Semifield root phenotyping

Root traits for deep nitrate uptake

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Semi-field root phenotyping: Root traits for deep nitrate uptake

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Conflict of interest
The authors declare no conflict of interest.

Summary statement
Root traits of winter wheat were constructed from minirhizotron image data. Root traits predicted 24% of \(^{15}\)N tracer uptake, which was injected at anthesis at 1.8m depth, and explained genotypic variation in tracer uptake.

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Abstract
Deep rooting winter wheat genotypes can reduce nitrate leaching losses and increase N uptake.

We aimed to investigate which deep root traits are correlated to deep N uptake and to estimate genetic variation in root traits and deep $^{15}$N tracer uptake.

In two years, winter wheat genotypes were grown in RadiMax, a semi-field root-screening facility. Minirhizotron root imaging was performed three times during the main growing season. At anthesis, $^{15}$N was injected via subsurface drip-irrigation at 1.8m depth. Mature ears from above the injection area were analysed for $^{15}$N content. From minirhizotron image-based root length data, 82 traits were constructed, describing root depth, density, distribution and growth aspects. Their ability to predict $^{15}$N uptake was analysed with LASSO regression.

Root traits predicted 24% and 14% of tracer uptake variation in the two years. Both root traits and genotype showed significant effects on tracer uptake. In 2018, genotype and the three LASSO-selected root traits predicted 41% of the variation in tracer uptake, in 2019 genotype and one root trait predicted 48%. In both years, one root trait significantly mediated the genotype effect on tracer uptake.

Deep root traits from minirhizotron images can predict deep N uptake, indicating potential to breed deep-N-uptake-genotypes.

Introduction
Deeper rooting crops expand the soil depth from which nitrogen (N) can be taken up. This increases the N use efficiency of cropping systems and decreases leaching losses (Dresbøll and Thorup-Kristensen 2014). The primary form of mineral N in temperate soils is nitrate, which is highly mobile in the soil water solution, as it is a negatively charged molecule (Allred et al. 2007). Therefore, nitrate percolates with excess precipitation, and reaches deep soil layers easily. If subsequent crop roots do not penetrate to these soil layers, nitrate leached so deep that it is close to the bottom of the root zone will be lost from the cropping system. If we expand the
rooting depth of crops, we can increase the uptake of leached nitrate (e.g. Thorup-Kristensen 2006) and increase total crop N uptake (Thorup-Kristensen et al. 2009). The importance of deep roots for N acquisition in a leaching situation was suggested in the “steep-cheap-deep” ideotype concept (Lynch 2013).

A two-year Danish field experiment found significant variance in root depth between cultivars and that deeper roots extracted more N from deep soil (Rasmussen et al. 2015). These findings are supported by other studies that measured the deep root N uptake of crops through $^{15}$N injection into deep soil layers (Kristensen and Thorup-Kristensen 2004a, b; Saengwilai et al. 2014; Chen et al. 2019). These studies found significant variation between species (Kristensen and Thorup-Kristensen 2004b, a) and within species (Saengwilai et al. 2014; Chen et al. 2019) in N uptake, showing a direct link of tracer uptake to rooting depth and a genotypic effect.

Little is known about the extent of genotypic differences impacting the rooting depth of winter wheat cultivars. Thus, it is unclear to what extent rooting depth could be optimised through breeding. To investigate this, it is imperative to study root traits under field conditions, and follow the root growth throughout the crop growth cycle (Rich et al. 2020). However too few field studies have compared roots of mature winter wheat cultivars at depths below one meter. These studies found that the maximum rooting depth of winter wheat cultivars had a variation between 0.1m to 0.4m (Rich et al. 2016; Rasmussen, Dresbøll, and Thorup-Kristensen 2015; Botwright Acuña and Wade 2012). Targeting this variation in rooting depth through breeding programs, could be an effective method for increasing nitrogen efficiency in farming systems (Thorup-Kristensen et al. 2020).

Despite earlier breeding efforts not targeting roots (Bingham et al. 2012; Gioia et al. 2015), recent breeding strategies have proposed to focus on increasing root depth and deep root density (Foulkes et al. 2009; Cormier et al. 2016). Richards et al. (2010) suggests trait-based breeding programmes. But it is unclear which specific root traits are most directly linked to deep soil nitrate uptake. In general, deep root traits that have been studied are maximum root depth
(Rasmussen et al. 2015), root density at a specific depth (Kristensen and Thorup-Kristensen 2004b, a; Wasson et al. 2012; Chen et al. 2019; Svane et al. 2019b), or the density of the entire root profile (Wasson et al. 2017). These traits are rather unspecific, not directly connected to the function of nitrate uptake, and subject to spatial and temporal variation. We need to know more specifically which traits facilitate nitrate uptake from deep soil.

The significance of different root traits for deep nitrate uptake depends on multiple factors. Nitrate is easily transported to the roots by both diffusion and mass-flow, due to its high solubility and mobility in the soil solution (Allred et al. 2007). Hence, little root density at depth is required to facilitate nitrate uptake from deep soil. Roots of annual crops penetrate the deep soil layers only at late stages of their growing season. This limits deep root function to a short period during the last stages of crop growth. Typically, N uptake is limited after anthesis (Barraclough et al. 2014), and is affected by genetic variation, which is likely caused by grain sink-strength (Bingham et al. 2012; Barraclough et al. 2014). Furthermore, root function is a multifactorial trait, and focusing on just one parameter might not give reliable results. Thus, a combination of depth, density, distribution and growth together may give the best prediction of deep nitrate uptake. These attributes have inherent trade-offs, based on the metabolic costs to the plant to sustain the root system. For efficient breeding strategies, we need to know which traits are most important for deep nitrate uptake. In addition, understanding the interactions of root traits with soil conditions and aboveground traits is required.

Previous field studies of roots have often been limited in scope as data collection through root observation is labour intensive and often destructive. Therefore, deep root activity has often been studied through above-ground proxy traits, which are easier and cheaper to access. However, as Wasson et al. (2012) discuss, it may be problematic to use these types of proxy traits as they may not be deep root specific. Using stable isotope tracers injected at specific soil depths could allow for above ground measurements with root depth distinctive characteristics. As tracer uptake is a
relatively cheap and easy measurement method, it might be a more optimal tool to phenotype the potential of deep N uptake based on aboveground measurements.

Combined phenotyping of deep rooting and deep tracer uptake can help clarify the relationship between the two. The non-destructiveness of the minirhizotron method allows for tracer experiments, where the tracer is injected into the soil during the growing period, and the crop is harvested at maturity. Root observation can be obtained over the whole growing period. Recent developments in software-based image analysis enable the production of high-throughput belowground phenotyping data (Smith et al. 2020; Svane et al. 2019; Wasson et al. 2016). Therefore, the belowground minirhizotron method combined with an aboveground tracer uptake measurement can help to translate high-quality, image-based root data to plant-based uptake functions.

To understand root functions, we must improve our interpretation of the data obtained from minirhizotron images. Single measurements of root depth are vulnerable to stochastic chance in an inhomogeneous environment such as soil. The chance of a root hitting the minirhizotron surface at a specific area is limited, especially, at the lower boundary of the root system, where root density is low. Increasing the observation area might increase the reliability of the trait measurement but is a trade-off to depth-specific observations. In addition, roots tend to branch out in areas of low resistance. This has been considered one of the draw-backs of the minirhizotron method, as the insertion of the minirhizotron tube likely causes areas with low resistance around the imaging area (van Noordwijk et al. 1985; Volkmar 1993). The resulting root clusters contribute to noise in the data and may not be well related to high rooting density in the surrounding soil. Further, due to the mobility of water and nitrate towards the roots, high root density is not needed for efficient uptake and high root density will increase root zone overlap rather than water and nitrogen uptake (Lynch 2013; Zhang et al. 2020). Therefore, methods for noise and overlap reduction should be considered for the interpretation of minirhizotron images.
The RadiMax facility (Svane, Jensen, and Thorup-Kristensen 2019) offers the possibility to screen large numbers of genotypes (> 100) in semi-field conditions until full maturity. We injected $^{15}$N tracer into the soil at a specific depth to study deep N uptake by winter wheat. Our target depth was the lower boundary of the root profile, based on the assumption that this is where we will find the highest variation in the uptake capacity of deep N. Tracer uptake was then correlated to root traits from minirhizotron images to establish their relationship to measured deep N uptake. The specific aims of this study were 1) to define deep root traits that predict deep N uptake, 2) to investigate the genotype variation in selected root traits and $^{15}$N uptake under semi-field conditions, and 3) to assess the use of isotope tracers as a method allowing us to study deep root uptake through aboveground sampling and measurement.

We tested two main hypotheses: (1) Root observations predict deep $^{15}$N uptake variation. (2) These root observations explain the genotype effect on $^{15}$N uptake.

**Material and Methods**

**RadiMax**

Two winter wheat experiments were conducted in two beds of the semi-field root-screening facility RadiMax (Svane et al. 2019b) in 2018 and 2019. Each bed was 40m long and 9.7m wide. The bed’s bottom was sealed with concrete forming a V-shaped, 23.5˚ slope and spanning from 0.8m to 3m soil depth.

On one side of the slope, each bed was equipped with minirhizotron PMMA plastic tubes (5.5m long and 0.07m wide), situated close to the bottom of the bed. Above each minirhizotron tube, different genotypes of winter wheat were grown with 0.25m distance between lines allowing us to obtain minirhizotron images for each line of plants. Time Domain Transmission sensors (Acclima Inc., USA) were installed at 0.5, 1.0, 1.5, 2.0 and 2.5m depth in the deepest part of the beds, and at two locations in each bed, within the two beds used for the study (Fig. 1). The sensors logged volumetric water content (VWC) and soil temperature every 5 minutes. Along the long side of the bed, following the bottom of the slope, pressure-compensated drip-irrigation
lines were placed at ten different soil depths at 0.2m depth intervals, starting at 1.01m. Additional Time Domain Transmission sensors were located 0.25m above each dripline (Fig. 1).

Meteorological measurements were taken hourly at a weather station, ca. 600m west of the facility. Reference evapotranspiration (ETo) was calculated based on the FAO Penman-Monteith equation for hourly time steps (Allen et al. 1998, 2006). Temperature sum was expressed as Degree Days (Pedersen et al. 2010) with T\text{Min} = 0 \degree C, (Kage et al. 2000).

Movable rainout-shelters were used to create drought conditions from June onwards (see Sub. Tab.1). In 2018, the dripline irrigation system was used for subsurface irrigation aiming to keep the soil water content around field capacity throughout the reproductive growth phase. The management objective was to keep the soil at volumetric water content of ca 20% (pF=1.8), measured at individual locations on the slope. In 2019, subsurface irrigation was not applied.

**Tracer injection**
Tracer injection was performed by an adapted method described by Chen et al. (2019). The $^{15}$N tracer (Ca($^{15}$NO$_3$)$_2$, 98% enriched) was applied at end-anthesis at 25 mg $^{15}$N per m dripline by injecting a solution with 12.9 mg $^{15}$N L$^{-1}$ and a total water amount of 2 L m$^{-1}$ through the upper subsurface drip-irrigation line at 1.81m depth on the side of the bed with minirhizotron installations (Fig. 1, A Tab.1). Due to tracer dispersion, we assumed the injected area above the dripline to be 0.3m wide, spanning over the entire length of the bed. Based on this assumption an application of 85 mg $^{15}$N m$^{-2}$ tracer injection was calculated. To ensure equal distribution of tracer along the dripline, we flushed the dripline with tracer solution, until we collected a volume of solution corresponding to the total volume of the dripline at the end of the facility. After this, the valve at the end of the dripline was closed to allow pressure in the dripline to build up, and then a tracer solution was injected into the soil through the drippers. After the tracer solution was applied, the valve at the end of the dripline was opened again, and the dripline was flushed with fresh water to remove residual tracer solution from the system.

**Plant samples**

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Modern winter wheat varieties and advanced breeding material from Denmark were selected for the experiment. All commercial varieties were younger than 25 years, with the majority being released within the past ten years. The advanced breeding material stems from two Danish breeding companies. All varieties were adapted to Nordic climate conditions and used for bread or fodder wheat production and of similar phenology. The plants were sown with 300 plants m\(^{-2}\) and in 0.25m row width. In 2018, 95 varieties with two to four replicates were grown. In 2019, 120 varieties with two replicates were grown (A Tab.1).

At harvest, ten ears from a 0.3m wide area right above the dripline used for tracer injection were harvested, dried at 105 °C for 48h, milled to 250µm (Cyclone Mill Twister, Retsch, Germany) and analysed for \(^{15}\)N content at the Stable Isotope Facility, UC Davis by continuous flow isotope ratio mass spectrometry (Sercon Ltd., Cheshire, UK). Enrichment of plant samples is expressed as δ values relative to air (Sharp 2007).

The remaining ears were harvested, threshed and oven dried. Grain yield was estimated by the harvested area and grain dry weight. Grain protein content was measured by near infrared reflection (NIR) spectroscopy (Intratec grain analyser, Foss, Hilleroed, Denmark), and divided by the nitrogen to crude protein conversion factor of 6.25 for cereals (ISO 16634-2:2016 2016).

Grain N-uptake was calculated by multiplying grain yield and protein content.

**Root images and image analysis**

Roots were imaged by a multispectral camera unit (Svane et al. 2019a) at three time-points in the late growing season of winter wheat each year. The time-points were at early booting, BBCH 39-41, late anthesis/early grain filling, BBCH 68-71 and phenological maturity, BBCH 87 (A. Tab. 1). For easier references, the imaging time-points are from here on referred to as BBCH_40, BBCH_70 and BBCH_87, respectively, despite small differences in the phonological stage. Images were taken with 35mm interval distance. In 2019 and in May 2018, the total tube length of 5.5m was imaged. In June and July 2018, imaging started at 1.3m soil depth, and resulted in a total tube length of 4.25m.
Analysis of root images was performed using a convolutional neural network (Smith et al. 2020), which was adapted to the RadiMax facility (Svane 2019; Guo et al. 2020). The software detects pixels that represent roots, which are converted into root length per image.

**Construction of root trait estimates**

From the image-based data, in total 82 root traits were calculated (Fig. 2, A. Tab. 2). They represent different calculations of root depth, density, distribution, and growth.

Varying approaches were established, to handle method specific challenges and to increase accuracy and stability of root estimates based on root length data obtained from minirhizotron images.

Upper areas of the root profile were left out of the analysis, by applying cut-offs to the profile to focus on deep roots specifically (Fig. 2 a, Svane, Jensen, and Thorup-Kristensen 2019). Another approach calculating root traits used cumulative root profiles (Fan et al. 2016; Pierret et al. 2016). From these curves, distribution parameters were calculated at which depth 50% or 75% of the cumulated root length was present (Fig. 2a). Those distribution parameters were additionally further used as cut-off points. Linear regressions were fitted to image-based data for the deep section of the root profile, of which intercept and slope parameters were extracted (Fig. 2 b, Bodner et al. 2013; Cardinael et al. 2015). Root growth between two time-points was calculated by subtracting root length in depth intervals (Fig. 2c). As soil moisture changes and age change the reflectance of roots in the multispectral imaging way (Lobell and Asner 2002; Nakaji et al. 2008), late season root systems might be under-detected in the root segmentation model trained for young roots in wet soils (Svane et al. 2019a). To compensate for the potential losses in detected root length at late imaging time-points, only positive changes were accepted. Maximum root depth (MRD) was estimated by the average depth of the three deepest consecutive images with a root length above a certain minimum threshold (Fig. 2 d, Svane, Jensen, and Thorup-Kristensen 2019). Another approach calculating rooting depth was applied by threshold calculations of minimum root length required at the bottom of the root profile to increase the area

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of images taken into analysis (Fig. 2 e, f). To reduce the effect of root-clustering, a non-linear approach was applied by transforming root length per image to the square root of root length. All Traits were calculated for each time-point separately.

**Statistical analysis**

**Effect of root traits on $^{15}$N uptake**

Relationships between measured traits, including the calculated root traits, and $\delta^{15}$N data were analysed and candidate traits were identified by least absolute shrinkage and selection operator (LASSO) regression with leave-one-out cross validation (Webb et al. 2011). All root traits were included in the regression, with each plant row representing one replicate. Delta $^{15}$N was log-transformed to obtain normal distribution of residuals. All traits ($X_i$) were scaled by subtracting mean ($\mu_i$) and dividing by standard deviation ($\sigma_i$) for each trait (Eq. 1).

$$\frac{(X_i - \mu_i)}{\sigma_i} \tag{1}$$

The regression was run with three different scenarios with varying sets of predictors: 1) only belowground traits, 2) only above ground traits, 3) below- and aboveground traits. On top of this the “bed” was also included as a potential block effect in all three scenarios. For each scenario, 100 iterations were run. Parameters selected in at least 70% of the runs were chosen for further analysis. For each run, a random training set with 70% of the total data was created. Prediction precision ($R^2$) was tested by correlating the LASSO-model predictions against the actual data from the test set given as the remaining 30% of the total data. Average and standard deviation of the $R^2$ was calculated over all 100 iterations. The two experimental years were analysed separately with $n=249$ and $n=236$ observations in the respective years. Variable selection was performed using the glmnet package (Friedman et al. 2020).

**Genotype effect**

To determine the genotype effect of root traits on $\delta^{15}$N, a mediation analysis was performed with the candidate root traits as mediators (Fig. 3). Due to spatial variation effects in the facility (Guo et al. 2020), only replicated genotypes were included in the mediation analysis. This was done, to allow for higher reliability of the genotype effect in the model. The direct effects $\alpha$ and $\gamma_1, \ldots, \gamma_n$ of
the genotype (ID) and the root traits (trait\_1,\ldots,\text{trait}\_n) on $\delta^{15}$N was estimated in a linear normal model with spatially autocorrelated residuals (Eq. 2), where the residuals are modelled as the sum of independent errors and a random term ($A$) with exponentially decreasing correlations between each row ($x$) within beds (Pinheiro et al. 2020). The mediated effects $\beta_1,\ldots,\beta_n$ of the genotype on the root traits were tested in similar spatial correlation models (Eq. 3).

$$\log(\delta^{15}N)_{yi} = \alpha(\text{ID}) + \gamma_1 \cdot \text{trait}_{1,i} + \cdots + \gamma_n \cdot \text{trait}_{n,i} + A(x_{i,\text{bed}},) + \text{error}$$ \hspace{1cm} (3)

The model (Eq. 2) was reduced by removing the candidate root traits that were not significant on the 5% significance level. The resulting predictions $\hat{y}_i = y_i^{direct} + y_i^{trait_1} + \cdots + y_i^{trait_n}$ of $y = \log(\delta^{15}N)$ using the reduced model were accordingly decomposed into the sum of the direct and indirect effects given by

$$\hat{y}_i^{direct} = \hat{a}(\text{ID}_i) \hspace{1.5cm} \hat{y}_i^{trait_n} = \hat{\gamma}_n \cdot \hat{\beta}_n(\text{ID}_i)$$ \hspace{1cm} (4)

Following the approach by Lindeman, Merenda, and Gold (1980) the relative importance of the direct and indirect effects on $^{15}$N uptake were found as the partial $R^2$ averaged over all possible orderings of the direct and indirect effects. Here the partial $R^2$’s were found by using Eq. (2) for a given effect together with the preceding effects in a given ordering, see Grömping (2007, Eq. 7).

Statistical computations were done in R (R core team, 2020) using the nlme package (Pinheiro et al. 2020).

**Results**

**Meteorological conditions during experimental phase**

Although the temperature sum over the whole growing period was similar in the two years (Fig. 4 a, b), the temperature conditions of the two years were quite different: 2018 was colder in spring and very warm from mid-April and onwards. High temperatures during the reproductive phase made winter wheat ripen one week earlier in 2018 compared to 2019 (A. Tab. 1). Higher temperatures in the late growing season of 2018 also result in an increase of ETo. Further, in the time period from tracer injection to harvest, the average relative humidity was 67.6% in 2018,
being 8.3% lower than in 2019. While the total cumulative sum of ETo from March to harvest did not differ much between the years (Fig. 4 a, b), ETo was 48mm higher between May and harvest in 2018 than in 2019, reflecting the exceptionally warm and dry summer months in 2018.

This was also reflected in higher water consumption in 2018, as soil water content decreased to permanent wilting point (pF=4.2) at 12% VWC at 1m soil depth in the beginning of July. In 2019 drying at the same depth was delayed and stopped at 14 % (≈pF 3.5) (Fig. 4 c, d). Significant water extraction up to 2m was observed in both years, plateauing around the same level with 18% and 22% VWC at 1.5m and 2.0m soil depth, respectively (Fig. 4 c, d).

Soil temperature in spring (March to April) was lower in 2018 than in 2019, but increased at all measured depths, resulting July soil temperatures being 1 °C (at 2.0m) to 2.5 °C (at 0.5m soil depth) higher in 2018 compared to 2019 (Fig. 4 e, f).

The use of rainout shelters was initiated at a later stage in 2019 than in 2018, allowing for ca. 50 mm higher precipitation in spring and summer than in 2018 (Fig. 4 a, b, A. Tab. 1). Different subsurface irrigation management influenced soil water content (Fig. 4 c, d). While in 2018, the area around the dripline at 1.8 m was maintained between 20 – 30% VWC until harvest, water content decreased rapidly from June onwards in 2019, levelling off in July around pF 3.5 (14% VWC). In 2019, the water content measured at the injection dripline (1.8m) decreased faster and levelled off at a lower level than the water content measured at 1.5m depth in the deepest part of the facility (Fig. 4d). In 2018, the water content at the tracer-injection dripline was kept approximately 10% higher than the measurement at 1.5m depth from end-June onwards (Fig. 4c), due to capillary rise from the irrigation system below.

**Root profile**

Average root length distribution over soil depth varied between years and observation dates (Fig. 5). There was a general shift from roots mainly being found in the upper 1.5m in May towards deeper soil layers in June. July data show a decrease in root length above 1.6m, which we suspect is due to fading of root colour and a change in root-soil contrast due to the drying of the soil.
Average rooting depth, when measured as maximum rooting depth (MRD) (A. Tab. 2) was 1.7m at BBCH_40 both years, 1.8m and 1.9m at BBCH_70 and 1.8m and 2.0m at BBCH_87 of 2018 and 2019 respectively, indicating limited root growth after anthesis and deeper rooting in 2019.

A higher root length at depth was observed in 2019 compared to 2018. Average root length below 1.3m soil depth was 0.6m and 0.9m in 2018 and 2019, respectively.

**Tracer uptake**

Significant tracer uptake was measured in winter wheat ears and was higher than standard wheat $^{15}$N signature with $\delta^{15}$N of approximately 2.0 (data measured in 2018 from a local experiment with winter wheat, n=64) in both years (A. Fig. 1). Delta $^{15}$N was on average 2.5 times higher in 2018 compared to 2019. Also the variation of log-transformed $\delta^{15}$N values between single lines was higher in 2018 with a variance of 1.79 compared to 1.14 in 2019.

**Root trait selection**

Despite all root traits being calculated from the same minirhizotron image data, the root traits captured different aspects of the root system as they did not show full correlation to each other (A. Fig. 2). Distribution parameters, regression parameters and growth parameters showed to be least correlated with the other traits. June and July traits were more related to each other than to May derived traits (A. Fig. 2).

Root traits were selected for deep nitrate uptake by comparing traits to tracer uptake. Of a total of 82 root trait constructions (A. Tab.2) plus bed, the LASSO regression with belowground only predictor set selected three root trait candidates in 2018 and one root trait and the block effect bed in 2019 which predicted up to 24% of the variation in $^{15}$N uptake (Tab. 1). The selected trait candidates represent a combination of root length and root depth measurements from different time-points. Noise cancelling approaches, including summed square root of the image based data or fitting of a linear model, were used in two of the total four selected trait candidates. In 2018, the selected trait candidate with the highest model estimate was an intercept trait, based on a linear regression of the lower part of the root profile. Both years also include one of the
“TRDcomb” traits, which is the depth at which a root length threshold is met, averaged over three increasing threshold lengths.

In 2018, the selected trait candidates were from all three growth stages, the selected trait candidate in 2019 was from the BBCH_70 stage. Model predictability was low in 2019 (range of R² from all three scenarios = 13.6 - 14.2 %), but markedly higher in 2018 (range of R² from all three scenarios = 9.2 – 27.3 %). In both years, adding aboveground traits, which by themselves were not predictive for ¹⁵N uptake, to the model did not increase the model predictability significantly. In 2019, bed showed strong predictability, and was the only parameter selected in the scenario 3.

Genotype effect
The mediation analysis showed both direct and indirect effects of genotype on δ¹⁵N. We found a significant effect of genotype on all LASSO-selected root trait candidates on δ¹⁵N uptake in both years (p < 0.001) (Tab. 2). The combined model explained 41 % of the total variation of δ¹⁵N in 2018 and 48 % in 2019 (Tab. 2). In both years, one root trait candidate mediated the effect of genotype on δ¹⁵N significantly, in 2018 it was iD75_intercept_BBCH_40 (γ: p < 0.001), in 2019 it was ssqrt_TRDcomb_BBCH_70 (γ: p < 0.027) (Tab. 2). In 2018, 84.1 % of the variation explained by the model was due to the direct genotype effect, which was likely caused by the high number of factor levels. In 2018, the indirect effect of the significantly mediating root trait, the intercept estimate iD75_intercept_BBCH_40, explained 1.8% of the root effect. The other two threshold traits explained 6.2 and 7.9%, with TDR_40_BBCH_70 and TDRcomb_BBCH_87 respectively. In 2019, the indirect effect of ssqrt_TRDcomb_BBCH_70 explained 1.8 % of the variation.

Discussion
Tracer uptake
We showed that winter wheat genotypes differ in deep N uptake and that deep placed ¹⁵N tracer experiments can be used for phenotyping these differences in root activity at the lower boundary.
of the root profile. The tracer technique used in this experiment allowed us to measure deep root functioning using aboveground samples. The variation in δ¹⁵N was plant-row specific (A. Fig. 1) and exceeded variation reported on isotope fractionation processes based on plant physiology (Sharp 2007). Therefore, the high variation of ¹⁵N in the plants must have derived from tracer uptake and should relate to aspects of deep root activity and nutrient acquisition.

**How directly is ¹⁵N uptake related to root activity?**

Root trait candidates predicted up to 24% of variation in tracer uptake. The LASSO regression was performed on a pool of trait candidates, which showed different aspects of deep root development (A. Fig. 2). Other parts of the variation might have derived from other plant traits of deep N uptake not included in the analysis, from environmental effects in the facility, and from general variation in the measurements of root traits or ¹⁵N uptake itself. The high predictive power of bed in year 2019 (Tab. 1) indicates that at the low level of uptake present in year 2019, environmental impacts as south or north direction, as well as potential small differences in tracer application became dominating. Further, we applied a linear model to a potentially non-linear relationship.

Although roots were present at depth in 2019 and exceeded the root length at depth in 2018 (Fig. 4), variation in tracer uptake was limited in 2019. The difference between the years in ¹⁵N uptake, the predictability via root traits and the predictor variables might be due to water availability and water demand at the tracer injection depth and may decrease the generalization power of the model at this stage.

The fast reduction in water availability at the depth of tracer injection in 2019 might have caused reduced tracer uptake and limited row specific differences in 2019. While in 2019, the labelled soil dried out fast after injection, continued sub-irrigation and high transpiration demand in 2018 facilitated continued mass flow and transport of ¹⁵N. In 2019, mass flow ceased around the dripline, as the impermeable membrane along the slope seals the facility off from additional soil water, creating a water deficit gradient. The reduced mass flow in 2019 might have caused
residual tracer left in the soil water and a low tracer recovery. Modelling the hydraulic properties in the facility will help us better understand the water dynamics at play.

As the tracer injection time-point coincided with anthesis, tracer uptake is dependent on post-anthesis N uptake capacity of the genotype, so that we measured the ability for post-anthesis N uptake, rather than total deep N uptake ability. In a field experiment comparing wheat varieties, Barraclough et al. (2014) found on average 16 to 31% of the total N uptake occurred post-anthesis, depending on N fertilization level and genotype. Hence, variation in tracer uptake capacity may be influenced by genetic variation in post-anthesis N uptake capacity.

**Linking root traits and $^{15}$N uptake**
Deep root density traits were most predictive for $^{15}$N uptake and their effect relates to N uptake characteristics, since N is transported to the roots by water flow and diffusion (Allred et al. 2007). This makes a dense root network more effective at nutrient uptake, though investment in unnecessarily dense deep root structures will be a waste of resources for the crop.

Depth specific root traits were less predictive for $\delta^{15}$N compared to other root trait candidates, which might be due to the root observation method and tracer injection placement. Tracer injection was made in the deeper 20% of the total root zone rather than at actual MRD, which was ca. 0.2-0.3m below tracer injection. This suggests that depth alone did not account for the variation in tracer uptake, but that root length measurements in the deeper part of the profile were more relevant for N uptake than total depth. In addition, Svane (2019) did not find rooting depth to significantly predict deep root activity related measurements under semi-field observations, while deep root density showed a better correlation to biomass production under drought stress.

The stronger effect on tracer uptake of deep density than root depth traits may show the stronger importance of deep root density, but it could also be the effect of the low measurement accuracy of root depth measurements, related to the small image area upon which the trait calculation is based. Especially at depth, previous studies have shown a large impact of the soil environment on root growth (White and Kirkegaard 2010; Gao et al. 2016). Further, Guo et al. (2020) found
spatial variation to be dominating over root traits in the RadiMax facility. Although we used an auto-correlation approach to take spatial variation into account, this highlights the need for further investigations of root-soil interactions in different environments.

We cannot discern whether the higher accuracy of density related root traits in predicting $^{15}$N uptake compared to root depth related traits is a real effect or an artefact of our method. Either deep root density is more important for $^{15}$N uptake, or we may be better at measuring deep root density than at measuring rooting depth, and therefore find higher accuracy of deep root density than of rooting depth in predicting $^{15}$N uptake, even if rooting depth is really the most important factor. Square root calculations, used for noise-cancelling, were included in most selected traits, where relevant. This indicates general uncertainties in image based root data and root zone overlap and root competition for water and nitrate in soil volumes with clustered roots, causing a non-linear relationship between root density and root function for these resources. This might further imply that a larger observation area on the minirhizotron surface could make results more statistically robust, as discussed in Svane (2019). We need a better understanding of the interactions between deep root density and the ability of the deep roots to efficiently exploit N available deep in the root zone (Plett et al. 2020).

No root trait candidates related to changes in root length between measurement time-points were selected by the LASSO regression. This may be because this trait was actually irrelevant for deep N uptake, or because we took imaging measurements in a four-week interval, which might have been too long to catch relevant changes. For more predictive time-series root traits, more frequent root imaging after anthesis may be needed, with an improved segmentation model with a better ability to identify older roots on a background of dryer soil and differentiate them from newer roots.

Traits identified from any of the three phenological stages, BBCH_40, BBCH_70 and BBCH_87 were selected by the LASSO regression as root trait candidates predicting $^{15}$N uptake. In 2019, only one trait from data imaged at BBCH 70 was selected. As the tracer was injected at BBCH
68, and most tracer uptake occurring shortly after injection it is not surprising that BBCH_70 root traits were most predictive for tracer uptake. This is especially the case in 2019, where the soil was drying out after injection, leaving tracer uptake around the BBCH_87 imaging to diffusion. The different depth interval imaging strategy in 2018, where imaging started at 0.8m at BBCH_40 but at 1.3m at BBCH_70 and BBCH_87, may have caused the selection of fewer BBCH_70 than BBCH_40 traits. As some traits were calculated based on the total profile, BBCH_70 and BBCH_87 traits were affected by the deeper starting point of the imaging, and hence were less predictive. These considerations imply that external factors of soil water conditions and imaging depth might have impacted the prediction power for tracer uptake more than specific growth stages of the plants at the imaging time-point. We can, however, not discern whether a more frequent imaging strategy and a resulting time-series model on the root front might result in a better prediction.

**Genotype effect on $^{15}$N uptake and root traits**

As we could relate some of the $^{15}$N variation to root observations, we tested if and how much the selected root trait candidates could explain the genotype specific variation as an indication for root trait focused breeding efforts.

Genotype showed strongly significant effects on root trait candidates and $\delta^{15}$N in both years, indicating that we can measure deep root related genetic differences. In both years, one root traits had significant mediation effects ($\gamma$) on $\delta^{15}$N ($p<0.05$), meaning that after correction for genotype effects those root traits increased predictability of $\delta^{15}$N uptake. In 2018, two additional root traits added further accuracy to the prediction of $\delta^{15}$N, which was higher than the mediating root trait. This indicates that these root traits carried prediction for $\delta^{15}$N uptake which was included in the variation of genotype. Both, significance in the mediation analysis and substantial predictability of $\delta^{15}$N by root traits shows that the isotope tracer method can be used to identify genotype specific root traits that affect deep N uptake.
Due to the high number of factor levels and the low replication number, the strong correlation of modelled and predicted $\delta^{15}$N observations especially in 2019 should be taken with caution. The higher direct effect of genotype on $\delta^{15}$N than on root traits in 2018 further indicates that additional genotypic traits apart from those recorded by image based root data influenced $^{15}$N uptake.

Aboveground measurement for below ground traits
With the specialised root screening facility RadiMax, we were able to measure deep root activity by tracer uptake, a specific aboveground measurement, while the common aboveground traits grain yield and N uptake did not sufficiently predict deep N uptake. The set-up of the facility allows for deep isotope injections at any time-point without disturbing the soil, while at the same time being as close to real field conditions as possible. Thereby we do not need to rely on less related traits (Wasson et al. 2012), or on specific site and weather conditions (Kirkegaard et al. 2007). It has been shown that $^{15}$N tracers can be used for specific aboveground measurements of deep root activity, when injected at the deep end of the root profile, where roots seem to be young and active with a high affinity to N (Kristensen and Thorup-Kristensen 2004a; Chen et al. 2019).

Adding the commonly used aboveground traits: grain yield, protein concentration and total grain N uptake to the LASSO regression analysis only marginally increased the predictive ability of the resulting model (Tab. 1). The fact that the common aboveground traits were less predictive for tracer uptake may just originate from the overshadowing effect of other processes in these broad traits of cumulative nature (Wasson et al. 2012). As our three aboveground traits, which are some of the most common breeding criteria, do not increase predictability of tracer uptake and hence deep N uptake, our results indicate the need and potential of breeding specifically for deep root related traits.

Potential for breeding deep and late N uptake?
Previous breeding strategies, aimed at increasing grain yield and protein content, have indirectly focused on pre-anthesis N uptake, as there is a higher contribution of pre-anthesis N uptake to grain yield and grain protein than post-anthesis N uptake (Barraclough, Lopez-Bellido, & Hawkesford 2014; Kichey et al. 2007). The existing genotypic variation in post-anthesis N uptake (Barraclough et al. 2014) implies that there is a potential for further improvements through breeding programs. This could further increase the potential of deep roots to contribute to winter wheat N uptake and grain yield performance in suited environments.

Phenotyping strategies must consider the inherent trade-off between the need to scan a large gene pool at low costs per data point, versus more replicates for stronger statistical stability. As little information exists on the deep N uptake capacity of specific genotypes, identifying genotypes that might potentially impact N uptake is needed before large scale trials to identify the effect sizes of individual genotypes are conducted. Therefore, we decided that scanning a large gene pool with few replicates, to identify genotypic candidates was the most relevant strategy. The use of a low number of replicates and small sample area resulted in high variation for both tracer uptake and root traits for our genotype specific measurements. Wasson et al. (2014) discussed methodological or plant plasticity related characteristics being the reason for high in-genotype variation of deep root traits under field conditions. In their study, four replicates per genotype were used, which is double the amount to the present study. The area of our measurements of root and tracer related traits was small with 0.075m² for ¹⁵N uptake and 0.006 to 0.062m² on the minirhizotrons for root traits. Increasing both replicate number and sample area, may be needed in future experiments, to further investigate the genotypes showing to be promising in this study.

The choice of genotypes used in our experiment was rather narrow and may not have captured the potential genotypic differences in deep N uptake. The genotypes in our experiment are all modern northern European genotypes, mostly originating from two Danish breeding companies. Also at larger scale the gene pool of modern winter wheat genotypes is limited (Wissuwa and Mazzola 2009) and wheat has generally been bred without regards to root traits. It might be
valuable to include more diverse genotypes into future screening, to better understand the biology and the potential of breeding for deeper root growth in wheat. Including more extreme genotypes into studies will help extremes from both shallow and deep root profiles, which might increase our understanding of the potential for deep N uptake. Other studies looking into a more diverse gene pool found genetic variation in deep root traits of up to 27% (Botwright Acuña and Wade 2012). Rich et al. (2016) found consistently deeper roots in Indian wheat genotypes which had been developed under deep root favouring conditions. However, to include deep rooting into breeding programmes, exploring the potentials within modern elite material is more realistic, at least in the short term.

**Perspectives**

**Root phenotyping methodology**
The RadiMax facility is a highly specialized root phenotyping facility, which allows for the unique opportunity to use tracer injections at specific depths as a deep root phenotyping tool. Employing deep tracer injections in phenotyping studies elsewhere will require specific efforts, such as trenching or the construction of specific facilities, and will be less applicable as a high-throughput phenotyping method. However, we deem several aspects from the work from the RadiMax facility relevant for other studies. Recent advancements in root image analysis software (Smith et al. 2020) enables high-throughput phenotyping studies. While the image analysis has often been a bottle-neck in phenotyping studies, we are now challenged by finding the best interpretation of these data. In our study several root traits calculated from the length data showed improved prediction for deep N uptake. Especially noise-cancelling approaches and deep intercepts performed better compared to absolute root length data. Further, including somewhat larger areas on the minirhizotron tube improved performance compared to single depth measurements. We also see potential in time-dependent modeling of root data.

**The implications of deep roots for N uptake**
Even small improvements in deep rooting could cause large reductions of nitrate leaching due to the widespread cultivation area of winter wheat in temperate climates. We found genotypic
variation in the uptake of $^{15}$N tracer from 1.8m depth, indicating the potential to increase deep N uptake by breeding processes. World-wide, winter wheat is considered one of the three most important crops grown on ca 30% of the area used for cereal production (FAO 2021), with a significant share of arable land in the temperate regions, where N leaching has caused environmental concerns (Di and Cameron 2002). By combining the genetic potential with appropriate management choices, the difference in deep root traits might become even more effective (van der Bom et al. 2020).

**Conclusion**

We conclude that modern winter wheat genotypes varied significantly in deep $^{15}$N tracer uptake, and that root traits across all observations were able to predict up to 24% of the variation in uptake of deep $^{15}$N tracer. It was found that root traits, obtained from minirhizotron images, explained variation in $^{15}$N tracer uptake: Root density below 1.4m depth was most predictive of deep N tracer uptake, while root depth and distribution traits had additional effects on predicting $^{15}$N uptake.

We found genotypic variation in the uptake of $^{15}$N tracer from 1.8m depth, which was partly explained by root traits, indicating breeding potential for deep N uptake.

Given the high uncertainties inherent to root observations under field conditions, using deep placed $^{15}$N to study deep root activity is likely to be the most promising strategy for deep root phenotyping. Thereby the belowground trait can be measured aboveground allowing for high-throughput phenotyping in a facility that enables deep $^{15}$N injection into the soil. We therefore see tracer studies as an applicable root phenotyping tool, which can help our understanding of root functions over root morphology. The results indicate the potential to increase deep N uptake by plant breeding and offers opportunities in the potential to improve nitrogen use efficiency and reduce N leaching.
Tab.1: LASSO-Regression (n=100) with 70% training and 30% test data-set, randomly drawn. $R^2$ is average, parenthesis is standard deviation (n=100), prediction power of trained data for $^{15}$N uptake. Listed traits are those selected in minimum 70% of the iterations, ordered after average estimate size. Belowground tested only root traits and block effects (bed), above ground only yield, N-uptake traits and block effects (bed), and all includes both above and below ground traits.

<table>
<thead>
<tr>
<th>Year</th>
<th>Traits</th>
<th>$R^2$</th>
<th>(Standard deviation)</th>
<th>Selected parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>2018</td>
<td>Belowground</td>
<td>0.245</td>
<td>(0.076)</td>
<td>TRD$<em>{40}^{BBCH,70}$, TRDcomb$</em>{BBCH,87}$, iD75_intercept$_{BBCH,40}$, bed, grain_yield</td>
</tr>
<tr>
<td></td>
<td>Aboveground</td>
<td>0.092</td>
<td>(0.061)</td>
<td>grain_yield, TRD$<em>{40}^{BBCH,70}$, TRDcomb$</em>{BBCH,87}$, iD75_intercept$_{BBCH,40}$</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>0.273</td>
<td>(0.079)</td>
<td>bed, grain_yield, TRD$<em>{40}^{BBCH,70}$, TRDcomb$</em>{BBCH,87}$, iD75_intercept$_{BBCH,40}$</td>
</tr>
<tr>
<td>2019</td>
<td>Belowground</td>
<td>0.141</td>
<td>(0.068)</td>
<td>bed, ssqrt_TRDcomb$_{BBCH,70}$</td>
</tr>
<tr>
<td></td>
<td>Aboveground</td>
<td>0.142</td>
<td>(0.061)</td>
<td>bed, nitrogen_uptake_NIR</td>
</tr>
<tr>
<td></td>
<td>All</td>
<td>0.136</td>
<td>(0.062)</td>
<td>bed</td>
</tr>
</tbody>
</table>

Tab. 2: Mediation analysis of root traits on the genotype effect of $^{15}$N uptake.

<table>
<thead>
<tr>
<th>Year</th>
<th>Parameter</th>
<th>p-value</th>
<th>Significance of parameters of the total effect model (parameter $\alpha$ and $\gamma$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2018</td>
<td>iD75_intercept$_{BBCH,40}$</td>
<td>&lt; 0.001</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>TRD$_{40}^{BBCH,70}$</td>
<td>&lt; 0.001</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>TRDcomb$_{BBCH,87}$</td>
<td>&lt; 0.001</td>
<td>***</td>
</tr>
<tr>
<td>2019</td>
<td>ssqrt_TRDcomb$_{BBCH,70}$</td>
<td>&lt; 0.001</td>
<td>***</td>
</tr>
</tbody>
</table>

$R^2$ of the total effect and decomposition of $R^2$ for direct (ID) and indirect effects (root traits) predicting $^{15}$N uptake (* indicates significantly mediating root traits)

<table>
<thead>
<tr>
<th>Year</th>
<th>Parameter</th>
<th>Total effect</th>
<th>Percentage due to direct effect</th>
<th>Percentage due to Indirect effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>2018</td>
<td>ID</td>
<td>84.1</td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>iD75_intercept$_{BBCH,40}$</td>
<td>41.04</td>
<td>6.2</td>
<td>1.8</td>
</tr>
<tr>
<td>2019</td>
<td>ID</td>
<td>48.63</td>
<td>98.2</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>ssqrt_TRDcomb$_{BBCH,70}$</td>
<td>48.63</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1: Conceptual view from the side (a) and top (b) of the facility and the $^{15}$N isotope injection set-up through a dripline perpendicular to the plant rows and minirhizotrons. Valves on both ends of the injection line enable fine steering of the water flow through the dripline. Isotope sample from an area of 0.3m above the injection line was taken at harvest.
Fig. 2: Calculation principles of varying root traits. Cut-offs (a) based on relative cumulative root length (x) were inserted into the total root profile. A linear regression (b) of the deeper part of the root profile estimate slope (a) and intercept (b). Root growth between two month was calculated by total positive change between the two profiles (c). Maximum root depth is the average depth (µ) of the three deepest images with a root length above a specific threshold T (d). Rooting depth (x) was calculated based on a root length threshold (T) from the bottom of the profile (e). The mean (µ) of different rooting depth based on differing thresholds (T₁-T₃) gives the combined threshold root depth (f). Abbreviations for root traits in parenthesis, with further defining parameters in italic.

Fig. 3: Mediation analysis of the genotypic effect of root traits on $^{15}$N uptake. Parameter estimate $\alpha$ is the direct genotype (ID) effect on $\delta^{15}$N, $\beta$ the genotype effect on root trait candidates (trait₁, trait₂, ..., traitₙ).
…, trait, and the γ parameter estimates describes the direct effect of root trait candidates on δ^{15}N.

Fig. 4: Weather and soil conditions in the facility during the main growing season of experiment in 2018 (left) and 2019 (right). (a, b) Cumulated Precipitation (blue line) and Degree Days (orange line), and cumulated Evapotranspiration (black line). Soil volumetric water content (c, d) and soil temperature (e, f) profile in the center of the facility (grey shades, four depths, n=4) and around the Tracer injection line (blue grey shade, n=4).
Fig. 5: Average root length per 3.5 cm image interval for each imaging campaign (n_{2018} = 249, n_{2019} = 235). Purple shades are from the 2018 experiment and green shades from the 2019 experiment. Black line is the total average from all six imaging campaign. Horizontal line shows the depth of tracer injection at anthesis (close to the June imaging).

Table legends
Tab.1: LASSO-Regression (n=100) with 70% training and 30% test data-set, randomly drawn. R^2 is average, parenthesis is standard deviation (n=100), prediction power of trained data for 15N uptake. Listed traits are those selected in minimum 70% of the iterations, ordered after average estimate size. Belowground tested only root traits and random effects (bed), above ground only yield, N-uptake traits and random effects (bed), and all includes both above and below ground traits.

Tab. 2: Mediation analysis of root traits on the genotype effect of 15N uptake.

Appendix legends
A. Fig. 1: Distribution of grain 15N uptake over the facility in two years. Dark line represents the moving average from n=10 closest neighbors.
A. Fig. 2: Level of correlation between root traits of all estimated traits including both years. The color intensity expresses the level of correlation, with red being positive and blue being negative correlations.
A. Tab. 1: Agronomic and experimental details of the two years.
A. Tab. 2: List of root traits, their abbreviation used, and how they are defined. Comments on references, or remarks to specific traits. Measure is an indication of specific root architecture or function measured by each trait.

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A. Tab. 3: List of winter wheat genotypes grown in this study, with a description of their origin, and breeding status.

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