these stayed high until the harvest 12 days after N application.

In general, experimental studies on timing of fertilizer application are rare, even more so those that focus on effects on the root system (Peng et al., 2012; de Boer et al., 2016). Peng et al. (2012) found similar results regarding early N-deficiency in field-grown maize, namely an initial increase in root length but severe growth limitation thereafter. De Boer et al. (2016) hypothesized that a delay of N fertilization after a harvest event on a *Lolium perenne* grassland could increase root biomass and N uptake, but found no effect for a delay up to 12 days and instead found that fertilization timing had a bigger impact on N dynamics in relation to heavy rainfall events and N leaching. Although this study was done in a slightly different agricultural setup, it shows that the timeframe of response of roots to fertilizer events is an important aspect for improving nutrient use efficiency. The fact studies that focus on root traits on a temporal scale are rare is most likely due to difficulties of non-invasively quantifying root traits, although

nowadays there is a multitude of options available, such as rhizoboxes, mini-rhizotrons, X-ray CT, and MRI and PET scans (Atkinson et al., 2019). These methods remain labor-intensive, although recent advances in machine learning look promising for reducing the labor required. Despite timing of fertilizer application being more common in agricultural studies (Limaux et al., 1999; Recous and Machet, 1999; Scharf et al., 2002), the focus in these studies is often on shoot traits, final grain yield or nutrient budgets and nutrient uptake. For example, Scharf et al. (2002) found no yield decline in maize if N fertilization was delayed until growth stage V11 (11^{th} leaf collar visible) and only a small yield decrease at V12-16 (12^{th}-16^{th} leaf collars visible). However, this is not directly comparable to our experimental setup, as we chose very poor initial nutrient conditions to amplify visible effects of fertilization on root growth. These conditions did not directly mimic agricultural fields, in which generally higher stocks of soil N are available. For example, soil total N concentration in croplands in southwestern Germany are around 1-2 g/kg (Chen et al., 2009) compared to 0.17 g/kg in our experimental soil. Thus, care needs to be taken with extrapolating our results into to the field.

4.2 Biomass allocation

As hypothesized, biomass decreased in all treatments compared to the control as expected, but this effect was stronger for delayed N than for delayed P application (Fig. 1). This could be because seedlings have a higher N requirement (compared to P) for early growth. Nonetheless, this cannot explain the marginal difference between N fertilization at 2, 3, or 4 weeks**.** This key result underlines that plants require N very soon after germination and that a delay of two weeks was not different in its effect from that of four weeks. It could be that plant recovery was faster the more N-deficient the plants were, as supported by similar N concentrations in the leaf tissue and overall shoot biomass across all N-late treatment levels, which again could be due to more efficient N uptake and utilization. A number of studies have found that plants are able to maintain similar metabolic and photosynthetic rates under very different N availabilities, and this is often due to an increase in nutrient use efficiencies (Geiger et al., 1999; Temperton et al., 2003).

Furthermore, RMFs were similar across all treatments, indicating no increased biomass allocation to roots. This was contrary to what we expected, because an often observed response to nutrient deficiency is an increase in biomass allocation to the root system (Hermans et al., 2006; Poorter et al., 2012b). According to the resource optimization hypothesis, plants will adapt to the most limiting resources by optimizing their traits to increase uptake of this resource, hence often adjusting their root to shoot ratio under varying environmental conditions. (Weiner, 2004) states that allocation is better understood as a function of size than time (an allometric perspective). It could be that in our timeframe, RMF was relatively non-plastic. However, modular plasticity, i.e. local adaptation of plant organs as Weiner (2004) defines it, was observed in late N treatment plants which invested relatively less root biomass in the top 10 cm layer, and more into deeper layers (Fig. 2). This fitted our second hypothesis that root responses are different for N and P, such that we found evidence for resource optimization for spatial root foraging. Moreover, plants that received N after 4 weeks had significantly increased root biomass at a depth of 40-50 cm, which might also be the extra root production seen in the VRL increase due to N-deficiency after 30 DAS (as seen in Fig. 3b). Because N, applied as nitrate in our experimental setup, is more mobile in the soil, a significant amount of N will most likely have leached to deeper layers of the rhizobox, due to watering from the top. Thus, plants would benefit from investing root biomass in deeper layers as well. However, we did not see a similar response for root investment in top layers, in which most of the fertilizer P would accumulate, for late P compared to the control. This could be either due to less stressed plants and a less pronounced P-deficiency root response, or adaptive responses in other root traits such as root hairs. Root hairs are known to be essential for acquiring P because P is highly immobile in the soil due to adsorption and precipitation, and uptake by bulk flow and diffusion is very low (Gahoonia et al., 1997; Haling et al., 2013). Unfortunately root hairs were not quantifiable in our rhizobox setup.

The similarity of RMFs between all treatments could have various causes. One might be that rhizoboxes force the roots to grow in a more two-dimensional space, hence the roots are restricted in their growth.

Another rhizobox study using wheat (*Triticum aestivum* L.) also found no difference in RMF under low/high P availability (Bauke et al., 2017). Container size can have a large impact on plant growth by a reduction of net photosynthesis (Poorter et al., 2012a). Poorter et al. (2012a) also suggests to not exceed plant biomass by 1 g L^{-1}, which was not the case for even our largest control plants, but nevertheless plant roots can accumulate at container edges (also see Poorter et al. 2012a). Another explanation could be the use of crops, in our case *H. vulgare* cv. Barke released in 1996, which have been bred for optimal yields in high nutrient input agriculture. Decades of selection in wheat (*Triticum aestivum* L.) indirectly selected for smaller, but more N uptake-efficient root systems (Aziz et al., 2017). Moreover, crops could also have indirectly been selected for less plasticity in the root systems due to breeding under homogeneous, high nutrient conditions (Grossman and Rice, 2012). Lastly, there might have been a difference in RMFs at the time plants were N/P-deficient, but allocation shifted from the root system to the shoots when the lacking nutrient was applied, thus obscuring previous root investments. This would make sense in light of the resource optimization hypothesis, although VRLs over time does not seem to support this shift in allocation (Fig. 3a,b). Overall, this hypothesis is not testable with our experimental setup, and would require multiple harvest times before and after nutrient application to test successfully.

4.3 Nutrient uptake

Total shoot nutrient uptake followed similar trends to the visible root length and biomass. However, leaf nutrient concentrations showed two main different patterns. First, we see an increase in N concentrations, and decrease in C/N ratios, the later the P (P2W compared to P3W/P4W) is applied (Fig. 6a**)**. However, biomass production was also reduced when P was applied after three or four weeks (Fig. 1). This together points towards more severe P-limitation as P application was delayed (as expected), but also lower P-utilization efficiency because total P-uptake was not significantly different at harvest (Fig. 6f). Hence N accumulated in the leaves, but this did not translate into more growth depending on the severity of the initial P-deficiency (i.e. time passed until P-fertilization). Secondly, all late N treatments had lower

N concentrations than the control group due to a lack of initial N availability. Interestingly, the timing of 2, 3, or 4 weeks N application had no significant effect on N/P concentrations or total N/P uptake (Fig. 6a, b, e, f). It could be the plants in these treatments were thus severely deficient in N that P uptake was also hindered. While P concentrations were not significantly higher than the control, N:P ratios clearly indicated less N uptake relative to P (Fig. 6d). Concluding, if N is applied in sufficient amounts, P availability matters for total N uptake and plant growth, but if N is not applied in sufficient amounts, P availability does not matter for either total N uptake, total P uptake or plant growth. This means that N is more important than P (at least in early plant development): If plants have no N, P uptake does not improve the plants growth.

Another interesting observation concerns the application of both N and P after 3 weeks (negative control; F), which had a trend towards increased aboveground biomass compared to N3W (Fig. 1). This treatment was significantly different from P3W, thus indicating more severe N-limited conditions than P-limited conditions. However, it was also not completely similar to N3W, and significantly different from N4W, regarding shoot biomass and total shoot N and P uptake (Fig 1; Fig. 6e,f). Liebig's law of the minimum states that the nutrient that is the least available compared to the plant requirements will limit the plants growth. Hence, plants will invest most in the acquisition of the limiting nutrient. However, alternate hypotheses exists, such as the multiple limitation hypothesis (Ågren et al., 2012), which argue that nutrient limitation is rather a spectrum with a smooth transition between either N or P limited plant states, although at extreme deficiencies plants follow similar responses to Liebig's law of the minimum. While in our experimental setup, N was certainly the primary limiting nutrient when both N and P were deficient, a trend of the negative control (where both N and P were applied after 3 weeks) acquiring more biomass than when only N was applied late could mean that a rather balanced, simultaneous application of N and P improves nutrient uptake and consequently biomass accumulation.

4.4 Relevance for agronomic practice

Within agronomic practice, the early root system in annual crop species is an important factor for proper crop establishment. The initial plant response to severe nutrient deficiency is to increase their (seminal in the case of monocots) root length. However, this is not a long-term strategy, as the carbon investments need to be balanced between the aboveground biomass (for primarily photosynthesis and later on seed production) and root biomass (for nutrient and water uptake, and anchorage and rhizodepositions). Obviously, in an agricultural context, prevention of yield loss is the most important reason for not delaying fertilizer application, but in an environmental quality context later application is mostly beneficial (Fageria and Baligar, 2005; Beillouin et al., 2018). We showed that initial N deficiency actually stimulated root length increase up to a certain point, which was coupled with a significant overall biomass decrease at the end of the experiment. Certainly, this threshold should be avoided at all cost, and a more complete picture would aid in this. For example, biotic interactions, such as intraspecific competition, can increase root investments (Hodge, 2009) and plant-microbe interactions can alter RSA responses (Vacheron et al., 2013). On the other hand, abiotic conditions, such as water scarcity/drought, can interact with N deficiency and lead to root investments in deeper layers as both resources are commonly found there (Wasson et al., 2012; Lynch, 2013). Lastly, banding of fertilizer is a practical example to make clever use of this early root proliferation response, and has been shown to improve nutrient use efficiency, uptake and yield (Ma et al., 2013). Ma et al. (2013) found root proliferation in localized fertilizer application areas, which lead to an increase in nutrient use efficiency and eventually yield compared to broadcast fertilizer application.

5. Conclusions

Timing of fertilizer application significantly affected plant performance, and this was more the case when N was applied late than for P. Plants were less affected by P-deficiency, most likely due to higher seed and soil P stocks. If plants had sufficient N then P availability mattered for overall plant N uptake, but if plants lacked N then P availability did not matter. Thus, early N availability plays a more important role than early

P availability. Late P application did decrease root length and overall plant biomass, but without any clear dynamic root responses. N-deficiency clearly increased overall root length from germination up to 10 DAS, but severely limited growth overall afterwards. After four weeks, plants that were still N-deficient increased their root length a second time, but root length decreased again after the missing N was applied. This study emphasizes the advantages of additional more mechanistic details supplied by dynamic root observations studies, when fertilizer timing is of interest, and provides a basic understanding of the timeframe of spring barley root investments under N/P-deficiency and consequent supply.

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5.3 Paper 3: Measuring Plant Root Traits Under Controlled and Field Conditions: Step-by-Step Procedures

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Chapter 1

Measuring Plant Root Traits Under Controlled and Field Conditions: Step-by-Step Procedures

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Abstract

In this chapter, we present methods that we routinely use to measure plant root traits in the field and under controlled environmental conditions (using rhizoboxes). We describe procedures to (1) collect, wash, and store root samples, (2) acquire images of washed root samples, and (3) measure root traits using image analysis. In addition, we also describe sampling methods for studying belowground productivity, soil exploration, and root distribution in the first soil layers at the community level (soil coring and ingrowth core method). Because the use of rhizoboxes allows a nondestructive and dynamic measurement of traits hardly accessible in the field, a section of this chapter is devoted to the acquisition and analysis of images of roots growing in rhizoboxes.

Key words Root traits, Phenotyping, Rhizobox, Soil coring, Ingrowth core method, Root washing, Root staining, Image analysis

1 Introduction

In plant ecology, trait-based approaches are a powerful way to investigate how population and community changes impact ecosystem processes [1]. Among all the possible traits that can be measured on individual plants, those focusing on roots are of major importance. Belowground, the spatial configuration of root systems [2], the morphological features of individual roots [3], their capacity to acquire water and nutrients and release exudates into the rhizosphere [4], as well as their ability to interact with soil organisms [5] are important drivers of ecosystem processes such as plant productivity, carbon storage, nutrient cycling, and soil formation/stabilization [1, 6, 7]. A summary of traits commonly used in functional root research is provided in Fig. 1.

In comparison with traits measured on aboveground plant parts, root traits are particularly difficult to measure, mainly because of the belowground location of roots. In addition, the impossibility

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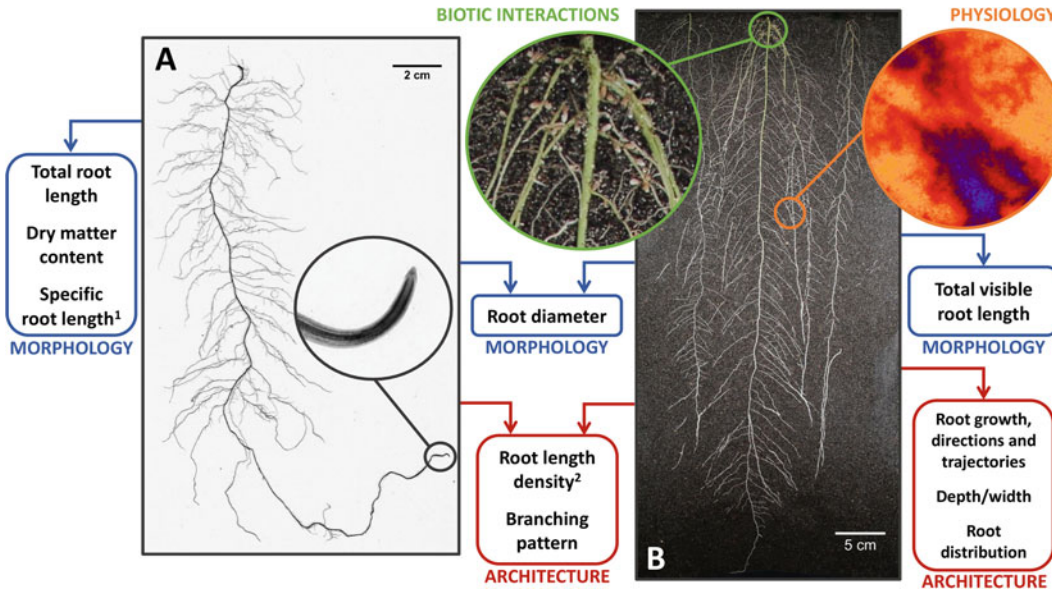

Fig. 1 Categories of root traits commonly measured on washed root samples (**a**) or on roots growing in a rhizobox (**b**). Biotic traits are illustrated by a picture of a *Trifolium pratense* root system showing a large amount of N_2-fixing nodules. Physiological traits are illustrated by a picture showing pH variations in the rhizosphere of tomato roots using planar optodes. [1]The specific root length is calculated as the ratio of total root length to root dry weight. [2]The root length density is calculated as the ratio of total root length to soil volume

to use simple root morphological markers to separate roots of different species in plant communities growing in the field or in pots makes the task even more challenging [1]. When plant scientists and ecologists wish to measure functional root traits in the field, they often extract soil cores and bring them to the laboratory to isolate the roots for further analyses [8]. Under controlled experimental conditions, however, a variety of root traits can be nondestructively measured using rhizoboxes [9–11]. Because of their transparent front window, rhizoboxes allow one to study root growth and physiology over time. Although rhizoboxes have a finite size constraining root development and hence duration of experiments as well as limiting the analysis of root system architectures in two spatial dimensions, their use is common in functional root research and allows for the nondestructive and dynamic measurement of biotic and physiological root traits that are not easily accessible in the field [12, 13]. An example might be the use of planar optodes for the quantitative 2D imaging of rhizosphere pH, O_2, and CO_2 dynamics [14, 15].

In this chapter, we present methods allowing the measurement of root traits in the field and under controlled environmental conditions (using rhizoboxes). In addition, we also describe sampling methods for studying belowground productivity, soil exploration,

and root distribution in the first soil layers at the community level. Although minirhizotrons are interesting methods to provide root length density estimates and fine root dynamics information in situ (e.g., root turnover) [16], their use is not described in this chapter. When plant root traits have to be measured, we describe methods that we routinely use in our laboratory to (1) collect, wash, and store root samples, (2) acquire images of washed root samples, and (3) measure root traits using image analysis. Methods describing how images of roots growing in a rhizobox can be acquired and analyzed are also included in this chapter.

2 Materials

2.1 Collecting Roots from Individual Plants

1. A garden fork or a spade.
2. Containers for transporting and washing the monoliths collected in the field (e.g., buckets).
3. A canister filled with tap water.
4. Plastic trays.
5. A scalpel or sharp dissecting scissors.
6. Containers to store the roots belonging to the target plants (e.g., plastic bottles).
7. Permanent markers (ink pens).

2.2 Collecting Roots from Soil Cores

1. Sharpened soil corers/augers.
2. Hammers.
3. A sharp knife and a ruler (for dividing the core into several layers).
4. Plastic bags.
5. Plastic labels.
6. Permanent markers (ink pens).
7. Cold boxes.

2.3 Collecting Roots with the Ingrowth Core Method

1. Opaque plastic ground sheet + material to hold the ground sheets in place in the field.
2. Shovels and spades.
3. Containers to store the soil collected from the field.
4. Sieves (mesh size: 2–5 mm).
5. Soil augers/corers (diameter: 5 cm).
6. Hammers.
7. Plastic trays.
8. A calibrated precision scale.

9. PVC tubes (diameter: 4–5 cm, length: 45 cm) + caps.
10. Mesh bags (polyamide fiber, mesh size: 1 mm, diameter: 4 cm, length: 45 cm).
11. A tool to compress the soil inside the mesh bags (e.g., a wooden stick).
12. Plastic labels (different colors).
13. Pliers.
14. Knives or sharp scissors.
15. Plastic bags (to store the ingrowth cores).
16. Permanent markers (ink pens).
17. Cold boxes.

2.4 Root Washing

1. A sink equipped with a soil collector.
2. A nozzle linked to a tap with a hose.
3. Nontransparent plastic trays.
4. Sieves (mesh sizes: 1–2 mm and 200–500 μm).
5. Tweezers.
6. A sharp knife and a template (for dividing the soil inside a rhizobox into several layers). A template could be a plastic-coated sheet of paper cut at the dimension of the soil layer.
7. A scalpel or sharp dissecting scissors.
8. Clean containers to store the roots after washing (e.g., plastic bottles).
9. Transparent plastic trays.
10. A colored background to put below the transparent plastic tray.
11. A soft paintbrush.
12. A desk light.
13. A Petri dish.
14. A plastic or a glass funnel.
15. Some filters (*see* **Note 1**).
16. A glass bottle used to recover the water after filtration.
17. Permanent markers (ink pens).

2.5 Root Staining

1. Some glass beakers for staining the root samples (one per sample).
2. Permanent markers (ink pens).
3. Staining solution: 1.7 mM neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride). Add about 200 mL of distilled water to a 1 L volumetric flask or a 1 L graduated cylinder. Weigh 0.5 g of neutral red and transfer it to the flask/

cylinder. Add distilled water to a volume of 1 L. Mix the solution at room temperature. Transfer the solution to a glass bottle. Seal the bottle with a cap. Store at 4 °C.

4. Plastic trays.
5. A plastic or a glass funnel.
6. Some filters (*see* **Note 1**).
7. A glass bottle used to recover the neutral red solution after filtration (waste bottle).
8. Tweezers.
9. Containers to store stained root samples (e.g., plastic bottles).

2.6 Acquiring Images of Washed Root Samples

1. An optical flatbed scanner equipped with a dual lighting system (e.g., Epson Perfection V800 Photo).
2. A plastic tray.
3. A scalpel or sharp dissecting scissors.
4. A waterproof transparent plastic tray (*see* **Note 2**).
5. Plastic tweezers.
6. Transparent plastic sheets cut at the dimension of the transparent plastic trays.
7. A syringe without the needle.
8. A plastic or a glass funnel.
9. Some filters (*see* **Note 1**).
10. A glass bottle.

2.7 Measuring Root Dry Weight

1. A drying oven.
2. A desiccator.
3. A calibrated precision scale.
4. Tweezers.

2.8 Acquiring Images of Roots Growing in a Rhizobox

If direct images of roots are taken with a scanner or a digital camera, only item 1 is needed. If roots are drawn on transparent acetate sheets, only items 2–4 are needed.

1. An image acquisition system relying on a scanner or a digital camera (*see* **Note 3**).
2. Transparent acetate sheets of the same size as the transparent front window of the rhizoboxes (1 sheet per rhizobox).
3. Ink pens of different colors.
4. A common flatbed scanner (reflective lighting system).

3 Methods

In this section, we present methods for measuring morphological, architectural, and biotic root traits on plants growing in the field or under controlled environmental conditions (using rhizoboxes). The main steps of these different protocols are presented in Fig. 2. In addition, we also describe sampling methods allowing the analysis of root productivity and root distribution at the community level (e.g., soil coring, ingrowth core method).

Please make sure to follow all waste disposal regulations when disposing waste materials.

3.1 Collecting Roots from Individual Plants (Soil Monolith Method)

1. In the field, select the plant on which root traits are to be measured.
2. With a garden fork or a spade, demarcate a soil monolith around the target plant. The size of the monolith will mainly depend on the plant species studied (age, size, root distribution pattern, etc.). Typically, soil monoliths can be 30–40 cm large and 30–50 cm deep [3].
3. Dig out the soil monolith and put it in a container.
4. Fill the container containing the soil monolith with tap water.
5. Wash the monolith until the roots are visible.

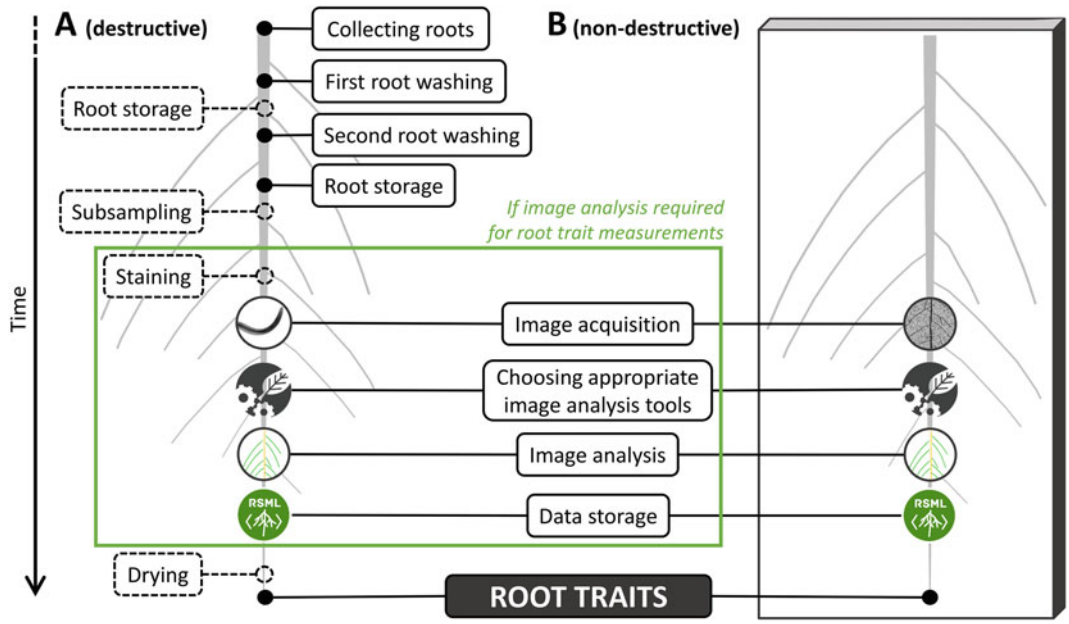

Fig. 2 Step-by-step procedures for measuring root traits on washed root samples (**a**) or on roots growing in a rhizobox (**b**). Boxes surrounded by a dashed line denote facultative steps (depending on the root traits that one wishes to measure). Logos of the Plant Image Analysis Database and the Root System Markup Language (RSML) were kindly provided by Guillaume Lobet

6. Trace the roots back to the shoot of the target plant. Remove the roots that do not belong to the target plant.
7. Put the roots of the target plant in a tray filled with tap water.
8. With a scalpel or sharp dissecting scissors, separate the roots from the shoot.
9. Store the roots inside a container labelled with the name of the sample and/or its identification number (*see* **Note 4**). Unwashed roots or roots that are not perfectly washed can be stored under humid and chilly conditions (e.g., 4 °C) for up to 3 days. For longer time periods, the samples should be stored in a freezer (−20 °C) (*see* **Note 5**).

3.2 Collecting Roots from Soil Cores

Soil coring is a technique commonly used in the field to study belowground productivity, root morphology, root distribution, and soil exploration by roots in the first soil layers at a given time point (*see* **Note 6**). With this technique, roots of several plant species are collected as fragments no longer connected to their parent. Because roots are heterogeneously distributed in the soil, a large number of samples are often required [16].

1. With a sharpened soil auger/corer, take a soil core at a spot where you wish to sample roots. Depending on your research question, you might want to collect roots only from the first soil layers or deeper (>0.5 m) into the soil. If deep and/or large cores are to be taken, more sophisticated mechanical devices will be required (e.g., soil corers mounted on vehicles) [16].
2. If you are interested in the vertical distribution of roots in the soil, divide your entire core into several depth layers (use a sharp knife and a ruler).
3. Store the samples in labelled plastic bags. If you divided the cores into several layers, store them in different plastic bags. Add an additional label annotated with the sample name and/or its identification number inside each bag (*see* **Notes 4** and **7**).
4. In the field, store the samples in a cool box. In the lab, unwashed roots or roots that are not perfectly washed can be stored under humid and chilly conditions (e.g., 4 °C) for up to 3 days. For longer time periods, the samples should be stored in a freezer (−20 °C) (*see* **Note 5**).

3.3 Collecting Roots with the Ingrowth Core Method

In a field experiment, the ingrowth core (IGC) method allows the measurement of fine root productivity over time. This is done by installing the so-called ingrowth cores (i.e., mesh bags filled with sieved soil, IGCs) into pre-drilled holes. Roots of a plant community are allowed to grow into the IGCs for a given time period.

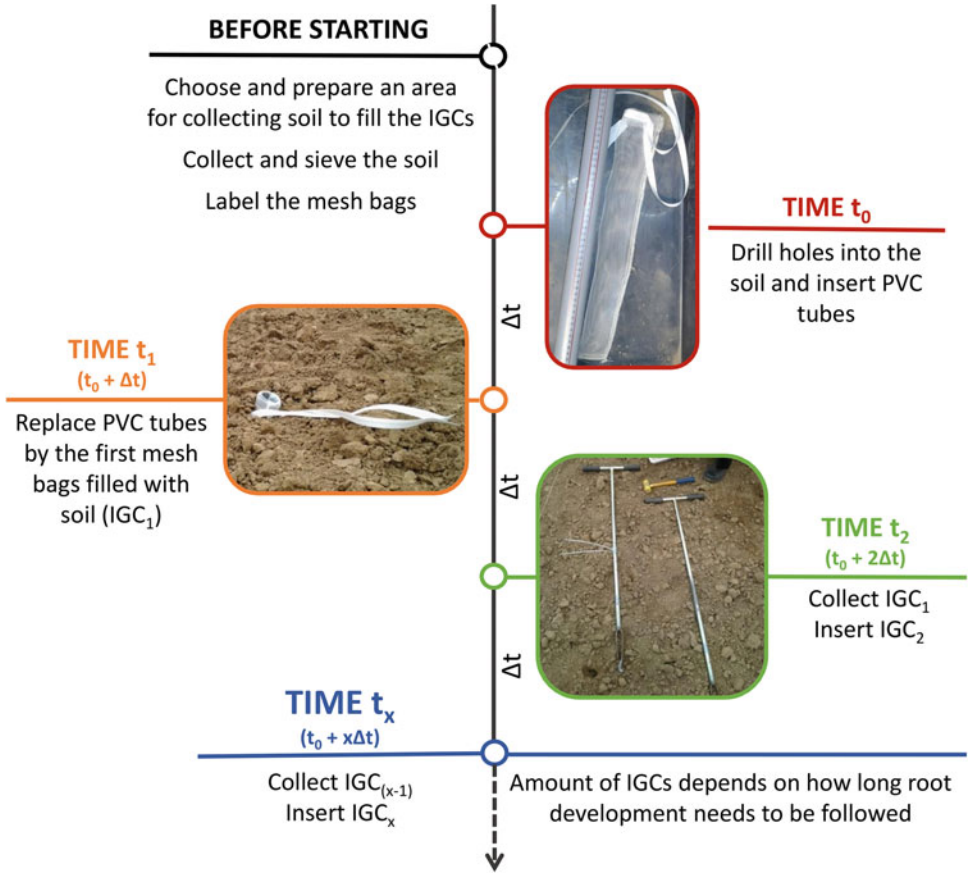

Fig. 3 Time line showing the main steps of the ingrowth core method used for measuring new root growth in the field. *See* Subheading 3.3 of this chapter (Collecting Roots with the Ingrowth Core Method)

Then, the IGCs are collected and fine root productivity is measured [17, 18]. In systems characterized by fast root proliferation (e.g., managed grasslands), the ingrowth time period is often short (e.g., 2 weeks), and the root decay inside the IGCs is considered negligible [19]. Therefore, the total amount of root material inside one IGC is considered to represent the total fine root productivity at a given location, for a given time period, and a fixed core volume [17]. At the plot level, fine root productivity can be followed continuously by activating new IGCs located in another part of the field plot at the end of each time period. A time line presenting the main steps of the IGC method is shown in Fig. 3. Hereafter, we present a protocol using an ingrowth time period of 2 weeks.

1. Choose an area adjacent to the main experiment with homogenous soil properties. Cover the soil surface of the chosen area with an opaque plastic sheet in order to suppress plant growth and obtain soil material free from living roots. After several months, collect soil from the topsoil layer (0–30 cm deep).

Store the soil in a container for transportation. Sieve the soil in order to remove any organic material (*see* **Note 8**).

2. Determine the dry bulk density of the soil at the location of your experiment. To do so, collect a number of soil cores from the study area. The volume of each core must be known. Bring the cores to the lab. Spread the soil samples in plastic trays, and let them dry at room temperature until constant mass is reached. Weigh the dry soil samples and determine the dry soil bulk density (ratio between the dry mass of a core and its volume).
3. Locate the spots where fine root productivity is to be tracked at the location of your experiment. Depending on the site of the area and the period of time you want to assess, you will need to sample a certain number of IGCs per plot. The exact locations need deciding before standing coring. Using a hand auger, drill holes into the ground at an angle of 45° to the soil surface. The holes should be deep enough to allow the insertion of the IGCs. Typically, 45 cm long IGCs allow the exploration of the first 30 cm of the soil. The number of drilled holes must be equal to the total number of IGCs that will be installed over the entire time frame of your experiment. Protect each hole by inserting one PVC tube. Cover the top of the PVC tubes with a plastic cap (*see* **Note 9**).
4. Calculate the amount of soil needed to fill one IGC (*see* **Note 10**). This value can be easily calculated by multiplying the volume of one IGC by the dry soil bulk density measured previously (point 2 of this subsection).
5. After 2 weeks, replace PVC tubes by IGCs at the locations where fine root productivity is to be measured for the first time period (2 weeks). To do so, take the PVC tube out of the soil, and insert it in an empty mesh bag. Put the mesh bag with the PVC tube inside back into the ground. Use the PVC tube as a funnel to fill the mesh bags with the appropriate amount of sieved soil (see **steps 1** and **4** of this subsection). During this process, gradually remove the PVC tube from the bag, so that only the mesh bag remains in the ground. A wooden stick can be used to press and compact the soil inside the mesh bag. Make sure that the soil compaction is similar between IGCs. When the mesh bag is filled with the required amount of sieved soil, close it with a knot and add a colored label on it (we advise you to use a different color for each time point) (*see* **Note 11**).
6. After 2 weeks, pull the IGCs out of the soil. Pliers might be useful at this step. The IGCs should be taken out carefully in order to avoid losing roots. If necessary, use a knife or sharp scissors to cut the roots growing outside the IGCs. Store the

IGCs in plastic bags, and keep them inside cold boxes until they are brought back to the lab. Be careful when labelling the samples collected from the field. For the IGCs, write the sampling name and/or its identification number in the bag's strap, as well as in an additional label connected to the IGC. Write the name of the sample on the plastic bag itself (*see* **Note 7**). Close the hole that remains in the soil after collecting an IGC.

7. In each plot, activate a new IGC as soon as one is removed from the soil. To do so, repeat **steps 5** and **6** of this subsection (*see* **Note 12**).
8. In the lab, unwashed roots or roots that are not perfectly washed can be stored under humid and chilly conditions (e.g., 4 °C) for up to 3 days. For longer time periods, the samples should be stored in a freezer (−20 °C). Mesh bags containing ingrowth cores can be stored in a freezer (−20 °C) as well (*see* **Note 5**).

3.4 Root Washing

The washing of roots is organized in two steps. The objective of the first washing is to remove most of the mineral particles and organic material that do not adhere to the roots (**steps 1**–**8** of this subsection). The operations described for the first root washing are best performed over a sink equipped with a soil collector underneath. For very sandy soils, this first washing is often enough to get clean roots. In most cases, a second washing will be required in order to separate the roots from small debris and other contaminants (**steps 9**–**16** of this subsection).

1. Fill a clean tray with a thin layer of tap water (this tray will be used to store all the roots collected during the washing of one sample). Hereafter, we will refer to this tray as "tray 1." If roots are from ingrowth cores, start with **step 2**, and skip **steps 3** and **4** of this subsection. For washing entire root systems growing in rhizoboxes, go to **step 3**, and skip **step 2** of this subsection. If roots are from soil cores taken from the field or from soil layers taken from a rhizobox, go to **step 4**, and skip **steps 2** and **3** of this subsection.
2. If soil samples are from mesh bags, start by opening the bags at the top. If roots are stuck in the mesh or in the lace of a bag, take these roots first, and store them in the tray 1. When a mesh bag is open, start washing the sample above a 1–2 mm sieve using running water. Place a tray or a collecting plate below the sieve, and regularly check for the presence of roots that might not have been retained by the sieve. Wash the ingrowth core progressively, from the top to the bottom of the core. Using tweezers, remove all visible roots from the sieve/tray/collecting plate, and place them in tray 1. Fine roots can be easily removed from the sieve by immersing the mesh in a thin layer of water (fine roots will float).

3. If roots were growing in a rhizobox, start by carefully removing the front window. If the soil does not have to be divided into layers to study the vertical root distribution, incline the rhizobox at a 30–40° angle to the horizontal, hold the plants at the top, and start washing the roots with running water. Move progressively from the bottom of the rhizobox to the top. When most of the soil is gone, place the roots in tray 1. If you wish to study the vertical root distribution, divide the soil into several layers soon after opening the rhizobox (use a sharp knife and a template). Then, go to **step 4** of this subsection, and follow the instructions for washing the different layers.
4. If roots and soil are intimately mixed but are not contained in a mesh bag or a rhizobox, directly place one sample inside a 1–2 mm sieve. Put a tray or a collecting plate below the sieve, and start washing the roots using running water. Manually remove big mineral and/or organic particles from the sieve. Wash the roots progressively, and regularly check for the presence of roots that might not have been retained by the sieve. Using tweezers, remove all visible roots from the sieve/tray/collecting plate, and place them in tray 1. Fine roots can be easily removed from the sieve by immersing the mesh in a thin layer of water (fine roots will float).
5. Filter the content of tray 1 through a 200–500 μm sieve. Rinse tray 1 with tap water and filter the suspension.
6. Place the sieve containing the roots in a tray, and incline the sieve at a 45° angle to the horizontal.
7. Under running water, finger-massage the roots, and remove the rest of the mineral and organic soil particles that do not strongly adhere to the roots.
8. Using tweezers, remove the roots from the sieve, and store them in an appropriate container. At this stage, roots can be stored under humid and chilly conditions (e.g., 4 °C) for up to 3 days. For longer time periods, the samples should be stored in a freezer (−20 °C) (*see* **Note 5**).
9. In order to remove debris and contaminants that strongly adhere to the roots, a second washing is often necessary. Start by putting one of your samples in a tray filled with a thin layer of distilled water (*see* **Note 13**). Hereafter, we will refer to this tray as "tray 2."
10. Deposit a transparent tray on your working table, and place a colored background behind it. Fine roots can be easily detected using a blue background. Fill the transparent tray with a thin layer of distilled water.
11. Transfer the whole or a portion of your sample into the transparent tray.

12. Using tweezers, manually separate the roots from the soil particles. A soft paintbrush might be helpful to remove small mineral particles adhering to the roots. Use a desk lamp to cast light on your sample. Store clean roots in a Petri dish filled with distilled water. Before starting to clean a new sample, replace the dirty water in the transparent tray by clean distilled water.
13. When you think that the totality of your sample is clean, check that you did not forget any fine roots by pouring the content of tray 2 into the transparent tray.
14. Recover the roots from the Petri dish using tweezers or by filtering the content of the Petri dish using a funnel and appropriate filters.
15. Store clean root samples inside appropriate containers. Write the name and/or the identification number of the samples on the containers.
16. Washed roots can be stored for short time periods at 4 °C in a 50% (v/v) ethanol solution. Roots can be stored in a freezer (−20 °C) for long time periods (*see* **Note 5**). Sometimes, roots have to be flash-frozen in liquid nitrogen and stored at −80 °C for further biochemical analyses.

3.5 Root Staining

Staining the roots with a dye is an efficient way to increase the contrast between roots and the background during scanning. Staining the roots is particularly useful if samples contain a large proportion of fine roots. However, this operation must be avoided if one wishes to do biochemical analyses on roots after scanning [20, 21].

1. Place the whole or a subsample of each clean root sample inside a glass beaker. Write the name and/or the identification number of the sample on its corresponding beaker.
2. Fill the beakers with the staining solution. Add an abundant volume of staining solution in order to fully immerse the roots. Store the beakers on a tray at room temperature (*see* **Note 14**).
3. After 24 h (*see* **Note 15**), recover the stained roots by filtration using a funnel and a filter. Collect the staining solution in a bottle.
4. Rinse the roots with an abundant volume of distilled water in order to remove excess dye.
5. Recover the roots from the filter using tweezers.
6. If you do not plan to acquire root images soon after staining, store the stained root samples in an appropriate and annotated container at 4 °C (short-term storage) or in a freezer (−20 °C, long-term storage) (*see* **Note 5**).

3.6 Acquiring Images of Washed Root Samples

1. Create a method for root image acquisition. Ideally, root images should be acquired as 8 bits per pixel gray level images using the transparency unit (TPU) of the optical scanner. Setting a resolution value appropriate for your sample is of great importance to perform accurate measurements using image analysis (*see* **Note 16**). If you do not know the root diameter distribution in your sample, setting the resolution to 400 dpi is a good starting point.
2. Fill the nontransparent plastic tray with a thin layer of distilled water, and spread your root sample in the water. The roots do not have to be well spread at this stage.
3. If structural information are not of interest, use a scalpel or sharp dissecting scissors to cut the roots into small segments (1–3 cm). It will reduce overlapping between roots, and it will make the spreading of the roots on the scanning area easier.
4. When acquiring images of washed root samples, two situations can be encountered depending on whether the spreading of roots inside the transparent tray and image acquisition are performed at the same location (*situation 1*) or at two different locations (*situation 2*). For *situation 1*, both the spreading of roots inside the transparent tray and image acquisition are performed on the scanner glass. When *situation 2* is encountered, it is important to find a way to keep the roots in place when the tray is moved to the scanning area. *Situation 2* is often met when several people work together to prepare the samples and acquire root images. For *situation 1*, start with **step 5** and skip **step 6** of this subsection. For *situation 2*, start with **step 6** and skip **step 5** of this subsection.
5. *Situation 1.* Position the transparent tray on the scanner glass, and fill it with a thin layer of distilled water. Depending on the size of your sample, transfer the whole or a portion of the roots (subsample) inside the transparent tray. Several images can be acquired for large root samples. Evenly spread the roots on the scanning area using plastic tweezers. Try to avoid overlapping between roots by not exceeding a root length density of 1 cm cm^{-2} on the scanning area.
6. *Situation 2.* Position the transparent tray on a table, and fill it with a thin layer of distilled water. Depending on the size of your sample, transfer the whole or a portion of the roots (subsample) into the transparent tray. Several images can be acquired for large root samples. Evenly spread the roots using plastic tweezers. The use of a colored background is very helpful for improving visibility of roots. Try to avoid overlapping between roots by not exceeding a root length density of 1 cm cm^{-2} on the scanning area. Once the roots are evenly spread, deposit a transparent plastic sheet on top of the water

layer, and remove the excess liquid from the tray using a syringe. Take the transparent tray and position it on the scanner glass.

7. Acquire an image (e.g., tiff, jpeg) and save it on your computer.
8. Remove the roots from the tray. Roots can be removed from the tray using plastic tweezers or by filtration using a funnel and appropriate filters. Recover the water in a glass bottle. Put the roots back in their container for storage. Store the roots in a freezer (−20 °C).
9. Proceed with the next sample (*see* **Note 17**).

3.7 Measuring Root Dry Weight

1. Dry the roots in an oven (60 °C) until constant mass is reached (e.g., 48 h, a longer time period will be required for thick and/or lignified roots).
2. Let the samples cool down in a desiccator at room temperature.
3. Weigh the samples using a calibrated precision scale.

3.8 Acquiring Images of Roots Growing in a Rhizobox

When roots are observed through the transparent front window of a rhizobox, two main strategies can be used to acquire root images and follow the development of roots over time.

1. The first strategy is to acquire direct images of roots with a scanner or a digital camera. This option is interesting if you do not want to lose information (root system architecture, root distribution, root diameters, presence/absence of nodules, presence/absence of root hairs, etc.). With a well-designed image acquisition system, root images can be quickly and easily acquired (<30 s/rhizobox). A description of our image acquisition system is provided in **Note 3**.
2. A second strategy is to manually draw the roots on a transparent acetate sheet using ink pens. A specific color can be used for each observation date, root type, or root order. This option is particularly interesting if you are only interested in following the root system architecture of plants over time. Be aware that some traits (e.g., root diameters) cannot be accurately measured with this technique. At the end of the experiment, the acetate sheets can be scanned using a common flatbed scanner. The resulting images can then be analyzed using image analysis software tools.

3.9 Root Image Analysis

1. Follow the method described in **Note 18** to select one or several root image analysis software solutions that meet your needs. Read **Note 19** if you are interested in counting the number of nodules along the roots of legumes using digital images of roots growing in a rhizobox.

2. Analyze your images following the instructions provided by the developers of the software tools (*see* **Note 20**).
3. Store and analyze your data (*see* **Note 21**).

4 Notes

1. Clean tea bags work fine for recovering the roots by filtration.
2. Transparent and waterproof plastic trays are easy to build yourself. For an A4 optical scanner glass, a 20 × 25 × 2 cm (width × depth × height) tray could be made out of transparent acrylic glass sheets (PMMA, polymethyl metacrylate). First, cut one 2–3 mm thick sheet into five pieces: one 20 × 25 cm piece for the bottom, two 2 × 25 cm pieces for the long edges, and two 2 × (20–2 × thickness) cm pieces for the short edges of the tray. Second, glue the pieces together using a PMMA glue (colorless, polymerization adhesive, UV/light curing) following manufacturer's instructions. Check if the tray is waterproof before using it on the scanner. PMMA trays can easily be scratched, so care must be taken during their manipulation.
3. Our image acquisition system is composed of a metallic frame holding the rhizobox vertically, a digital camera equipped with a 28 mm lens and connected to a computer, a camera holder, and two LED tubes (4300 K, 60 cm length). The camera, the camera holder, the LED tubes, and the frame holding the rhizobox are installed inside a closed box whose internal walls are entirely covered with dark sheets. The camera directly faces the rhizotron and is supported by a camera holder located 54 cm away from the transparent front window. The left and right sides of the transparent front window are illuminated by LED tubes positioned laterally (raking lighting). This setting provides a uniform lighting all over the height of a rhizobox and allows us to avoid reflection on the transparent front window during image acquisition. Images are acquired using the remote live view and shooting option of our camera before being saved on our computer (both jpeg and uncompressed raw images). We advise the reading of [22] for a presentation of inexpensive methods to acquire images of roots growing along a transparent front window.
4. In order to avoid mistakes when analyzing samples, it is sometimes easier to code sample names using identification numbers (or a combination of numbers/letters). This strategy might be particularly useful if you work with people that are not familiar with the names you give to your samples, or if your sample names are long and/or similar to each other. A file storing the sample names and their corresponding identification number

must be created and safely archived. In such file, we also advise you to write the level of each experimental factor for each sample. When data are analyzed using statistical software tools (e.g., R statistical software), the identification numbers of the samples can then be automatically replaced by the name of the samples and/or the factor levels using the information stored in the file created previously.

5. Because freezing compromises root cell integrity, it might influence root diameters. Therefore, root diameter measurements should be performed on fresh root samples (i.e., soon after their collection).
6. When working with soil cores coming from species-rich plant communities, studying root biomass distribution at the species level is one of biggest challenges because we cannot rely on simple root morphological markers to sort the roots of different species. To overcome this drawback, a quantitative DNA-based technique relying on the amplification of species-specific markers has been developed to calculate the relative species abundances in mixed root samples (see [23] for more information).
7. Always label samples twice and use permanent markers to identify the cores and the plastic bags used in the field.
8. In order to make the sieving easier, we recommend to air-dry the soil material at room temperature.
9. Instead of using PVC caps, the tubes can be closed using a plastic sheet hold in place with rubber bands.
10. Once you know the amount of soil you need to fill one mesh bag, we advise you to go to the field with plastic bags containing the exact amount of sieved soil needed for each mesh bag. A beaker marked with a line showing the desired volume of soil required for each mesh bag can also be used in the field.
11. Marking each activated IGC with a colored tape/strap makes their localization easier in the field, particularly when IGCs are surrounded by a dense vegetation.
12. If the goal is to follow fine root productivity for 8 weeks, each experimental plot should contain four IGCs. Every 2 weeks, one IGC is collected and a new one is activated. Only one IGC at a time is activated inside each plot. Therefore, the number of IGCs used depends on how long root development needs to be followed.
13. Adding sodium chloride (NaCl) and/or liquid soap to the water often helps separating the roots from the soil [3]. This technique must be avoided if other analyses have to be performed on roots (e.g., biochemical analyses).

14. An orbital shaker can be used to agitate the roots during staining.
15. See [20] for a discussion about the effect of the staining time period on root length measurements with WinRHIZO™.
16. Because objects appear as a set of square dots (pixels) on digital images, the resolution (usually expressed in dots per inch) is an important factor affecting the accuracy of length and diameter measurements based on image analysis [20]. Because the diameter of a single root varies from one species to another, as well as between different root types and root orders within individuals, care must be taken when one has to set the resolution of the images acquired with an optical scanner. For root diameter measurements, the minimum resolution ($\mathrm{Res}_{\mathrm{min}}$, dpi) can be estimated with Eq. 1, where D_{min} and P_{min} are the diameter of the finest root (μm) and the required minimum number of pixels transversally crossing the finest root on a given image, respectively. For accurate root diameter measurements, we suggest to have $P_{\mathrm{min}} \geq 8$.

$$\mathrm{Res}_{\mathrm{min}} = 25400\frac{P_{\mathrm{min}}}{D_{\mathrm{min}}} \quad (1)$$

17. If images of stained roots are acquired, we advise you to regularly replace the water layer inside the transparent tray by clean distilled water because the dye might leak out of the roots. This is particularly true when the root cell integrity is compromised by long-term storage in a freezer (−20 °C).
18. Depending on the research questions you want to address, one or a combination of root image analysis software packages will be necessary to extract the relevant biological structures from the captured images and compute static and dynamic features to describe your root systems locally (at the individual root level) and/or globally (at the whole root system scale). In order to choose the best software package(s) to analyze your images, you first have to identify your needs. To do so, we suggest you to ask yourself the following questions: [1] What traits/variables would you like to measure? [2] Do you have to deal with time series images? [3] What is the operating system of your computer (Windows, Mac, Linux)? [4] What automation level do you need (fully automated, semi-automated, manual)? [5] What are the properties of your images (format, size, resolution, color/grayscale, presence/absence of soil around the roots, etc.)? [6] Do you need a free software package or can you afford a commercial one? [7] With which file format would you like to save and store your data? [8] How portable should the data be? Once you have answered all these questions, we

advise you to use the Plant Image Analysis Database (http://www.plant-image-analysis.org/) to identify all the root image analysis software packages meeting your needs [24]. Finally, try the tools you selected with your own images, and find out the best one(s) to perform your analyses.

19. Because of the soil surrounding the roots, digital images of roots growing in a rhizobox are not easy to analyze. If a poor contrast exists between roots and background, images might have to be analyzed manually. In order to count the number of nodules visible through the transparent front window of a rhizobox, we analyze root images using Fiji [25]. Nodules are counted manually using the multipoint and ROI manager tools.
20. If you use WinRHIZO™ to analyze your images, we recommend the use of a global thresholding method to detect the roots. The threshold value can be chosen manually or automatically. In order to avoid missing roots, we advise you to adjust the threshold interactively for some parts of the image by using the "interactive modifications to gray level pixel classification" command. More information about the accuracy of the methods proposed in WinRHIZO™ can be found in [21].
21. The large diversity of software solutions available to analyze root images can be seen as an opportunity to use complementary tools to analyze a set of images. Unfortunately, it can be difficult to compare measurements acquired with different platforms because [1] each platform has its own representation of a given root system architecture and [2] the exported data do not share a common structure. To overcome these problems, Lobet and co-workers recently introduced the Root System Markup Language (RSML) as a convenient way to store, share, and increase the transferability of root architecture data [26]. At the time of writing, the RSML has been implemented in seven image analysis tools: SmartRoot [27], RootNav [28], RhizoScan [29], Root System Analyser [30], RooTrak [31], EZ-Rhizo [32], and GLO-RIA [33]. In addition, open-source packages have been developed in several languages (R, Excel, Python, Java, and C#) in order to analyze data saved with the RSML format [26]. Within the R statistical environment, the functions of the archiDART package allow the batch processing of RSML files containing 2D or 3D RSA data [34]. These functions are able to compute global RSA traits, root growth rates, root growth directions and trajectories, and lateral root distribution. It also contains plotting functions developed to visualize the dynamics of root system growth. This R package also allows the batch processing of files produced with Data Analysis of Root Tracings [35].

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5.4 Paper 4: Accuracy and bias of methods used for root length measurements in functional root research

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Accuracy and bias of methods used for root length measurements in functional root research

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Summary

1. Functional root traits are becoming a key measure in plant ecology, and root length measurements are needed for the calculation of root traits. Several methods are used to estimate the total root length (TRL) of washed root samples [e.g. modified line intersect (LI) method, WinRHIZO^{TM} and IJ_Rhizo], but no standardized comparison of methods exists.

2. We used a set of digital images of unstained root samples to compare measurements given by the LI method and automated methods provided by WinRHIZO^{TM} and IJ_Rhizo. Linear regression models were used to detect bias. Both linear regression models and the Bland-Altman's method of differences were used to evaluate the accuracy of eight methods (1 manual, 2 semi-automated and 5 automated) in comparison with a reference method that avoided root detection errors.

3. Length measurements were highly correlated, but did not exactly agree with each other in 11 of 12 method comparisons. All tested methods tended to underestimate the TRL of unstained root samples. The accuracy of WinRHIZO^{TM} was influenced by the thresholding method and the root length density (RLD) in the pictures. For the other methods, no linear relationship was found between accuracy and RLD. With WinRHIZO^{TM} (global thresholding + pixel reclassification; RLD = 1 cm cm^{-2}), the Regent's method and the Tennant's method underestimated the TRL by 7·0 ± 6·2% and 4·7 ± 7·9%, respectively. The LI method gave satisfactory results on average (underestimation: 4·2 ± 6·0%), but our results suggest that it can lead to inaccurate estimations for single images. In IJ_Rhizo, the Kimura method was the best and underestimated the TRL by 5·4 ± 6·1%.

4. Our results showed that care must be taken when comparing measurements acquired with different methods because they can lead to different results. When acquiring root images, we advise to (i) increase the contrast between fine roots and background by staining the roots, and (ii) avoid overlapping roots by not exceeding a RLD of 1 cm cm^{-2}. Under these conditions, good length estimates can be obtained with WinRHIZO^{TM} (global thresholding + pixel reclassification). The Kimura method in IJ_Rhizo can be an alternative to WinRHIZO^{TM}.

Key-words: functional ecology, IJ_Rhizo, ImageJ, line intersect method, root length, washed root samples, WinRhizo^{TM}

Introduction

Often referred to as 'the hidden half' (Eshel & Beeckman 2013), plant roots serve multiple functions simultaneously and are an important driver of ecosystem processes (Gregory 2006; Bardgett, Mommer & De Vries 2014). Root systems provide anchorage, a network for water absorption and nutrient uptake, and alter the physicochemical properties of the rhizosphere via the exudation of a great diversity of low and high molecular weight metabolites into the soil (Delory *et al.* 2016; Mommer, Kirkegaard & van Ruijven 2016; Rellán-Álvarez, Lobet & Dinneny 2016). In addition, the rhizosphere houses a number of organisms developing interactions with roots that can have positive or negative effects on plant health (Hinsinger *et al.* 2009; Raaijmakers *et al.* 2009).

Although plant roots play a central role in ecosystem functioning (Bardgett, Mommer & De Vries 2014), plant scientists and ecologists face many technical challenges in measuring root traits. There are two main reasons for this: (i) the belowground location of roots that hampers direct observations (Pagès *et al.* 2010), and (ii) the impossibility of species identification based on simple root morphological markers in species-rich plant communities (see Mommer *et al.* 2008, 2010, 2011; Faget *et al.* 2013 for methods to identify roots of different species). Among all the possible root traits that can be measured on root samples extracted from soil cores, the specific root length (SRL, the root length per unit root biomass) and the root length density (RLD, the root length per unit volume of soil) are of particular interest (Bardgett, Mommer & De Vries 2014; Kramer-Walter *et al.* 2016; Weemstra *et al.* 2016). While SRL is a morphological trait that provides information about the amount of resources needed to increase the surface area

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between roots and soil (Kramer-Walter *et al.* 2016), RLD is an architectural trait describing the capacity of a root network to explore a given volume of soil and acquire limited resources (Hecht *et al.* 2016; Ravenek *et al.* 2016). As a prerequisite for calculating SRL and RLD, one has to know the total root length (TRL) in the studied samples.

The first method developed to compute the TRL from washed root samples was presented by Newman in 1966. In his study, Newman (1966) derived an equation estimating the TRL in a sample by counting the number of intersections between randomly orientated roots evenly distributed in a tray of known area and randomly oriented straight lines of known total length. A few years later, Marsh (1971) and Tennant (1975) simplified Newman's equation by using a grid of regularly spaced lines crossing the roots. This line intersect (LI) method became rapidly popular among plant scientists and ecologists because it is easy to use, not expensive and faster than any other manual length measurement method (Tennant 1975). The rapid development of computer hardware and software led scientists to develop image analysis algorithms able to compute the TRL from captured images of washed root samples (Ewing & Kaspar 1995; Kimura, Kikuchi & Yamasaki 1999; Pierret *et al.* 2013) and analyse 2D root system architectures (see the Plant image analysis database described in Lobet, Draye & Périlleux 2013).

Different image analysis methods rely on a different set of assumptions and care must be taken when choosing the best software package to analyse a set of digital images (Pridmore, French & Pound 2012). Next to the LI method, two software packages can be used to easily compute the TRL from scanned root samples: the commercial software package WinRhizo™ (examples of studies using WinRhizo™: Mommer *et al.* 2012; Pagès & Picon-Cochard 2014) and the open-source ImageJ macro IJ_Rhizo (Pierret *et al.* 2013). Even if previous studies showed that a strong linear relationship exists between measurements acquired with WinRhizo™ and IJ_Rhizo (Wang & Zhang 2009; Pierret *et al.* 2013), the agreement between the methods provided by these software packages and the manual LI method has not yet been investigated. In addition, the accuracy of these methods is poorly known and has been reported to be strongly related to the settings used for image acquisition and analysis (Bouma, Nielsen & Koutstaal 2000). Previous studies reported that differences between reference values and measurements acquired with WinRhizo™ (Himmelbauer, Loiskandl & Kastanek 2004) or the LI method (Goubran & Richards 1979) did not exceed 5% when the RLD on the scanning area was low (around 1 cm cm^{-2}).

Given the increasing number of studies dealing with root trait measurements and considering that different methods can lead to different results, we performed a comparative study of several methods commonly used in functional root research to estimate the TRL of washed root samples. We wanted to see if results computed with different methods can be safely compared or if different methods led to significantly different absolute measurements of the same quantity. We also aimed to assess to what extent researchers without access to expensive software and equipment may be able to rely on freely available software solutions (such as ImageJ and IJ_Rhizo). In this study, we used a set of 50 digital images of unstained roots collected from soil cores during a grassland field experiment (Jülich, Germany) to compare the length estimates given by the manual LI method (Tennant 1975) and the automated methods provided by WinRhizo™ and IJ_Rhizo (Pierret *et al.* 2013). We designed this study to answer the following questions:

1. Do the tested methods agree with each other?

2. Are the tested methods accurate?

Materials and methods

ROOT IMAGES

The roots analysed in this study were collected from soil cores in 2014 in a grassland experiment located in Jülich (Germany). After collection, the roots were carefully washed and stored in a freezer (−20 °C). Before image acquisition, the roots were gently defrosted and spread in a thin layer of water in a transparent plastic tray. The roots were cut into small segments (1–3 cm) in order to facilitate spreading and minimize overlapping. We did not use a dye to stain the roots prior to image acquisition because such an operation is not always possible, particularly when additional analyses/measurements have to be performed on roots after scanning (e.g. chemical analyses). For each sample, one grey-level image and one colour image were acquired with a flatbed scanner (Epson Perfection V800 Photo; Epson, Nagano, Japan). First, the roots were scanned in transparent mode at a resolution of 400 dpi. The root density in the tray was found to be between 0·1 and 2·9 cm cm^{-2}. The scans were then saved as 8 bits per pixel grey-level images. Then, the same roots were scanned in colour at a resolution of 800 dpi using the scanner's reflective lighting system. A white background with a green 2 × 2 cm grid was placed behind the roots before the second scanning. These scans were saved as 24 bits per pixel colour images.

ROOT LENGTH MEASUREMENTS

To evaluate the agreement between popular root length measurement methods, 50 images were randomly selected and analysed using one manual and five automated methods developed to estimate the TRL from washed root samples (Fig. 1). The grey-level images were analysed with the commercial software package WinRhizo™ and the free ImageJ macro IJ_Rhizo. The colour images were analysed manually using the modified LI method according to Tennant's guidelines (Tennant 1975).

Using WinRhizo™ Pro 2017a Pre-Release (Regent Instruments, Québec, Canada), the roots were separated from the background using a local thresholding method (Lagarde). This method was advised by Bouma, Nielsen & Koutstaal (2000) when unstained roots have to be skeletonized with WinRhizo™. Then, TRL was computed with two different methods: the non-statistical Regent's method (L|WinR) and the Tennant's statistical method (L|WinT).

The grey-level images were then also analysed with IJ_Rhizo (Pierret *et al.* 2013). Batch analyses of root images were performed with the following parameters: excluded border of 40, 50 or 60 pixels width; perform particle cleaning (size of the smallest particle: 1 mm^2; circularity of particles: 0·75); automatic thresholding. IJ_Rhizo computed the TRL derived from either the uncorrected skeleton (IJraw), the corrected skeleton (IJcorrected) or the Kimura skeleton (IJKimura). Briefly, IJraw uses the skeleton obtained after skeletonization of a

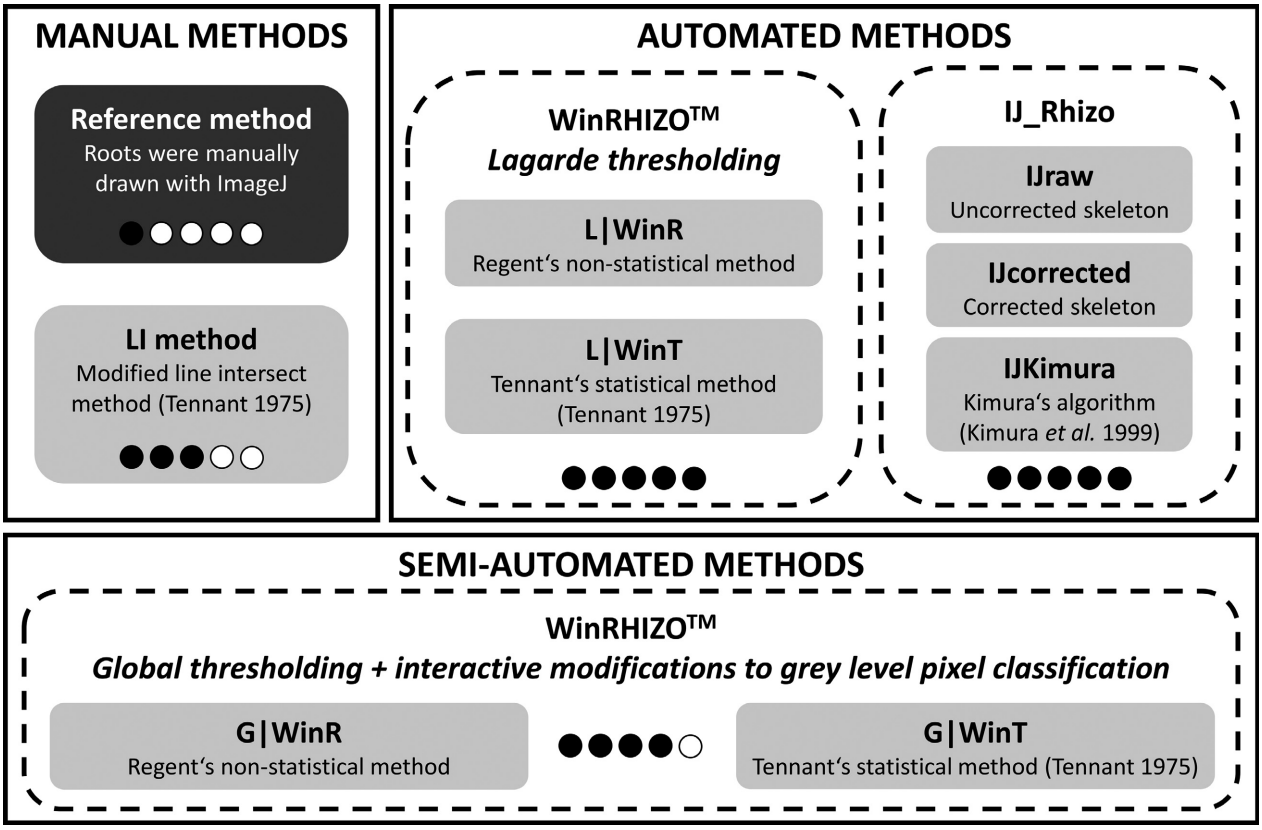

Fig. 1. Manual, semi-automated and automated methods used in this study for estimating the TRL of washed root samples. The methods tested in this study are written in black (light grey background). The figure provides a qualitative assessment of the speed of each method to compute the TRL of 15 images (RLD_{min} = 0·1 cm cm^{-2}, RLD_{max} = 2·7 cm cm^{-2}, RLD_{mean} = 1·1 cm cm^{-2}, RLD_{SD} = 0·7 cm cm^{-2}). Depending on the method, the analysis of 15 images can be fast (5 black dots, less than 1 h), slow (3–4 black dots, several hours) or very slow (1 black dot, several days). TRL, total root length; RLD, root length density.

thresholded image in ImageJ to estimate the TRL. Because this process removes more pixels from around thick roots than thin roots, IJcorrected adds a number of pixels to the uncorrected skeleton before estimating the TRL (Pierret *et al.* 2013). With IJKimura, pixels are discriminated and the TRL is estimated based on the number of diagonally and orthogonally connected pairs of pixels in a skeleton (Kimura, Kikuchi & Yamasaki 1999).

The colour images were analysed manually using the LI method modified by Tennant (1975) (Fig. 1). With the manual LI method, the TRL (cm) was estimated following eqn (1), where N is the number of intercepts between the roots and the grid, and D is the distance between two parallel lines of the grid (cm) (Rowse & Phillips 1974; Tennant 1975). Following Tennant's guidelines (1975), we used three different grid sizes depending on the TRL in each image: 0·5 × 0·5 cm (TRL < 75 cm), 1 × 1 cm (TRL between 75 and 275 cm) or 2 × 2 cm (TRL > 275 cm). When a 2 × 2 cm grid was used, we counted the number of intercepts between the roots and the grid using the colour images. When the LI method required a lower grid size, we superimposed a 0·5 × 0·5 cm or a 1 × 1 cm grid on the grey-level images using the Grid plugin (Wayne Rasband 2007) in ImageJ 1.50b (Schneider, Rasband & Eliceiri 2012).

$$TRL = \frac{\pi}{4} \times N \times D \qquad \text{eqn 1}$$

Because we also wanted to evaluate the accuracy of each tested method (i.e. the closeness of the outcome to an absolute and accurate value), we randomly sampled 15 pictures from the 50 images selected at the beginning of this study and we determined the TRL in each with a reference method (Fig. 1). To do so, all the roots were manually drawn with ImageJ and the TRL was calculated as the sum of the length of each individual segmented line (= reference method). Although time consuming, this method allowed us to avoid root detection errors and to have accurate absolute values for TRL with which the absolute values given by the methods tested in this study can be compared. In addition, we also wanted to test if the accuracy of WinRhizo™ was influenced by the thresholding method used for root skeletonization. To do so, grey-level pixel classification within the 15 images selected previously was performed with a Lagarde's local threshold (L) or with a global threshold (G). With the latter, a single threshold value was chosen automatically to classify all pixels of an analysed region. Then, this value was adjusted manually for some parts of the images following the procedure described in the Regent's technical support manual to avoid missing roots. With both thresholding methods, the TRL was computed with the Regent's method (L|WinR or G|WinR) and the Tennant's method (L|WinT or G|WinT). Therefore, the accuracy of one manual, two semi-automated and five automated methods was evaluated in this study (Fig. 1).

DATA ANALYSIS

First, we performed pairwise comparisons between six tested methods (WinRhizo™ with Lagarde thresholding vs. LI or IJ_Rhizo; LI vs. IJ_Rhizo) (Table 1, Fig. 2). In addition, the values obtained with each

method were compared to the values calculated with the reference method (Table 2, Fig. 3). When two methods were compared, the strength of the linear relationship between the TRL estimates was assessed by calculating the Pearson's product–moment correlation coefficient and performing a Model II linear regression (ordinary least products). We chose this type of regression model because the x values were not fixed by the experimental design and were thus subject to error (Ludbrook 1997, 2010b; Legendre & Legendre 1998). The regression models were fitted using the R package LMODEL2 (Legendre 2014) and were used to search for fixed and proportional bias. We considered that a method had a fixed bias if it gave values that were consistently higher (or lower) than a second method. In contrast, if the difference between length estimates given by two methods increased (or decreased) with the TRL in a picture (Ludbrook 1997), we considered this a proportional bias. For each fitted model, we calculated the 95% confidence interval (95% CI) of the slope and the intercept. Then, we tested if the regression line was significantly different from the line of equal outcomes ($y = x$). If the 95% CI for the intercept did not include zero, there was a fixed bias. If the 95% CI for the slope did not include 1·0, there was proportional bias (Ludbrook 1997, 2002, 2010b).

Second, we evaluated the agreement between the tested methods and the reference using the Bland and Altman's method of differences (Altman & Bland 1983; Bland & Altman 1986, 1999; Giavarina 2015) (Table 3, Fig. 4). The lack of agreement between each tested method and the reference was evaluated by calculating the 95% confidence limits (or limits of agreement). In this study, we considered that two methods agreed with each other if the following criteria were met simultaneously: (i) no bias could be detected and (ii) the calculated limits of agreement were narrow. To do so, we followed the guidelines published by Ludbrook (2010a). We started by plotting the absolute differences between the root length estimates given by a tested method and the reference (y axis) against the average lengths given by the two methods (x axis). The strength of the linear relationship between the absolute differences and the averages was evaluated by calculating the Pearson's product–moment correlation coefficient. We also performed Model I regression analyses (ordinary least squares) to estimate the regression coefficients of the best linear model linking the differences between root length estimates (y) and the average values (x). Because we were only interested in predicting y from x (and not x from y), Model I regression analysis can be used (Ludbrook 2010a, b). Ordinary least squares regression models were fitted using the lm function of R (R Core Team 2016). If the correlation coefficient and the slope of the fitted model were significantly different from zero, we considered that there was proportional bias. If there was no proportional bias, the limits of agreement were calculated following eqn (2), where $\bar{d}$ is the mean difference between a tested method and the reference, n is the number of observations, t is the quantile of the Student's t distribution ($\alpha = 0{\cdot}05$ and $n-1$ degrees of freedom) and s_{diff} is the sample standard deviation for the differences (Ludbrook 2010a). If there was a proportional bias, we constructed hyperbolic 95% confidence limits (prediction interval) around the fitted regression line using eqn (3) (Altman & Gardner 2000), where y_{fit} is a predicted value of y for a fixed value of x, t is the quantile of the Student's t distribution ($\alpha = 0{\cdot}05$ and $n-2$ degrees of freedom), s_{res} is the residual standard deviation of y about the regression line, $\bar{x}$ and s_x are the mean value and the standard deviation of x and n is the sample size. All statistical analyses were performed with R 3.3.0 (R Core Team 2016).

$$\bar{d} \pm \left(t_{1-\frac{\alpha}{2}} \times s_{\text{diff}} \times \sqrt{1+\frac{1}{n}} \right) \quad \text{eqn 2}$$

$$y_{\text{fit}} \pm \left(t_{1-\frac{\alpha}{2}} \times s_{\text{res}} \times \sqrt{1+\frac{1}{n}+\frac{(x-\bar{x})^2}{(n-1)s_x{}^2}} \right) \quad \text{eqn 3}$$

Results

PAIRWISE COMPARISONS OF SIX METHODS AND DETECTION OF FIXED AND PROPORTIONAL BIAS

Overall, we found a strong positive correlation between root length measurements acquired with the six different methods (Fig. 2 and Table 1). The correlation coefficients ranged from 0·985 (LI vs. IJ_RHIZO methods) to 0·999 (L|WinR vs. L|WinT). Our results showed that L|WinT gave on average greater length estimates than L|WinR (Table 1, Fig. 2a). When the RLD increased, we found that the manual LI

Table 1. Pairwise comparisons of methods commonly used for estimating the total root length of washed root samples

Methods			Linear regression		Bias		
x	y	Correlation (Pearson)	Slope (95% CI)	Intercept (95% CI)	Fixed	Proportional	Figure
L|WinR	L|WinT	0·999	1·03 (1·02, 1·05)	6·12 (0·37, 11·79)	Yes	Yes	2a
	LI	0·988	1·09 (1·04, 1·13)	−34·72 (−58·50, −11·97)	Yes	Yes	2b
	IJraw	0·996	0·86 (0·84, 0·88)	−11·10 (−21·60, −0·86)	Yes	Yes	2c
	IJcorrected	0·996	0·86 (0·84, 0·89)	−10·30 (−20·81, −0·04)	Yes	Yes	2d
	IJKimura	0·996	1·07 (1·05, 1·10)	−11·86 (−24·98, 0·94)	No	Yes	2e
L|WinT	LI	0·988	1·05 (1·00, 1·10)	−41·14 (−65·57, −17·78)	Yes	Yes	2f
	IJraw	0·995	0·83 (0·81, 0·86)	−16·21 (−28·96, −3·83)	Yes	Yes	2g
	IJcorrected	0·995	0·84 (0·81, 0·86)	−15·41 (−28·18, −3·01)	Yes	Yes	2h
	IJKimura	0·995	1·04 (1·01, 1·07)	−18·20 (−33·81, −3·03)	Yes	Yes	2i
LI	IJraw	0·985	0·79 (0·76, 0·84)	16·49 (−3·36, 35·38)	No	Yes	2j
	IJcorrected	0·985	0·80 (0·76, 0·84)	17·34 (−2·59, 36·31)	No	Yes	2k
	IJKimura	0·985	0·99 (0·94, 1·04)	22·42 (−2·17, 45·83)	No	No	2l

All correlation coefficients were significantly different from zero ($P < 0{\cdot}001$). The 95% confidence intervals (CIs) of the regression coefficients (ordinary least products) are shown in parentheses (lower limit, upper limit) and were used to detect bias. If the 95% CI for the intercept did not include zero, there was fixed bias. If the 95% CI for the slope did not include 1·0, there was proportional bias. Method abbreviations are explained in Fig. 1.

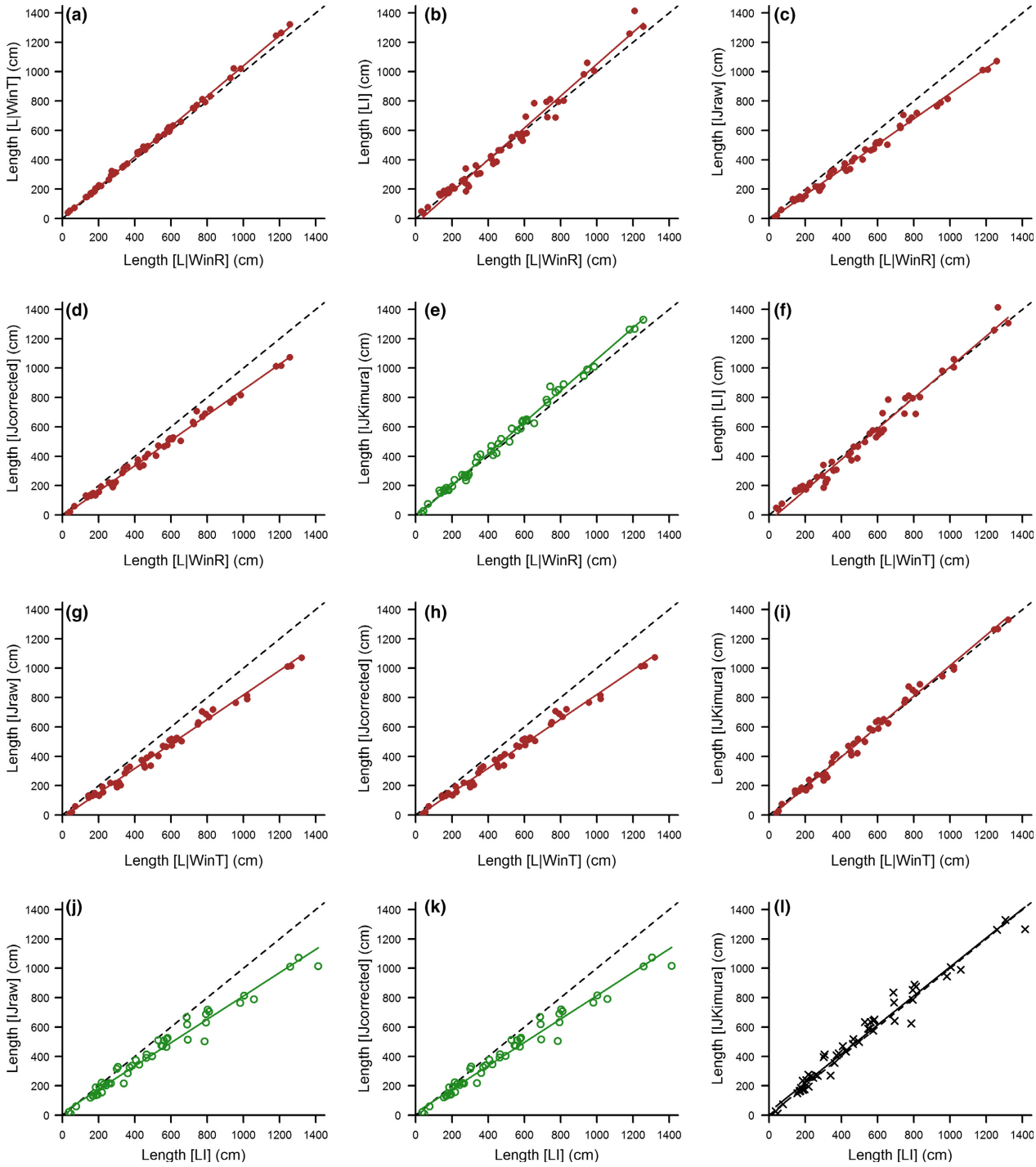

Fig. 2. Pairwise comparisons of methods commonly used for estimating the TRL of washed root samples. On each graph (a–l), both the ordinary least products regression line (solid line) and the line of equal outcomes (dashed line) were plotted. Filled brown dots, both fixed and proportional bias; open green dots, only proportional bias; black crosses, no bias. Abbreviations are explained in Fig. 1. TRL, total root length.

method (Fig. 2b) and IJKimura (Fig. 2e) tended to give higher length estimates than L|WinR. In addition, IJraw and IJcorrected gave lower values than L|WinR (Fig. 2c,d), L|WinT (Fig. 2g,h) and the manual LI method (Fig. 2j,k). When comparing L|WinR and L|WinT, both fixed and proportional bias were detected (Table 1, Fig. 2a). These two types of bias were also found when the manual LI method, IJraw and IJcorrected were compared to L|WinR and L|WinT (Table 1, Fig. 2b–d and f–h), as well as when root length estimates given by IJKimura were plotted against the estimates computed by L|WinT (Fig. 2i). We detected a proportional bias when the following methods were compared: L|WinR and IJKimura (Fig. 2e), LI and IJraw (Fig. 2j) and LI and IJcorrected (Fig. 2k). No bias was detected when the length estimates given by IJKimura

Table 2. Detection of fixed and proportional bias in eight methods used for estimating the total root length of washed root samples

Methods		Correlation	Linear regression		Bias		
x	*y*	(Pearson)	Slope (95% CI)	Intercept (95% CI)	Fixed	Proportional	Figure
Reference	L|WinR	0·996	0·86 (0·81, 0·91)	26·33 (3·23, 48·19)	Yes	Yes	3a
Reference	G|WinR	0·999	0·88 (0·86, 0·90)	17·77 (7·16, 28·11)	Yes	Yes	3b
Reference	L|WinT	0·992	0·87 (0·81, 0·94)	39·93 (7·82, 69·72)	Yes	Yes	3c
Reference	G|WinT	0·999	0·91 (0·88, 0·93)	14·74 (1·34, 27·73)	Yes	Yes	3d
Reference	LI	0·986	0·91 (0·83, 1·01)	12·51 (−32·46, 53·24)	No	No	3e
Reference	IJraw	0·991	0·73 (0·67, 0·79)	15·02 (−13·18, 41·08)	No	Yes	3f
Reference	IJcorrected	0·991	0·73 (0·67, 0·79)	15·50 (−12·80, 41·65)	No	Yes	3g
Reference	IJKimura	0·991	0·90 (0·83, 0·98)	21·41 (−14·85, 54·83)	No	Yes	3h

The 95% confidence intervals (CIs) of the Model II regression coefficients are shown in parentheses (lower limit, upper limit) and were used to detect bias. If the 95% CI for the intercept did not include zero, there was fixed bias. If the 95% CI for the slope did not include 1·0, there was proportional bias. All correlation coefficients were significantly different from zero ($P < 0·001$). Abbreviations are explained in Fig. 1.

were plotted against the estimates given by the manual LI method, meaning that the regression line was not significantly different from the line of equal outcomes (Fig. 2l).

AGREEMENT BETWEEN EIGHT TESTED METHODS AND THE REFERENCE METHOD

Both a regression analysis (Fig. 3 and Table 2) and a Bland-Altman analysis (Fig. 4 and Table 3) showed that all the semi-automated and automated methods used to estimate the TRL had a proportional bias when they were compared to the reference method. Looking at Fig. 4, the existence of such bias can be noticed by the significant positive correlation between the length differences and the TRL in a picture (see also Table 3). G|WinR, G|WinT and IJKimura were characterized by the lowest proportional bias, whereas IJraw and IJcorrected had the highest proportional bias (Tables 2 and 3). The regression analysis also showed that L|WinR, G|WinR, L|WinT and G|WinT were characterized by a fixed bias (Table 2). Interestingly, no bias was detected when the manual LI method was compared with the reference. Overall, all the tested methods tended to underestimate the TRL in the captured images (Figs 3 and 4).

The lack of agreement between the tested methods and the reference was also evaluated by calculating limits of agreement (i.e. the limits within which 95% of the population values should lie) (Fig. 4 and Table 3). Although no fixed or proportional bias was detected for the manual LI method, the limits of agreement for the population of differences were large (149 cm below or 91 cm above the value given by the reference method) (Fig. 4e). Because of the existence of a proportional bias for the semi-automated and automated methods, we determined hyperbolic 95% confidence intervals (prediction interval) around the regression lines (Table S1, Supporting Information). Our results showed that G|WinR (Fig. 4b) and G|WinT (Fig. 4d) had the narrowest prediction intervals, whereas L|WinT and IJKimura had the largest ones (Fig. 4c, h). Whether biased (semi-automated and automated methods) or not (manual LI method), all tested methods showed a lack of agreement with the reference method used in this study. This was demonstrated by (i) a significant deviation in the Model II regression lines from the line of equal outcomes for the semi-automated and automated methods (Fig. 3 and Table 2) and/or (ii) large 95% confidence intervals in Bland-Altman plots (Fig. 4 and Table 3).

When root lengths were computed with WinRhizo™, we found a statistically significant positive correlation between the per cent deviation of the estimates from reference values and the RLD (Fig. 5a–d). Our results were also strongly influenced by the thresholding method used for root skeletonization. The negative intercept values in Fig. 5a,c showed that small root samples were overestimated by WinRhizo™ when roots were skeletonized with the Lagarde thresholding method. This was particularly true when root lengths were calculated with L|WinT (Fig. 5c). This length overestimation of small root samples disappeared when a global thresholding associated with a local reclassification of grey level pixels was used (Fig. 5b,d). With the latter methodology, both the slope and the residual variance of regression models were lower than with the Lagarde method. In addition, the differences between estimates given by WinR and WinT were lower using this thresholding method (Fig. 5a–d). For a RLD of 1 cm cm^{-2}, G|WinR and G|WinT underestimated the TRL by 7·0 ± 6·2% and 4·7 ± 7·9%, respectively (predictions from linear models). When the TRL was estimated with the manual LI method (Fig. 5e) or with IJ_Rhizo (Fig. 5f–h), however, we did not find any significant correlation between the per cent deviation of the length estimates and the RLD in the pictures. On average, the manual LI method underestimated the TRL by 4·2 ± 6·0% (Fig. 5e). Both IJraw (24·3 ± 4·8%) and IJcorrected (24·0 ± 4·8%) strongly underestimated the TRL of the analysed samples (Fig. 5f–g). With regard to IJKimura, it underestimated the TRL in the samples by 5·4 ± 6·1% on average when assessing accuracy related to the reference method (Fig. 5h).

Discussion

In the first part of this study, we compared one manual (LI) and five automated methods (L|WinR, L|WinT, IJraw,

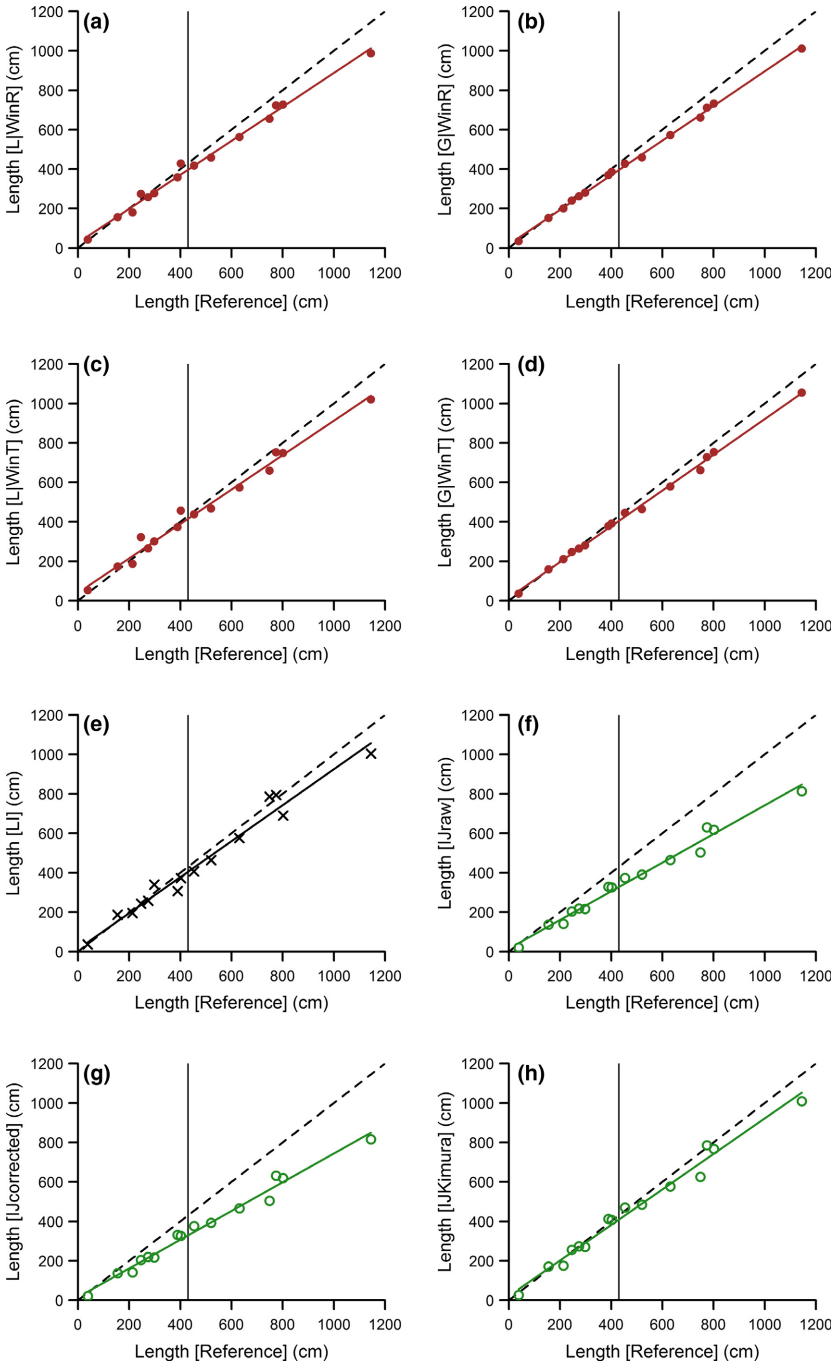

Fig. 3. Evaluation of the accuracy of eight methods used for estimating the TRL of washed root samples. On each graph (a–h), both the ordinary least products regression line (solid line) and the line of equal outcomes (dashed line) were plotted. Filled brown dots, both fixed and proportional bias; open green dots, only proportional bias; black crosses, no bias. The vertical line corresponds to a RLD of 1 cm cm^{-2}. Abbreviations are explained in Fig. 1. TRL, total root length; RLD, root length density.

IJcorrected and IJKimura) commonly used to estimate the total length of roots extracted from soil cores. Overall, we found a strong linear relationship between the lengths estimated by six tested methods. Previous reports showed that the lengths estimated with WinR and WinT are highly positively correlated (Wang & Zhang 2009; Pierret *et al.* 2013). Similar results were obtained when the root lengths computed by IJ_Rhizo were compared to those obtained with WinRhizo^{TM} (Pierret *et al.* 2013). Nevertheless, of 12 pairwise method comparisons performed in this study, 11 showed a significant deviation of the regression line from the line of equal outcomes ($y = x$). This result suggests that different methods of measurement can lead to different estimates of the same quantity. Supporting the results of Wang & Zhang (2009), we found that L|WinT tended to give higher length estimates than L|WinR. Interestingly, IJraw and IJcorrected gave lower length values than those computed with WinRhizo^{TM} (L|WinR and L|WinT) or the manual LI method. Supporting the findings of Pierret *et al.* (2013), we found that IJKimura tended to give greater length estimates than those computed with WinRhizo^{TM}.

In the second part of this study, we were interested to test the accuracy of all methods listed in Fig. 1. Our results showed that all the tested methods tended to underestimate the TRL. In addition, all the semi-automated and automated methods were characterized by a proportional bias when they were compared to the reference method. The existence of a proportional

Table 3. Bland-Altman analysis: estimation of 95% confidence limits (limits of agreement) for eight methods developed to estimate the total root length of washed root samples

Bland-Altman plot		Correlation	Linear regression		Proportional	95% confidence	
x	y	(Pearson)	Slope (95% CI)	Intercept (95% CI)	bias	limits	Figure
(Reference+L|WinR)/2	Reference-L|WinR	0·852***	0·15 (0·09, 0·20)	−28·44 (−57·40, 0·53)	Yes	Hyperbolic	4a
(Reference+G|WinR)/2	Reference-G|WinR	0·952***	0·13 (0·10, 0·16)	−18·95 (−32·20, −5·69)	Yes	Hyperbolic	4b
(Reference+L|WinT)/2	Reference-L|WinT	0·738**	0·14 (0·06, 0·21)	−42·90 (−83·03, −2·77)	Yes	Hyperbolic	4c
(Reference+G|WinT)/2	Reference-G|WinT	0·887***	0·10 (0·07, 0·13)	−15·49 (−31·91, 0·93)	Yes	Hyperbolic	4d
(Reference+LI)/2	Reference-LI	0·486^{ns}	0·09 (−0·01, 0·19)	−13·38 (−66·72, 39·96)	No	(−90·98, 149·03)	4e
(Reference+IJraw)/2	Reference-IJraw	0·927***	0·32 (0·24, 0·40)	−17·96 (−55·40, 19·47)	Yes	Hyperbolic	4f
(Reference+IJcorrected)/2	Reference-IJcorrected	0·925***	0·32 (0·24, 0·39)	−18·49 (−56·03, 19·05)	Yes	Hyperbolic	4g
(Reference+IJKimura)/2	Reference-IJKimura	0·609*	0·11 (0·02, 0·19)	−22·76 (−66·62, 21·10)	Yes	Hyperbolic	4h

The 95% confidence intervals (CIs) of the Model I regression coefficients are shown in parentheses (lower limit, upper limit). If the 95% CI for the slope did not include zero, there was proportional bias. In that case, we constructed hyperbolic 95% confidence limits (prediction interval) around the fitted linear model using eqn (3). When no proportional bias was detected, the limits of agreement were calculated using eqn (2). Abbreviations are explained in Fig. 1. ns, $P > 0·05$.

*$P < 0·05$.

**$P < 0·01$.

***$P < 0·001$.

bias means that the absolute difference between the values obtained with the tested methods and the reference increased with the amount of root material included in a sample. Previous reports showed that both the manual LI method (Tennant 1975; Goubran & Richards 1979) and image analysis algorithms (Zoon & Van Tienderen 1990; Smit *et al.* 1994) can underestimate the actual root length in a sample. With WinRhizo^{TM}, even the use of the Lagarde's thresholding method developed for pale roots can lead to a strong underestimation of the TRL for unstained root samples (Bouma, Nielsen & Koutstaal 2000). Several factors could explain the overall underestimation of the TRL.

First, the manual LI method and some image analysis algorithms assume a random arrangement of roots on the scanned area resulting in a uniform distribution of orientations (Newman 1966; Ewing & Kaspar 1995). Therefore, a preferential orientation of roots can lead to inaccurate estimations of the TRL, particularly with the manual LI method that tends to overestimate root lengths when the orientation of roots is not random (Ewing & Kaspar 1995). Among the methods tested in this study, both the Regent's method (Appendix C of the Regent's manual provided with the software; Himmelbauer, Loiskandl & Kastanek 2004) and IJKimura (Kimura, Kikuchi & Yamasaki 1999) have a low sensitivity to preferential root orientation. Because (i) care was taken to randomly spread the roots on the scanning area, and (ii) we mainly observed underestimation of the TRL, the influence of root distribution and orientation on the results did not seem to play a significant role in our study.

Second, the amount of root material included in a sample is also an important factor because a high RLD increases the occurrence of root overlaps (Fig. 6), leading to an underestimation of the TRL (Tennant 1975; Zoon & Van Tienderen 1990; Kimura, Kikuchi & Yamasaki 1999; Costa *et al.* 2000). Using WinRhizo^{TM}, both Bouma, Nielsen & Koutstaal (2000) and Himmelbauer, Loiskandl & Kastanek (2004) showed that the per cent difference between length estimates and reference values increased with increasing RLD. The same pattern was observed by Goubran & Richards (1979) with the manual LI method, and by Zoon & Van Tienderen (1990) using image analysis. Our results confirmed these observations for the WinRhizo^{TM} methods but not for the manual LI method. To avoid a too large underestimation of the TRL due to overlapping roots, some authors suggested to work with a RLD lower than 3 cm cm^{-2} (Bouma, Nielsen & Koutstaal 2000; Himmelbauer, Loiskandl & Kastanek 2004). In addition, if there is no interest in the study of root branching patterns, cutting the roots into small segments can decrease the number of crossings and adjoining objects when roots are spread all over the surface of the scanning area (Bouma, Nielsen & Koutstaal 2000). In our experiment, the roots were cut into smaller pieces and we worked with a RLD between 0·1 and 2·9 cm cm^{-2}. Because both WinR and the IJKimura are able to detect crossings and overlapping pixels, these methods have been advised by other people when the TRL has to be estimated for large root samples showing a high level of root overlaps (Appendix C of the Regent's manual provided with the software; Kimura, Kikuchi

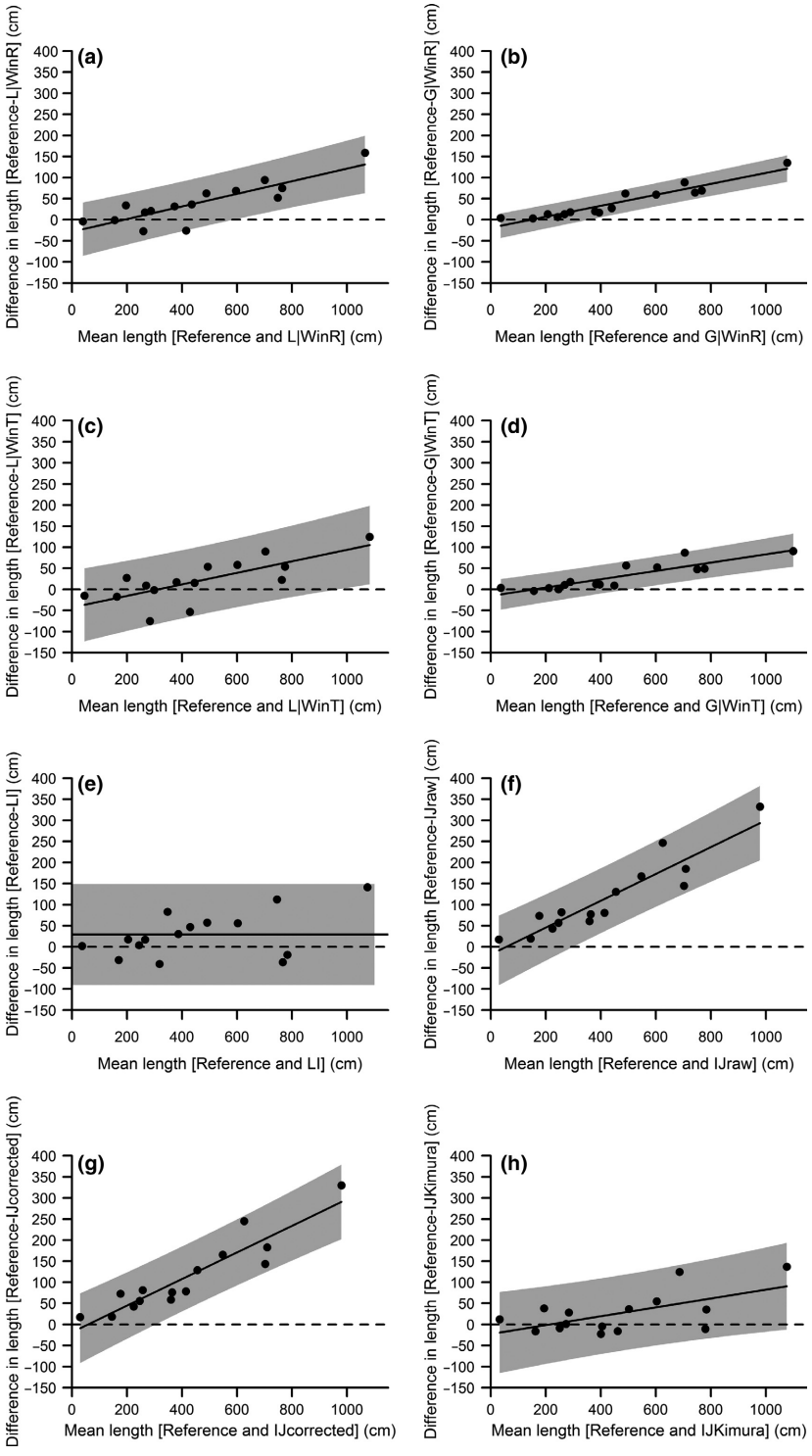

Fig. 4. Bland-Altman analysis: estimation of 95% confidence limits (limits of agreement) for eight methods developed for estimating the TRL of washed root samples. In each graph showing a proportional bias (a–d, f–h), the ordinary least squares regression line was plotted (solid line) and hyperbolic 95% confidence limits (prediction interval) were constructed around the fitted linear model (grey area) using eqn (3). When no proportional bias was detected (e), the limits of agreement were calculated using eqn (2) (grey area) and a horizontal solid line shows the mean difference. Both overestimation (negative differences) and underestimation (positive differences) can be observed in the figures. Abbreviations are explained in Fig. 1. TRL, total root length.

& Yamasaki 1999; Himmelbauer, Loiskandl & Kastanek 2004).

Third, considering that a significant part of plant root systems are made of absorptive fine roots (Pierret, Moran & Doussan 2005; McCormack *et al.* 2015), the underestimation of TRL by image analysis algorithms could also be explained by the fact that low diameter roots were not detected because of the poor contrast between fine roots and background (Fig. 6). Using the reference method or the manual LI method, however, the roots were visually detected on a screen and even fine roots were easily separated from the background (Fig. 6). Therefore, if the observed underestimation was caused by the removal of fine roots on thresholded images, one can expect greater length estimates using the reference method or the manual LI method. To increase the contrast between roots and background, some authors recommended the use of a dye to stain the roots (e.g. 3-amino-7-dimethylamino-2-methylphenazine hydrochloride; syn: neutral red; CAS 553-24-2),

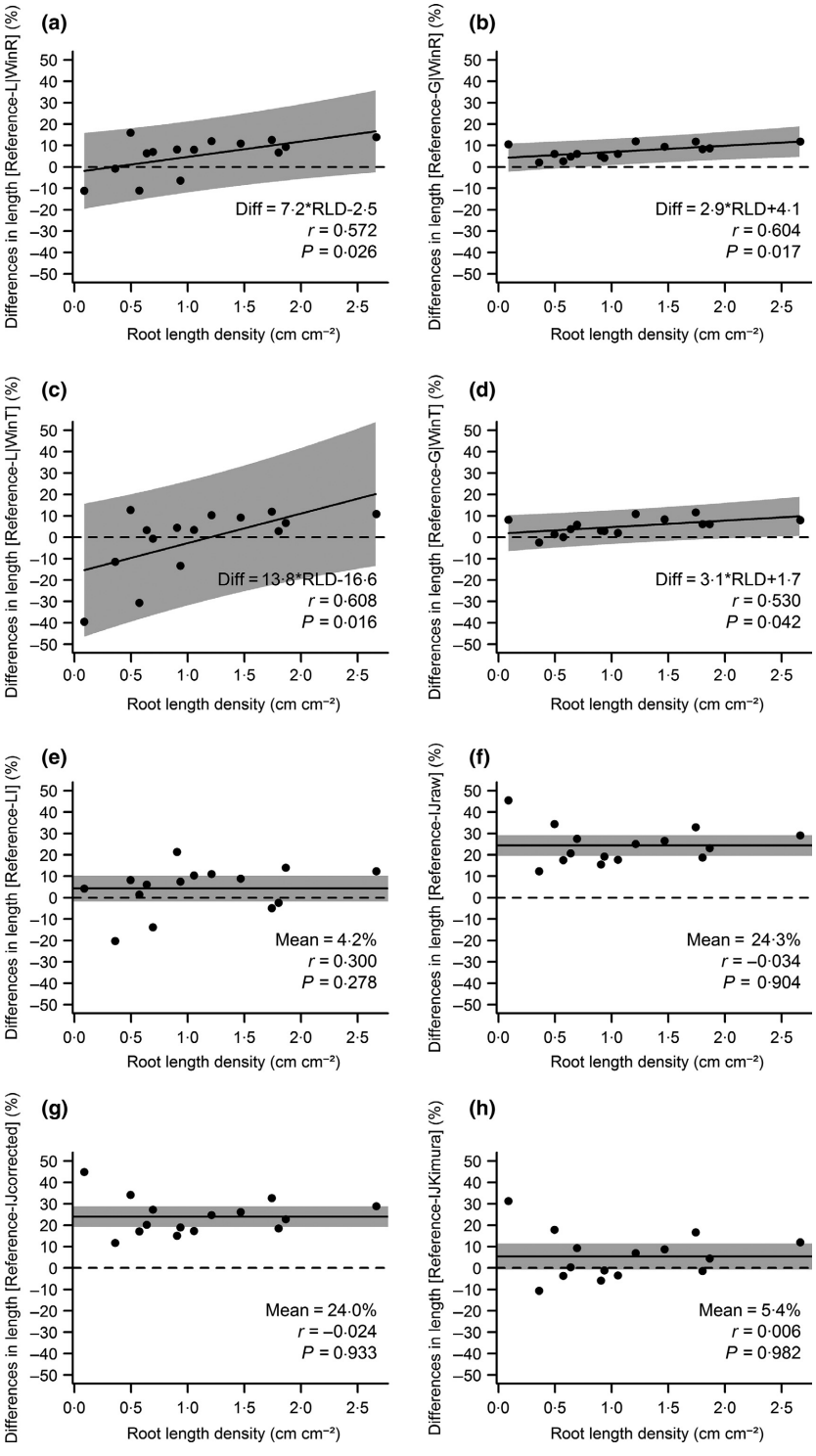

Fig. 5. Effect of RLD in the images on the percentage deviation of the estimates from the values computed by the reference method. The Pearson's correlation coefficient (r) and its associated P-value (P) are shown in each plot. When r was not significantly different from zero (e–h), the mean per cent difference (solid line) and its 95% confidence interval (grey area) were plotted. Otherwise, the Model I regression line (solid line) and the prediction interval (grey area) are shown (a–d). Both overestimation (negative differences) and underestimation (positive differences) can be observed in the figures. Abbreviations are explained in Fig. 1. RLD, root length density.

particularly when the samples contain a large proportion of fine roots (Wilhelm, Norman & Newell 1983; Bouma, Nielsen & Koutstaal 2000). In our study, we did not stain the roots because such an operation needs to be avoided when one wants to do chemical analyses of roots after the scanning. A compromise would be to stain only a subsample that will be used for root length measurement, and use the unstained part of the sample for chemical analyses. Collecting a representative subsample is not straightforward but protocols developed to estimate the TRL of large root samples can be found in the scientific literature (Goubran & Richards 1979; Schroth & Kolbe 1994; Costa *et al.* 2000).

Interestingly, the results computed with WinRhizo™ were strongly influenced by the thresholding method used to skeletonize the roots. When the RLD increased, the observed loss of accuracy was lower when the roots were detected with a global thresholding followed by a manual reclassification of grey-level pixels to avoid missing roots. In addition, estimates given

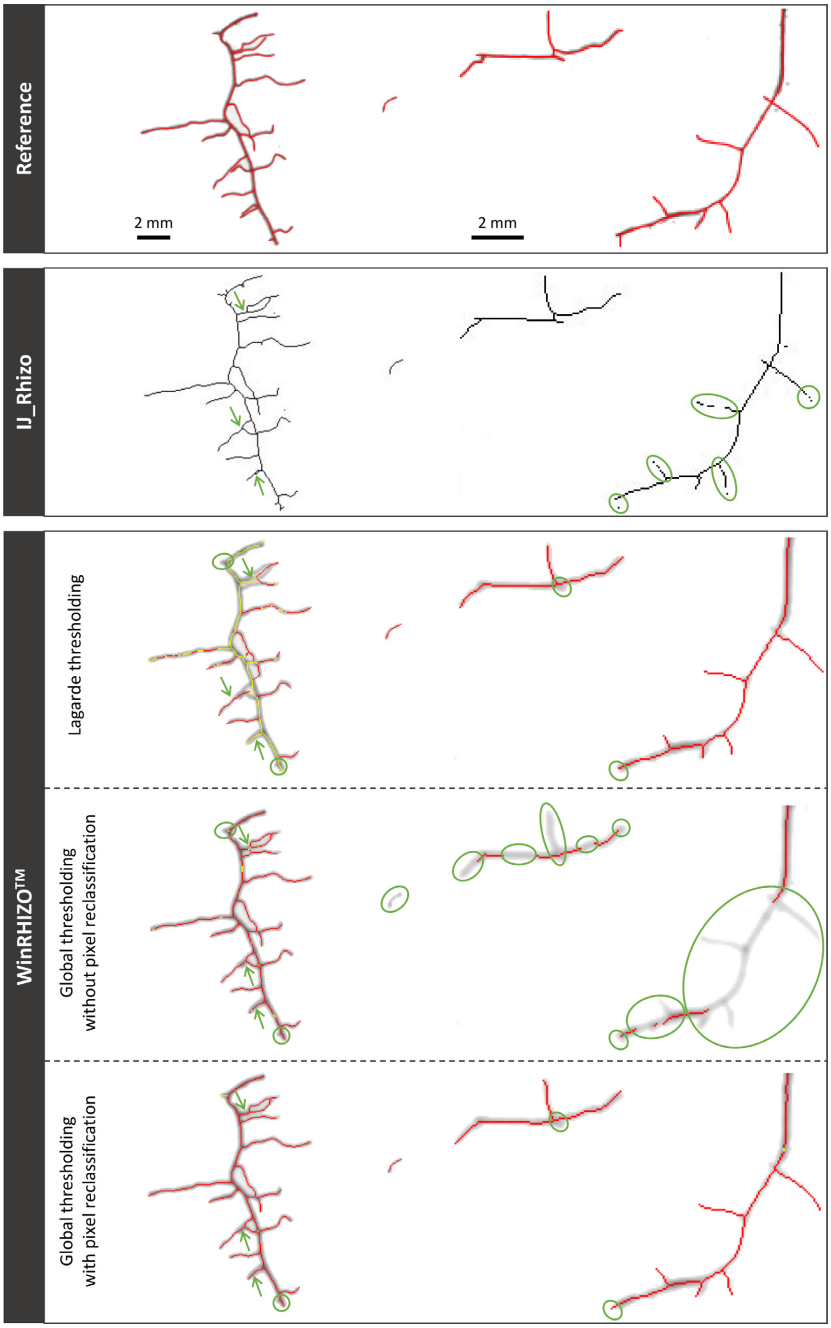

Fig. 6. Common root detection problems observed with the (semi-)automated methods discussed in this study. Reference, roots were manually drawn with ImageJ. Both WinRHIZO™ and IJ_Rhizo methods can misclassify pixels. Two causes are illustrated in this figure: (i) adjoining fine roots were detected as one single root of greater diameter (green arrows), and (ii) automated thresholding methods failed to detect fine roots (green circles).

by WinR and WinT were much closer using this thresholding method. Although the interactive modification in grey-level pixel classification increases the time required to analyse single images, root length measurements were more accurate and less variable using this approach. With regard to IJ_Rhizo, it has to be noted that the macro can be easily modified to test other thresholding methods available in ImageJ (Pierret *et al.* 2013).

Conclusion

In this study, we showed that the use of different methods to analyse the same images can lead to different results despite high correlation between the different methods. Therefore, care must be taken when measurements acquired on unstained root samples with different techniques are being compared. In addition, our results showed that all methods did not have the same accuracy and that using semi-automated or automated methods to estimate the TRL of unstained root samples can lead to underestimation.

Choosing a method for estimating the TRL of scanned root samples is not an easy task. Often, the selected method will be the result of a compromise between the desired level of accuracy and the time that one is able to invest in image analysis. Here, we advocate that the results obtained using image analysis will be influenced by the settings used for

image acquisition and the method used to compute root length. On average, the manual LI method gave satisfactory results but its large limits of agreement suggest that it can lead to inaccurate estimations for single images. In addition, the manual LI method was more time consuming than the other methods used in this study. One has also to keep in mind that the manual LI method has sources of errors that image analysis software packages do not have, such as involuntary omission of intersections, error in intercept interpretation using Tennant's guidelines, operator fatigue and between-operator variation. When possible, we recommend to increase the contrast between fine roots and background during scanning using a dye to stain the roots. In addition, we recommend to avoid overlapping between roots by not exceeding a RLD of 1 cm cm^{-2}. Under these conditions, the best results were obtained with WinRHIZO™ (global thresholding and interactive modification in pixel classification). Interestingly, we found that a good alternative to the commercial WinRHIZO™ software package is the IJKimura method provided with IJ_Rhizo. In comparison with WinRHIZO™, IJ_Rhizo offers two additional advantages: it is free and open source.

Authors' contributions

B.D., E.W. and V.T. conceived the ideas and designed the methodology; B.D., E.W., L.M., A.L., R.v.D. and R.W. collected the data; B.D. analysed the data; B.D. and V.T. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data accessibility

Raw data (Data S1) and R scripts (Data S2) used for data analysis are provided as online supporting information.

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Supporting Information

Details of electronic Supporting Information are provided below.

Table S1. Parameters used in eqn (3) for the calculation of the hyperbolic 95% confidence limits for differences (prediction interval) when there was a proportional bias.

Data S1. A text file containing the raw data used in this study. Abbreviations are explained in Fig. 1.

Data S2. A text file containing the annotated R script used to analyse the data.

6. Appendix

Information below is as of date of submission of the thesis, 11th July 2020.

Overview of articles included in this cumulative Ph.D. thesis

(in accordance with the guideline for cumulative dissertations in Sustainability Science [January 2012], in the following termed "the guideline")

Title of Ph.D. thesis: Improving agricultural nutrient use efficiencies: effects of crop rotation, high carbon amendments, and fertilizer application timing on barley

Papers included:

[1] van Duijnen, R., Roy, J., Härdtle, W., & Temperton, V. M. (2018). Precrop functional group identity affects yield of winter barley but less so high carbon amendments in a mesocosm experiment. *Frontiers in plant science*, *9*, 912.

[2] van Duijnen, R., Uther, H., Härdtle, W., Kumar, A. & Temperton, V. M. Timing matters: Distinct effects of nitrogen and phosphorus fertilizer application timing on the root system architecture of *Hordeum vulgare*. Submitted to *Journal of Plant Nutrition and Soil Science*, currently under review.

[3] Delory, B. M., Weidlich, E. W., van Duijnen, R., Pagès, L., & Temperton, V. M. (2018). Measuring plant root traits under controlled and field conditions: step-by-step procedures. In *Root Development* (pp. 3-22). Humana Press, New York, NY

[4] Delory, B. M., Weidlich, E. W., Meder, L., Lütje, A., van Duijnen, R., Weidlich, R., & Temperton, V. M. (2017). Accuracy and bias of methods used for root length measurements in functional root research. *Methods in Ecology and Evolution*, *8*(11), 1594-1606

Authors' contributions to the articles and articles publication status (according to §16 of the guideline):

Article #	Short title	Specific contributions of all authors*	Author status	Weighting factor	Publication status	Conference contributions
[1]	Precrop functional group identity affects yield of winter barley but less so high carbon amendments in a mesocosm experiment.	RvD: a-e JR: c-e WH: d-e VT: a-b, d-e	Co-author with predominant contribution	1.0	Published in *Frontiers in plant sciences* (IF = 4.402)	Bonares status seminar 2016
[2]	Timing matters: Distinct effects of nitrogen and phosphorus fertilizer application timing on the root system architecture of *Hordeum vulgare*	RvD: a-e HU: b-e WH: d-e AK: d-e VT: a-b, d-e	Co-author with predominant contribution	1.0	Submitted to *Journal of Plant Nutrition and Soil Science* (IF = 2.083). Currently under review	Bonares Conference 2018, ISRR 2018
[3]	Measuring plant root traits under controlled and field conditions: step-by-step procedures	BD: a-e EW: a-e RvD: a-e LP: d-e VT: a-e	Co-author with important contribution	0.5	Published in *Root development*	
[4]	Accuracy and bias of methods used for root length measurements in functional root research	BD: a-e EW: a-e LM: c-e AL: c-e RvD: c-e RW: c-e VM: a-b, d-e	Co-author with important contribution	0.5	Published in *Methods in Ecology and Evolution* (IF = 6.511)	
			Sum:	3.0		

*(a) = Conception of research approach; (b) = Development of research methods; (c) Data collection and data preparation; (d) Analysis/Interpretation of data or preliminary results; (e) Writing or substantive rewriting of the manuscript

Explanations

Specific contributions of all authors

RvD = Richard van Duijnen, VT = Vicky Temperton, WH ´Werner Härdtle, BD = Benjamin Delory, JR = Julien Roy, HU = Hannah Uther,

AK = Amit Kumar, LP = Loïc Pagès, EW = Emanuela Weidlich, LM = Leonie Meder, AL = Anna Lütje, RW = Rafael Weidlich

Author status

according to §12b of the guideline:

Single author [Allein-Autorenschaft] = Own contribution amounts to 100%.
Co-author with predominant contribution [Überwiegender Anteil] = Own contribution is greater than the individual share of all other co-authors and is at least 35%.
Co-author with equal contribution [Gleicher Anteil] = (1) own contribution is as high as the share of other co-authors, (2) no other co-author has a contribution higher than the own contribution, and (3) the own contribution is at least 25%.
Co-author with important contribution [Wichtiger Anteil] = own contribution is at least 25%, but is insufficient to qualify as single authorship, predominant or equal contribution.
Co-author with small contribution [Geringer Anteil] = own contribution is less than 20%.

Weighting factor

according to §14 of the guideline:

Single author [Allein-Autorenschaft]	1.0
Co-author with predominant contribution [Überwiegender Anteil]	1.0
Co-author with equal contribution [Gleicher Anteil]	1.0
Co-author with important contribution [Wichtiger Anteil]	0.5
Co-author with small contribution [Geringer Anteil]	0

Publication status

IF = ISI Web of science – Impact Factor 2019

Conference contributions (acronym, society, date, venue, website)

Bonares Status Seminar 2016	Bonares, November 14-15, 2016, Leipzig (Germany), https://www.ufz.de/index.php?en=41520
Bonares Conference 2018	Bonares, February 26-28, 2018, Berlin (Germany), http://www.bonares2018.de/
ISRR 2018	International Society of Root Research, July 8-12, 2018, https://www.ortra.com/events/isrr10/Home.aspx

Declaration (according to §16 of the guideline)

I avouch that all information given in this appendix is true in each instance and overall.