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PhD Thesis

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Differential effects on water and nitrogen uptake from deep soil layers by increasing crop rooting depth

Case studies on chicory and oilseed rape

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Preface

This thesis presents the research outcomes of my PhD from February 2018 to July 2021. The PhD project was a part of the "DeepFrontier" project funded by The Villum Foundation (grant number VKR023338). I was also financially supported by the China Scholarship Council (CSC) during this period. The overall aim of the PhD project was to unveil the hidden processes of accessing and utilizing deep placed water and nitrogen by crop plants.

I conducted three separate experiments during my PhD study. The first experiment conducted in 2018 showed the novel findings on water and nitrogen (N) uptake at different depths and their correlations to root intensity of chicory. Results showed that compared with N uptake, water uptake was more sensitive to increasing depth and decreasing root intensities. These findings have been summarized and published (Chen et al. 2021, Rhizosphere).

The two consecutive experiments conducted in 2019 and 2020 generated novel results on the contrasting patterns of water and N uptake at depths by oilseed rape under varying availability of the same soil resource. The findings were synthesized into one manuscript which is ready for submission. In addition, I did some measurements together with Camilla Ruø Rasmussen in 2020. The outcomes will be shown in her paper, where I will be one of the co-author. This paper is in preparation.

This new journey of mine in deep root exploration began four years ago. I was seeking for a PhD opportunity abroad, and found the works done by the group of Professor Kristian Thorup-Kristensen fascinating. I translated his vision in deep rooted systems into an opportunity to enhance resource use efficiency in China and reduce the use of external inputs such as fertilizers. I appreciate that Kristian found my passion and experience fitting into his ongoing research programs and accepted me as his PhD student (Feb 2018).

I had only few experience in root research, and was wondering how these "deep" root studies might be. Luckily, I had a chance to participate in a project meeting shortly after my arrival in Denmark in 2018, from which I was able to plan my research. My first experiment started in April 2018. Before that, I installed a few new sensors in the outdoor facility called "Root Towers". Without any coding skills, I spent days and nights getting the R scripts running. I got tremendous amount of help from my colleagues along the way - Camilla Ruø Rasmussen, Corentin Clementin, and Associate Professor Dorte Bodin Dresbøll on tracer mixing, injection and sampling, root imaging, and so on. Using the dual-labelling technique, I managed to observe different uptake

patterns of deep placed water and nitrogen. This method was further applied to the subsequent experiments. In 2019, I changed the level of fertilization and studied the responses of roots on taking water and nitrate from soil layers below 0.5 m; in 2020, the effects of water supply on deep water and nitrate uptake were investigated. I hurrahed for the great results, despaired at massive pest infestation, and worried about the water leakage. I will always remember these mixed feelings that dominated over my personal and work life.

Thanks to my supervisors and the DeepFrontier project, I had the chance to be involved in several international meetings. I met the top scholars in root research and had a few inspiring discussions with them. At the end of the PhD study, I participated in the 11th Symposium of the International Society of Root Research as a student ambassador. In this program, I learned how to organize an international meeting and trained my social networking skills.

I want to express my gratitude to my principal supervisor. Kristian, thanks for accepting me as a PhD student and being so patient even if I have challenged you so many times. I will never forget the insightful discussions on experiment design, data analysing, and manuscript writing.

I am so grateful to my co-supervisor, Dorte. Thank you for guiding me on the right track and for your warm words and supports when I was down. I might never have come through those hard times without your selfless supports.

The support, help, advice that I received from my colleagues are my treasures. I offer my thanks to Si, Tomke, Corentin, Olga, Simon, Abraham, Weronika, Nes and Niels. Thanks for the lovely morning talks and for bringing me so many joys. Thank you, Aymeric, Jason, Zahra, Signe and Susanne. Your help from either work or life side made this journey much easier. I want to express my appreciation to Eusun. Thank you for always offering selfless help whenever I need you. Special thanks to Camilla. Thank you for being there, teaching, comforting, and cheering me up. The exchange of opinions made the dubious working time so enjoyable. I will do my best to be a strong lady as you are.

感谢我在丹麦遇见的所有中国朋友们。感谢黄晶，任喧，李巧燕，谢谢你们在最后这段最迷茫的时期给我安慰和开导。感谢李胜兰，感谢你在我读博期间与我并肩作战，分享所有的开心和不开心。感谢我的挚友李虹霏，虽然我们不在一起，但你总是最能体会我的情绪，最能发现我的优点，最能及时给我安慰。得友如你，足矣。

我要感谢我的父母。 虽然你们可能并不明白我在做什么，但是你们无条件的信任和支持是我孤身一人在丹麦求学的坚实后盾。也许我并不优秀，但我明白，我始终是你们眼中最棒的小孩。

感谢我的男友，王琛。你的存在于我而言无异于一个奇迹。

这是一段经历的结束，也是全新生活的开始。

Every story has an end, but every end is a new beginning.

Guanying Chen / 陈冠英

June 2021 / 2021 年 6 月

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Summary

Water and nitrogen (N) are two most important factors determining plant growth and development. Adequate supply of these resources is crucial for maintaining yield, meanwhile inefficient use can lead to substantial loss of farm input and potentially to environmental pollution – e.g. N leaching. Deep-rooted crops have been highlighted for enhancing water and nitrogen uptake from deep soil layers. On the other hand, the potential of the increased rooting depth on water and nitrogen acquisition can be modified by the level of water and nitrogen supply. However, due to the lack of techniques to access and study deep roots and their functioning, water and nitrogen supply regulation on deep root growth, deep water, and nitrogen uptake remain unknown.

This thesis discusses current progress and challenges in studying deep water and nitrogen uptake and presents results from three experiments conducted from 2018 to 2020, where the target crops were grown in 4 m tall semi-field rhizotrons. Using a ^{2}H-^{15}N dual-labelling technique and chicory (*Cichorium intybus* L.) as a target crop, the first study revealed the disparities between water and nitrogen uptake at depths of down to 3.5 m. ^{2}H and ^{15}N fraction in transpiration water and leaf samples showed that root water uptake decreased drastically with the increased depth and reduced root intensity. In contrast, the nitrate uptake from 1.1 and 2.3 m was comparable. Furthermore, at the 1.1 and 2.3 m soil layers, the peak of ^{15}N accumulation was shown after ten days of injection, then decreased afterward. The fraction of ^{2}H-labelled water in transpiration water tended to increase until 20 days of labelling. The disparity of the patterns implied more rapid nitrogen uptake than water uptake at the labelled depths.

After getting a general idea of the similarities and discrepancies between deep water and nitrogen uptake, the effects of water and nitrogen application on water and nitrogen acquisition were investigated in the subsequent years. As with the first experiment, ^{2}H-labelled water and ^{15}N-labelled nitrate were applied at 0.5 and 1.7 m depth to track water and nitrogen uptake. Winter oilseed rape (*Brassica napus* L.), which can develop roots to more than 3 m during its lifecycle, was used as a model crop in the following experiments. During the experimental periods, oilseed rape developed roots below 2 m and extracted water and nitrogen from there. Neither water nor nitrogen supply altered the subsequent root growth, while the results showed that both high N application and topsoil water deficiency enhanced subsoil water uptake. Unlike water uptake, there was no implication that deep N uptake was sensitive to water supply, whereas the uptake efficiency of labelled-^{15}N was doubled by reduced N supply. The results address the importance of deep roots in acquiring soil

resources under sub-optimal conditions such as water and nitrogen deficiency, which should be considered when water and nitrogen management are made.

Overall, the studies revealed that deeper rooting, but not necessarily denser roots in the subsoil, was efficient at exploiting subsoil nitrogen. This potential was comparable under water deficiency and could be stimulated by nitrogen deficiency. Although deep rooting also played an essential role in enhancing water uptake when water deficiency occurred in topsoil layers, the contribution of deep roots to total water uptake can be limited to the increased soil depth and reduced root growth in the subsoil.

Resumé

Vand og kvælstof (N) er blandt de vigtigste faktorer, der bestemmer en plantes vækst og udvikling. For at opretholde et højt udbytte er tilstrækkelig tilgængelighed af disse ressourcer afgørende, mens ineffektiv udnyttelse kan føre til betydelige tab og potentielt til forurening af miljøet, f.eks. ved nitratudvaskning. Afgrøder med dybe rødder forventes at øge vand- og kvælstofoptaget fra dybe jordlag. Samtidig kan tilgængeligheden af vand og kvælstof påvirke roddybdens potentielle effekt på en øget vand- og kvælstofoptagelse. Viden om optagelse af vand og kvælstof fra dybe jordlag samt betydningen af vand- og kvælstoftilgængeligheden for dyb rodvækst er stadig begrænset, især pga. mangel på metoder til at studere dybe rødder og deres funktion.

Denne afhandling diskuterer den nuværende viden om vand og kvælstofoptagelse fra dybe jordlag og udfordringerne ved at studere dette og præsenterer resultater fra tre forsøg udført i 4 m høje rhizotroner fra 2018 til 2020. Ved brug af dobbeltmærkning med ^{2}H og ^{15}N i cikorie (*Cichorium intybus* L.) viste det første studium forskellene mellem vand- og kvælstofoptag i forskellige dybder ned til 3,5 m. Fraktionen af ^{2}H i transpirationsvandet og ^{15}N i bladprøver viste, at vandoptaget i rødderne faldt drastisk med stigende dybde og reduceret rodintensitet. I modsætning til dette var nitratoptaget fra 1,1 og 2,3 m dybde sammenlignelige. Det højeste optag blev fundet i begge dybder 10 dage efter injektionen og faldt derefter. Derudover blev der fundet en tendens til en stigning i andelen af ^{2}H-mærket vand i transpirationsvandet frem til 20 dage efter injektionen. De forskellige optagelsesmønstre indikerede et hurtigere optag af kvælstof sammenlignet med vand fra de mærkede dybder.

Efter at have opnået en generel forståelse for ligheder og forskelle mellem vand- og kvælstofoptag i dybe jordlag, blev effekterne af forskellige niveauer af vand og kvælstof på optagelsen af disse undersøgt de følgende år. Ligesom i det første forsøg blev ^{2}H-mærket vand og ^{15}N-mærket nitrat tilsat i 0,5 og 1,7 m dybde for at følge vand- og kvælstofoptaget. Vinterraps (*Brassica napus* L.), der kan udvikle rødder i mere end 3 m dybde i løbet af dens livscyklus, blev brugt som modelafgrøde i de følgende forsøg. I løbet af forsøgene udviklede vinterraps rødder til mere end 2 m dybde og vand og næring blev optaget derfra. Hverken vand- eller kvælstoftilgængeligheden ændrede den efterfølgende rodvækst, men resultaterne viste, at både høj N-tilførsel og mangel på vand i overjorden øgede optagelsen fra dybere jordlag. Ulig vandoptaget var der ingen indikationer på at optag af kvælstof fra dybe jordlag er afhængig af vandtilgængeligheden, mens optagelsen af ^{15}N-mærket nitrat blev fordoblet ved lavere kvælstoftilgængelighed. Resultaterne viste vigtigheden af dybe rødder i forhold

til at udnytte jordressourcerne under sub-optimale forhold som vand- og kvælstofmangel, hvilket skal tages med i overvejelserne når vand- og kvælstoftildeling planlægges.

Alt i alt viste studierne at dybere rodvækst, men ikke nødvendigvis øget rodtæthed i dybe jordlag, øgede evnen til effektivt at tage kvælstof op fra dybere jordlag. Dette potentiale var sammenligneligt under vandmangel og kunne stimuleres af kvælstofmangel. Selvom dyb rodvækst også spillede en væsentlig rolle for at øge vandoptaget når der var vandmangel i de øverste jordlag, kan bidraget fra dybe rødder til det totale vandoptag være begrænset af den øgede dybde og den reducerede rodvækst i dybe jordlag.

摘要

水和氮是影响植物生长和发育的两个最重要的因素。充足的水氮供应对保持产量有关键作用，而植物对水氮的低效利用会导致氮淋洗等环境问题。为了提高土壤深层的水氮吸收，深根作物已被广泛应用于生产实践中。与此同时，深根作物对深层水氮的吸收利用也受水分、氮素供应的调节。但由于缺乏适合研究深层根系及其功能的技术，目前在水氮供应对深层根系生长和深层水氮吸收的影响方面依然知之甚少。

本研究基于当前深层水氮吸收方面的研究进展和不足，利用置于户外的 4 米高的根箱开展作物种植与研究，在 2018 至 2020 年进行了三个相关试验。试验一以菊苣为试验作物，利用 ^{2}H-^{15}N 同位素示踪技术研究 3.5 米深的土层中根系对水和氮的吸收之间的差异。通过分析蒸腾水和叶片样本中的 ^{2}H 和 ^{15}N 的富集值，结果表明根系对水的吸收随着土壤深度的增加和根系密度的降低而急剧下降；与之相比而言，1.1 米和 2.3 米深的根系对硝酸盐的吸收没有显著差异。此外，在 1.1 米和 2.3 米深的土层中注射同位素后，叶片中 ^{15}N 的富集值在同位素注射后 10 天达到峰值，随后呈降低趋势；而蒸腾水中 ^{2}H 的富集值在在取样期内随着时间推移不断增加。这一结果表明，在同一深度， 根系对氮的吸收比对水的吸收更迅速。

在对深层水氮利用的相似性和差异性进行研究后，2019 和 2020 年，作者分别研究了不同水、氮处理对深根作物深层水氮利用的影响。这两个试验同样使用了同位素示踪技术，在 0.5 米和 1.7 米深的土层中施用 ^{2}H 标记的水和 ^{15}N 标记的硝酸盐，以跟踪冬油菜的根系对深层水和氮的吸收。在试验期间，冬油菜的根系深度达到 2 米以上，并有着活跃的水氮吸收。两个试验均于冬油菜花期前后开始， 此时改变水和氮的供应均未对冬油菜后续的根系生长产生显著影响。结果表明，高施氮量和表层土壤水分亏缺均可提高根系对深层水的吸收，而根系对深层氮的吸收不受表层土壤水分含量的影响。反之，低施氮量显著提高了冬油菜对 ^{15}N 的吸收效率。这些结果揭示了深层根系对提高水氮吸收的重要性，特别是在缺水、缺氮等胁迫条件下，深层根系对维持作物生长发育起到关键作用。

本研究表明，虽然土壤深层的作物根系密度较低，但依然有助于深根作物提高对深层土壤中的氮的利用。此外，表层土壤中水氮的亏缺并不会减少深层根系对氮的利用，并且在低施氮量下深层根系对氮的利用效率会显著提高。尽管土层表层的水分亏缺和高施氮量均能提高根

系对深层土壤水分的吸收，但相比于对氮的吸收，根系对水的吸收更易受到土壤深度与根系密度的影响。

Literature review

1. Water and nitrogen

Water and nitrogen are the most limiting factors for crop growth globally (Gonzalez-Dugo et al. 2010; Mueller et al. 2012). To meet the increasing food demand in a sustainable way, modern agriculture needs to improve use efficiency of these resources. While both water and nitrogen play a role in determining plant growth and productivity, these two resources are intricately related from the perspective of either plant physiology or soil availability (Quemada and Gabriel 2016; Sadras et al. 2016; De Pascale et al. 2018; Plett et al. 2020). Additionally, excessive water inputs enhance nitrogen leaching and lead to environmental problems (Cameron et al. 2013). Therefore, for more efficient production systems, it is necessary to take account into individual roles of the two resources and their interactions plant production into consideration.

Water and nitrogen – main and limiting resources

Water is crucial to the life of the plant. Seedling emergence is not possible without the presence of water. Many physiological processes, including cell enlargement, transport processes, enzyme functioning, and transpiration - the most water-consuming process, require the existence of water. In crop production, it takes 1000-3000 m^3 water to harvest one ton of cereal (FAO 2002). Although water is one of the most easily accessible resource for plant growth and development, it is frequently a limiting resource for agricultural production due to the imbalance between plant demand and water supply. Either water deficiency or waterlogging can trigger conditions that are adverse to the growth of plants and decrease the yields (Maryam and Nasreen 2012; Iqbal et al. 2020). Especially drought, the key constraint to global crop production, has reduced cereal production across the globe by 10% during 1964 - 2007 (Lesk et al. 2016). Faced with increasing food demand and the changing climate, it is crucial to improve crop productivity and water use efficiency under drought conditions.

Nitrogen (N) is one of the nutrients which is taken up in the largest quantity in plant tissues and plays an essential role in plant development. Plants utilize different inorganic forms of N, mostly nitrate (NO_3^-) and ammonium (NH_4^+), from the soil and then assimilate them into organic compounds, including amino acids, proteins, amides, nucleic acids, nucleotides, chlorophyll, coenzymes, hexosamines, etc. These compounds are critical to maintaining the functions such as photosynthesis. However, during the growing seasons, N deficiency can occur at any time when N availability cannot meet the plant demand. The initial stages of N deficiency usually appear as

a loss of green color; with the increase of the deficiency, the leaves die and drop, and growth of the plant ceases and further reduces the yield (Mills and Jones 1979). Crop production heavily relies on the extra input of fertilizer. The yearly estimation of global demand for N fertilizer is more than 110 million tons (FAO 2017). However, a large fraction of the applied N is prone to losses through leaching, ammonia (NH_3) volatilization, and denitrification from the soil/plant system, producing severe environmental problems (Raun and Johnson 1999; Cameron et al. 2013).

The transport and uptake of water and N

Absorption of water, dissolved mineral nutrients and conduction of them to the shoot are the primary functions of the root. These hidden belowground-processes can be further divided into i) movement from soil to root surface and ii) transport from root surface into the plant.

Mechanisms of water and N movement from bulk soil to the surface of roots have been identified previously (Barber 1966; Steudle 2001). Briefly, transpiration - the diffusion of vapour from the leaf to the air, increases the tension in the xylem, extending into the root systems. When the tension is higher than in the soil surrounding the roots, an inflow of water from the rhizosphere starts (Fig. 1a). The rate of water movement in soils depends heavily on soil hydraulic properties. This will be discussed in more detail in Chapter 3 and 4.

In the soil with the presence of roots, water flows between the particles, towards the root. After reaching the root surface, water is transported into the root cells and the xylem. It flows from the epidermis to the endodermis of roots through three different pathways (Fig. 1b) – (1) apoplastic (through the cell walls), (2) symplastic (through plasmodesmata), and (3) transcellular paths (across membranes) (Javot and Maurel 2002). Water moves via all three pathways at the epidermis and cortex. At the endodermis, the band-like thickening Casparian strip blocks the apoplastic pathway and forces water to cross the endodermis through the plasma membrane (Fig. 1b). Here, aquaporins in the membrane are thought to mediate root hydraulic conductivity, changing the permeability of roots. This regulation of root hydraulic conductivity allows the plant to change its root water uptake rate to adapt to variable water demand, e.g., between day and night, or under drought stress (Clarkson et al. 2000; Johnson et al. 2014). After passing through the endodermis, water enters the transport pathway within the xylem, which is a simple pathway with low resistance, compared with the movement through living cell layers.

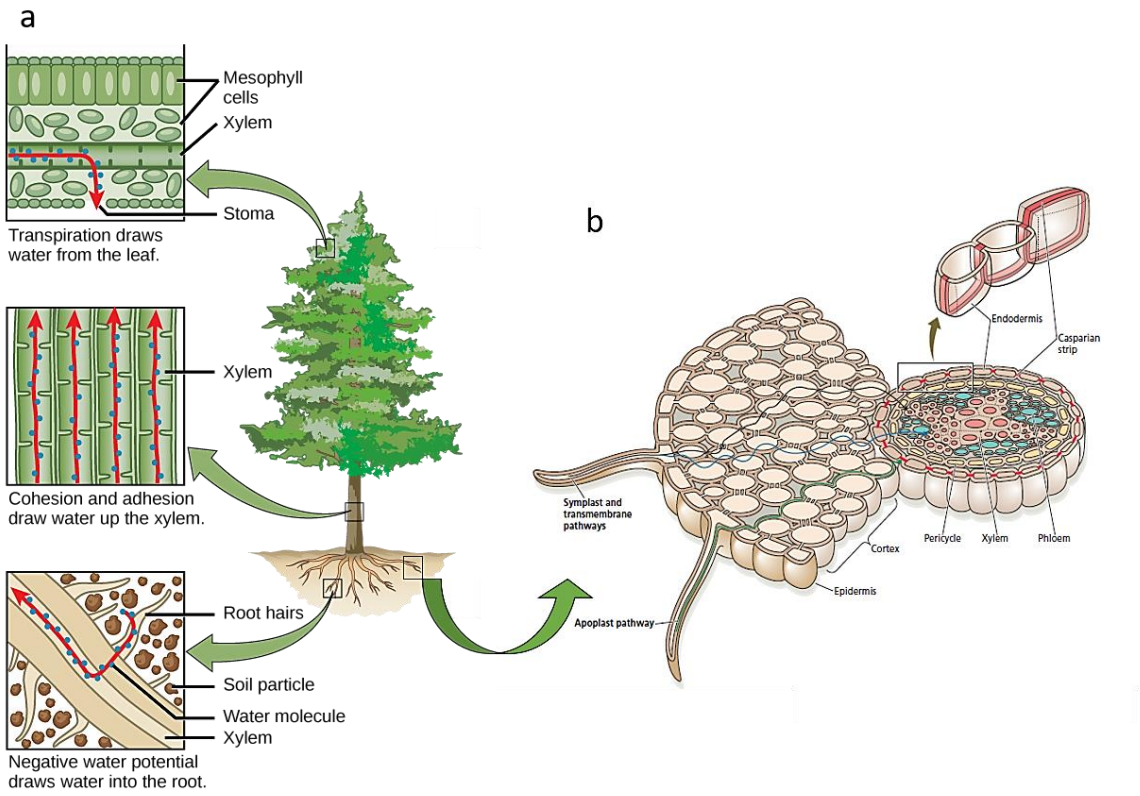

Fig. 1 Water movement from soil to root (a) and transport pathways in the living cells (b). (a) image credit: OpenStax Biology, (b) is modified from Taiz et al. (2015).

The main form of plant available N in the soil is NO_3^-, followed by NH_4^+ and organic forms (Nacry et al. 2013). The preferred form in which N is taken by plants depends on soil conditions and plant species. In general, plants adapted to higher pH and aerobic soils take more NO_3^- as it is the main N form in such soils. In contrast, at lower pH and in reducing soils, NH_4^+ is the predominant form of N (Maathuis 2009). N can be brought to the root surface with water flux, which is called *mass flow*. As roots extend through the soil, they intercept the adsorbable nutrients through direct contact, which is called *root interception*. The removal of N from the soil creates a concentration gradient between the surrounding soil and the root surface, making N move along the gradient to the root called *diffusion*. This diffusion mechanism continuously supplies N to the plant if the former two mechanisms does not meet the plant demand (Fig. 2a). The relative importance of these three mechanisms to N transport has been reported several times (Gregory et al. 1979; Barber 1995). Compared with mass flow and diffusion, root interception usually contributes little to N transport. The contribution rates of mass flow and diffusion depend on soil moisture, soil N concentration, and N forms. There is a remarkable difference in the mobility of NO_3^- and NH_4^+, the two primary available N forms. Negatively charged NO_3^- does not bind to negatively charged

soil colloids and makes few interactions with the organic matter, and consequently is very mobile. By contrast, positively charged NH_4^+ can strongly interact with the negatively charged soil particles and has lower mobility - 50 to 500 folds less than that of NO_3^-, whose diffusion coefficient is about 10^{-6} cm^2 s^{-1} (Forde and Clarkson 1999). Because of the high mobility of NO_3^-, the absorbed ions are rapidly replaced and do not create a significant gradient between the root surface and bulk soil. On the contrary, NH_4^+ with the relatively low mobility and its quick absorption, can lead to a lower concentration at the root surface than in the soil solution. Therefore NO_3^- is often assumed to move mainly to roots by mass flow (Jungk 2002), and NH_4^+ moves easier via diffusion. Mass flow and diffusion coincide and are interdependent. When mass flow is insufficient to cover plant demand, diffusion can become the major mode of N transport (Okajima and Taniyama 1980; Jungk 2002).

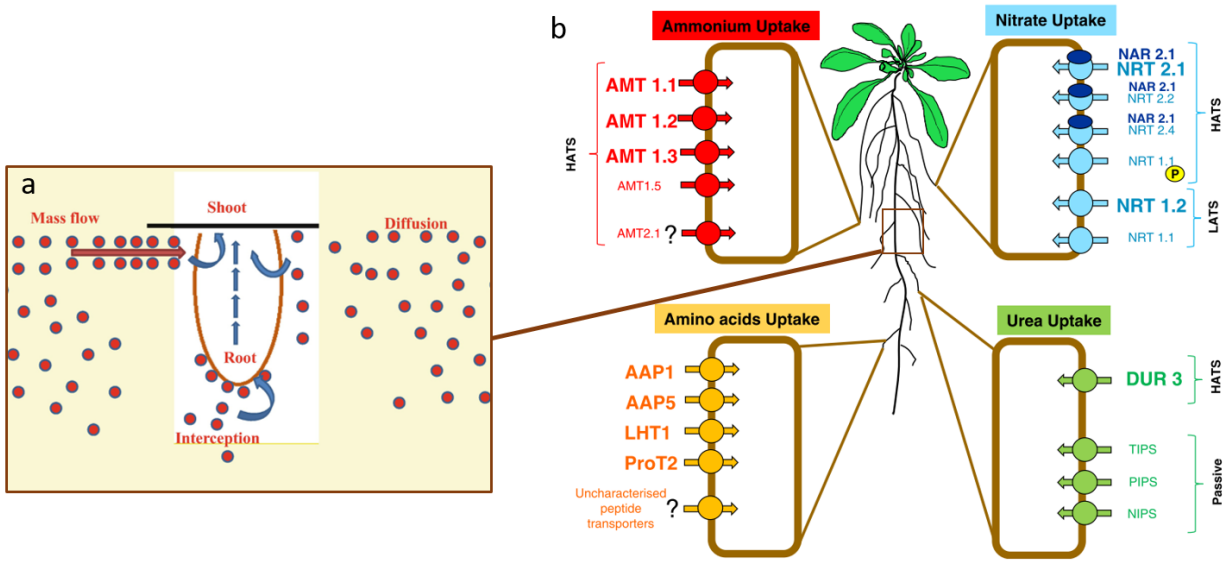

Fig. 2 Schematic representation of mechanisms involved in N movement (a) and membrane transporters involved in the root uptake of the four primary N sources in *Arabidopsis thaliana* plants (b). **HATS**: High-Affinity transporters; **LATS**: Low-affinity transporters. Transporters highlighted with big letters are those that play a major role in nutrient uptake. Question marks represent unconfirmed results. This figure is modified from Karthika et al. (2018) and Nacry et al. (2013).

Plants can use different chemical N forms, varying from inorganic compounds such as NO_3^- and NH_4^+ to organic forms such as amino acids and urea (Fig. 2b). As plants take up mainly NO_3^- and NH_4^+ (Nacry et al. 2013), here, the focus will be on the uptake of these two N forms. Two co-existing transport systems have been identified in plants to bring NO_3^- or NH_4^+ into root cells (Lea and Azevedo 2006; Masclaux-Daubresse et al. 2010). High-affinity transport systems (HATS) acting when the external N concentrations are low (as low as 1 μM), and low-affinity transport

systems (LATS) which are more active in a high concentration range (>0.5 – 1 mM) (Nacry et al. 2013). Influx rate by LATS increases with increasing external concentrations and is not saturable, while the NO_3^- or NH_4^+ uptake by HATS can be saturated (Wang et al. 1993; Touraine and Glass 1997). The relative contributions of HATS and LATS to N uptake are clearly different between NO_3^- and NH_4^+. HATS plays the central role in NH_4^+ uptake as NH_4^+ concentration in most soils is low, while both HATS and LATS are thought to be important in root NO_3^- uptake (Miller et al. 2007; Nacry et al. 2013).

The expression of transport systems is up-/down-regulated by external stimuli or stresses as well as nutritional status of the plant, which results in inconsistent N uptake rate (Masclaux-Daubresse et al. 2010). The N uptake rate also depends on plant species, cultivar, and growth rate (Gastal and Lemaire 2002). The N sources taken up by roots are assimilated into amino acids for further use by plants (Masclaux-Daubresse et al. 2010). Apart from some NO_3^- taken up by the roots that can be assimilated into the roots, a larger part of NO_3^- is transported to the shoot. There it is reduced to nitrite (NO_2^-) by nitrate reductase first, then to NH_4^+ by nitrite reductase and glutamine synthetase (GS). Later, via the GS/glutamine-2-oxoglutarate aminotransferase (GOGAT) cycle, the nitrate-derived NH_4^+, together with the NH_4^+ transported by the transporters, is assimilated into amino acids (Xu et al. 2012).

The interaction between N and water use

Soil water status affects use of soil N by plants, especially under water deficit or excess. Water deficit hinders plant growth, inhibits foliar development and expansion, and reduces N demand subsequently. In the longer term, water deficit leads to N deficiency, limiting photosynthesis and plant growth (Gonzalez-Dugo et al. 2010). After a dry season, the low level of plant growth and N uptake can result in an accumulation of NO_3^- in the soil, which can be leached over the following wet winter (Cameron et al. 2013).

At physiological levels, N can either move freely to roots with water by mass flow when there is active water uptake for transpiration, or by diffusion. However, either way requires the mobility of ions in the soil, which highly depends on soil water status. In dry soils, the delivery of dissolved N to the root surface will be less efficient (Chapman et al. 2012). Thus, water in the soil plays a fundamental role in both mass flow and diffusion. Although there is no evidence that transpiration-driven water flow affects NO_3^- transport across the membrane directly, NO_3^- concentration in the

rhizosphere increases due to the mass flow when water uptake is active, and the membrane NO_3^- transport may thereby be further enhanced (Plett et al. 2020).

On the other hand, N also plays an essential role in water use. Compared with no fertilization, N-fertilized crops usually grow more vigorously and have a larger leaf area, which increases transpiration and decreases soil evaporation. N fertilization can increase transpiration intensity under normal water supply but decrease the transpiration rate under water deficit (Li et al. 2009). N supply also affects root growth, morphology, and distribution of root systems (Lynch et al. 2012), which further affects water uptake.

The physiological regulation of N on water fluxes is more complex and needs further investigation. Previous studies showed that either N concentration in the xylem or soil N availability can regulate water fluxes to plants (Wilkinson et al. 2007; Matimati et al. 2014). Cramer et al. (2009) suggested that there are few possible mechanisms explaining such N regulations: 1) NO_3^- modulates root hydraulic conductance by controlling aquaporins in membranes, 2) nitric oxide (NO) production regulates shoot stomatal conductance. The regulation of NO_3^- on hydraulic conductance and aquaporins in *Arabidopsis* has been studied (Li et al. 2016), and it showed that the function of transceptors NRT2.1 (HATS), together with a shoot-to-root signal communicating shoot NO_3^- status regulated root aquaporins. Root hydraulic conductance was lowered in *Arabidopsis* mutant plants without NRT2.1 and also responded to reduced NO_3^- supply. However, NH_4^+ did not alter root hydraulic conductance and the expression of aquaporin in French bean (*Phaseolus vulgaris* L.) (Guo et al. 2007) and did not appear to induce the closing of stomata like NO_3^-. As indicated above, plant hydraulics can be modified by N form and amount, which have further impacts on plant water uptake. However, this regulation depends on plant species.

The above-mentioned evidence showed that strong interactions exist between water and N use, from both transport and uptake processes. Furthermore, water and N supply may affect the formation of root barriers, which indirectly affects water and N transport (Fig. 3). However, the metabolic and cellular processes of the formation of root barriers remain unexplored (Plett et al. 2020). Similarly, the signalling pathways which involves in water and N use and their interactions require further investigation.

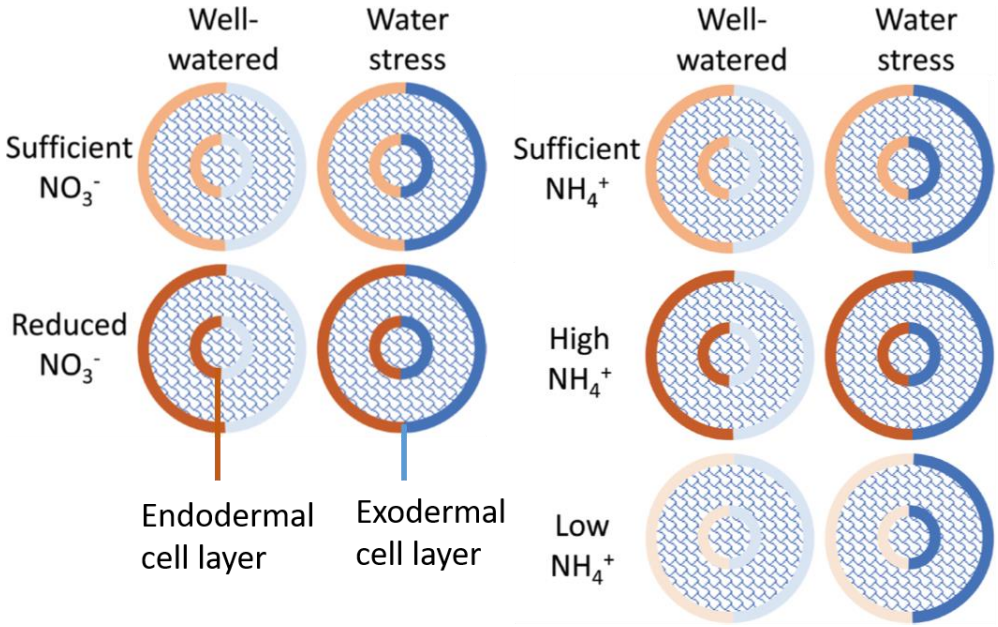

Fig. 3 The possible combined effects of water and N supply on the development of plant root barriers. Darker rings indicate increased suberin and lignin depositions, and reduced apoplastic water and N transport in outer or inner cell layers, while lighter rings depict the reverse. The effect of N (left) and water (right) is represented on opposite sides of each ring. Modified from Plett et al. (2020).

Pathways to improving N and water use

Enhancing root water and N uptake is crucial for resource-efficient crop production. Water use efficiency (WUE) and N use efficiency (NUE) have been widely used for assessing the efficiency of a given crop system, while their definitions vary from scales. In most studies, WUE is described as

$$WUE = Y/ET \quad (1)$$

where Y is the crop yield, and ET is the evapotranspiration (Hatfield et al. 2001). In irrigated cropping systems, it is more common to calculate water input efficiency (WUE_i) as,

$$WUE_i = Y/W_i \quad (2)$$

in which Y is the crop yield, and W_i is the incoming water (irrigation + precipitation) (Quemada and Gabriel 2016).

According to Moll et al. (1982), NUE is defined as the ratio between yield and soil available N, which can be further divided as uptake efficiency (NupE) and utilization efficiency (NutE),

$$NupE = Nt/ Ns \quad (3)$$

$$NutE = Gw/Nt \quad (4)$$

in which Nt is the total N in the plant, Ns is the total N supply, and Gw is the yield (Good et al. 2004).

In practice, the use of water and N by plants varies greatly depending on growing stages, duration of growth, root traits, soil properties, environmental conditions, agronomic practices. Field management strategies, such as modified N application and irrigation, early sowing, intercropping with catch crops or grasses, and optimizing plant density, are used to improve WUE and NUE, agricultural productivity, and minimize environmental damage (Thorup-Kristensen and Kirkegaard 2016; Rosolem et al. 2017; Liu et al. 2018). For instance, applying N fertilization only when soil water is sufficient is likely to improve the NUE of oilseed rape under drought stress (Rathke et al. 2006). Compared with a zero-N treatment, N applications increased both evapotranspiration and yield of wheat, and WUE of wheat was enhanced by more than 60% (Liu et al. 2018). Meanwhile, adjusted water management strategies such as deficit irrigation, improved irrigation schedule and technology increased WUE > 40% and NUE > 60% (Quemada and Gabriel 2016).

In addition to adjusting water and N application to match crop needs, agronomists have attempted to adopt ways to obtain more and deeper roots, with which the crops can explore more resources in a larger soil volume. Winter wheat (*Triticum aestivum* L.) with increased density or earlier sowing time enhanced N uptake, as increased density or advancing sowing time improved root length density and rooting depth (Dai et al. 2014; Rasmussen and Thorup-Kristensen 2016). Intercropping may also improve deep water and N use, as observed in a study where a barley (*Hordeum vulgare* L.) and /vetch (*Vicia sativa* L.) intercropping tended to produce more roots than the mono-cropped barley in the deepest soil layers, which benefits N uptake from soil (Ramirez-Garcia et al. 2015).

Genetic improvements of root traits can be valuable to increase water and N uptake from depth (Fig. 4). Overall, i) deeper root systems; ii) increased root length density in medium and deep soil layers; iii) reduced root length density in the topsoil and iv) decreased resistance to water movement from soil to root are thought to be four critical traits to increase water uptake (Wasson et al. 2012). Lynch (2013) has proposed the “steep, cheap and deep” ideotype for efficient N acquisition in maize (*Zea mays* L.), consisting of a few root traits that may improve N acquisition by deeper soil exploitation. Breeding crops with deeper and more bushy roots could also improve soil structure and carbon sequestration (Kell 2011). Therefore, breeding plants with deeper roots seems to be a promising way for efficient water and N use and sustainable agriculture.

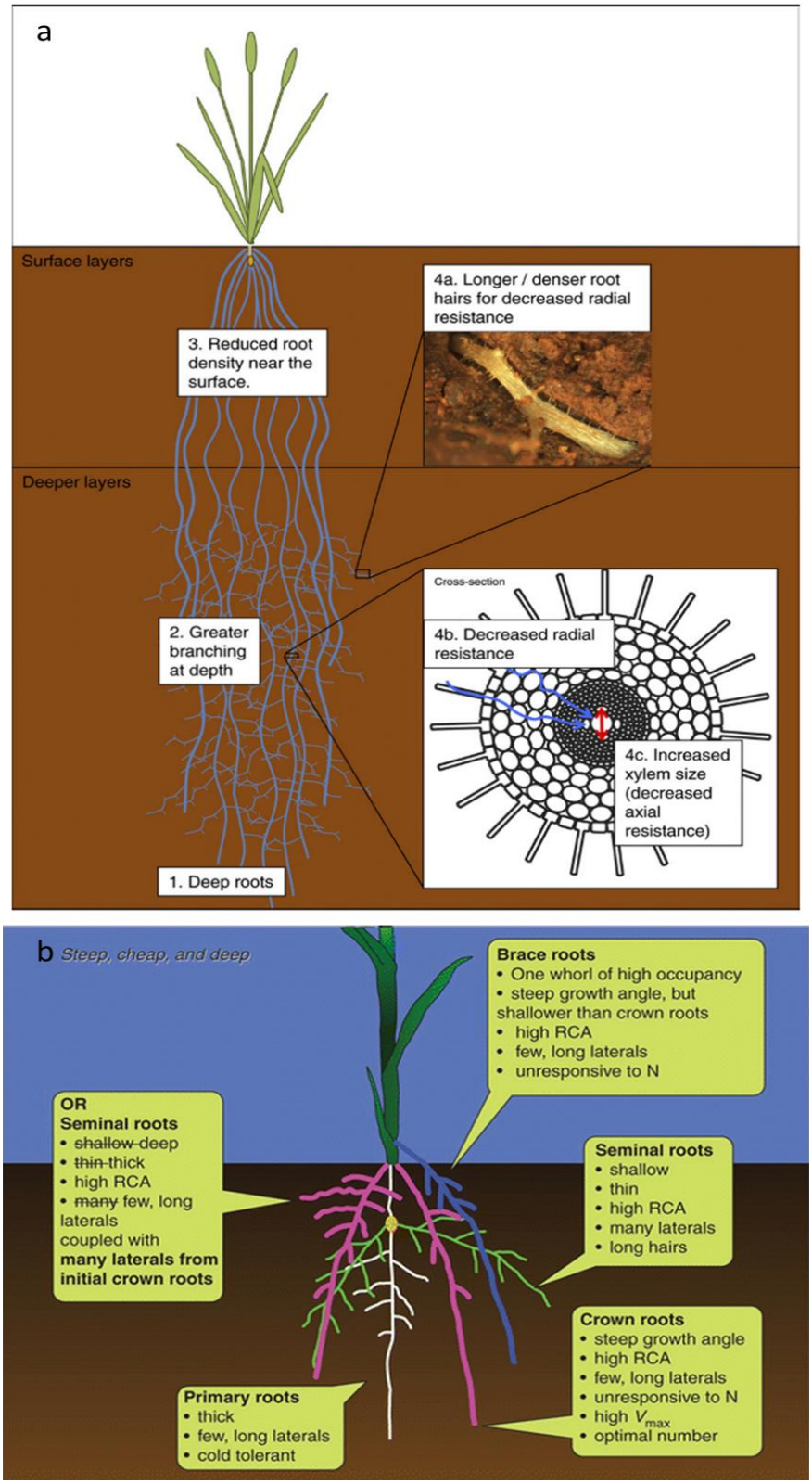

Fig. 4 Diagrams illustrating root traits (a) to increase water (Wasson et al. 2012), and (b) ideotype for optimal N uptake by maize roots (Lynch 2013).

2. Deep-rooted crops

Access to extra water at deeper soil layers can be valuable for plant development and yield, especially in dry seasons (Kirkegaard et al. 2007). Besides, the enhanced use of N reduces the risk of leaching while keeping crops adequately supplied with N and maintain the yield (Thorup-Kristensen et al. 2009). Water and N move downwards through the soil profile, especially outside the growing seasons and in periods of heavy precipitation. Therefore, water and N contents can increase in deeper soil layers. Thus benefits of increasing rooting depth on obtaining deep-stored resources are obvious (Thorup-Kristensen et al. 2020a). Yet, the potential of resource uptake by deep-rooted crops has not been adequately investigated. In this chapter, the definition, constraints and perspectives of growing deep-rooted crops will be discussed.

Definition of deep-rooted crop

Rooting depth varies between different plant genotypes, species, soil characteristics, ecosystems, and climates (Thorup-Kristensen et al. 2020a). Plant roots are able to grow deep in either natural or arable systems. Usually, woody plants have deeper roots than herbaceous ones. Canadell et al. (1996) summarized the maximum rooting depth of 253 woody and herbaceous species and found that over 190 species have roots at least 2 m deep (Fig. 5). The maximum rooting depth of trees was reported with an average of 7.0 m. Herbaceous plants' rooting depth was 2.6 m on average. Compared with natural ecosystems, crops tended to have shallower roots, with an average maximum rooting depth of 2.1 m. While this depth-level already surpasses the generic depth-scale for root research, i.e, <1 m, this might be also an under-estimation caused by the limitation of measurements (Schenk and Jackson 2002; Pierret et al. 2016). In fact, recent studies showed that several crop species are able to develop roots to deeper than 2.3 m (Dardanelli et al. 1997; Kristensen and Thorup-Kristensen 2004a).

Theoretically, there is no clear definition of "deep roots", given the plastic nature of root development and variable root patterns (Maeght et al. 2013). In practice, "deep roots" are defined as roots growing below the general depth where we can find most of the roots. Based on a global review of root profiles, Schenk and Jackson (2002) characterized the vertical root distributions by the depths above where 50% or 95% of all roots were found. They concluded that more than 90% of all profiles investigated had more than half of all roots in the top 0.3 m, and for terrestrial biomes, more than 95% of roots were found above 1.1 m. According to this, Maeght et al. (2013) proposed that "deep roots" should be defined as roots growing at soil depths of at least 1 m. In the

discussion below, deep-rooted crops refer to the crop species that are able to grow roots below 1 m.

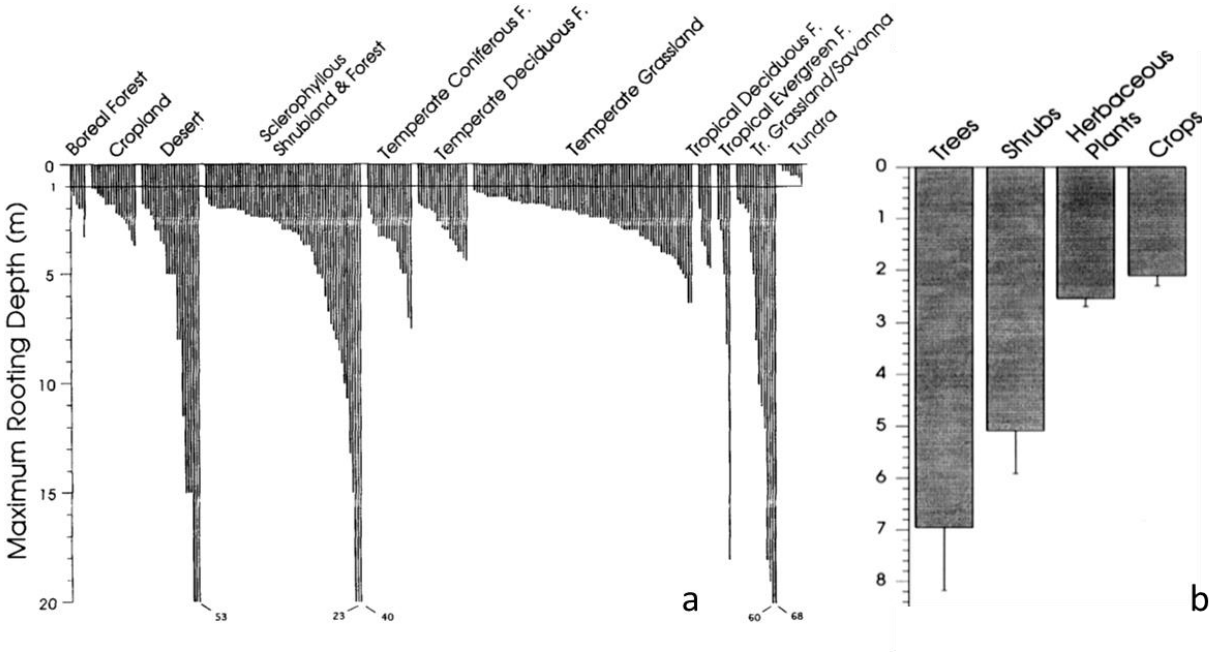

Fig. 5 (a) Reported species maximum rooting depth (m) grouped by terrestrial biome. Only the maximum value is plotted. (b) Reported maximum rooting depth (m) by three major functional groups (trees, shrubs, and herbaceous plants) and crops, mean, and SE are showed here. Modified from Canadell et al. (1996).

Constraints on deeper root growth for crops

Although it is common to find deep-rooted species in natural ecosystems, the root growth of crops in the subsoil (below tilled layer) is always constrained by various factors: (1) chemical constraints such as Al toxicity (Lynch and Wojciechowski 2015); (2) physical constraints such as soil compaction (Thorup-Kristensen et al. 2020a). There can be more constraints in the subsoil, including soil physical properties such as hypoxia, low temperature, low nutrient availabilities (Lynch and Wojciechowski 2015). Additionally, as main crops are mostly annual crops, the time for root development is relatively limited. It was claimed that the maximum root depth for annual crops ranged from 2 to 3 m, and these depths are only achieved in the sand and loamy sand soils, where no apparent limitations were found to root growth (Tennant and Hall 2001).

Compared with topsoil (the tilled or formerly tilled soil layer), where the soil is porous and usually more enriched in nutrients and oxygen, subsoil is less suitable for root growth. Soil structure and type vary from sites to sites, while it is common to find an increase in subsoil compaction in arable soils. The compacted and hard soil slow down root growth (Bengough et al. 2006). Moreover, the air-filled space is generally limited in the subsoil, which decreases the opportunities for root

development (Vartapetian and Jackson 1997). In spring and summer, soil temperature decreases with depth during the growing season, and the temperature at 2 m depth can be 10 ℃ lower than the soil temperature at the soil surface (McMichael and Burke 1998; Illston and Fiebrich 2017). For sunflower (*Helianthus annuus* L). and cotton (*Gossypium hirsutum*), lower root temperatures (20℃ for cotton and 10℃ for sunflower) may decrease root branching and metabolic activity and even harm the root cortex (McMichael and Burke 1998; Minchin et al. 2002). For oilseed rape, the optimum soil temperature for seedling root growth was about 25 ℃; its growth rate reduced to less than 50% of the maximum when soil temperature was lower than 16℃ (Kasper and Bland 1992). These inherent characteristics of subsoil often inhibit deeper exploration by roots.

The sub-optimal status in the subsoil may restrict root penetration, however, deeper rooting is achievable, and has been observed in various cases. In the experiments related to this thesis, the maximum rooting depth of oilseed rape reached 3 m in sandy loam soil in semi-field rhizoboxes (Chen et al., *Appendix* II). Compared with annual crops, biennial and perennial crops, e.g., chicory, intermediate wheatgrass (*Thinopyrum intermedium*), and lucerne (*Medicago sativa* L.), have a longer duration of growth thus can easily develop roots down to 3 m, increasing exploitation of subsoil (Li and Huang 2008; Thorup-Kristensen et al. 2020a; Chen et al. 2021). Even in soil with high impedance, roots can preferentially grow through cracks or macropores, which shows the potential of annual and perennial crops to develop deeper roots (White and Kirkegaard 2010).

In addition, the genetic modifications and proper management strategies also allow roots to develop and adapt to the subsoil. Specific root traits, e.g., radial thickening, may increase soil penetration, and the formation of root cortical aerenchyma would benefit the root growth by reducing metabolic cost and maintaining oxygen supply (Lynch and Wojciechowski 2015). The choice of the previous crop, sowing time, and other agronomic practices under specific environments can also improve the root performance in the subsoil and hence improve productivity and efficiency (Perkons et al. 2014; Han et al. 2016; Lilley and Kirkegaard 2016; Rasmussen and Thorup-Kristensen 2016). In conclusion, there are many constraints which inhibit deep root growth in agroecosystems, and deep rooting has not been adequately investigated. However, it is still possible and worth pursuing deeper roots, as deep roots bring potential benefits in various aspects.

Potential benefits of deep roots

Crops with greater rooting depth could improve the soil structure, water and N uptake, and carbon storage, as well as yields, and have impacts on soil fauna and microbial communities (Kell 2011; Maeght et al. 2013; Thorup-Kristensen et al. 2020a). These potential benefits may have been widely underestimated until recently. The function of deep rooting, especially on water acquisition, N capture, and carbon sequestration, has attracted recent research interest (Kell 2011; Wasson et al. 2012; Lynch 2013).

Rooting depth is positively related to water uptake from deep soil layers, especially in drought-affected regions. Annual crops can take up 50-100 mm water from below 1 m during the growing season (Nielsen and Vigil 2018). Under drought stress, 10.5 mm of additional water were taken from the 1.35 -1.85 m layer, which increased the final grain yield by 0.62 t ha^{-1} (Kirkegaard et al. 2007). Rasmussen et al. (2020) demonstrated that the deeper part of roots (> 1.7 m) of chicory, a biennial crop, has the considerable ability as compared to the shallow parts (0.5 m) to contribute to the total water uptake.

The development of roots in the deep layers is advantageous to increase N uptake and reduce N leaching from subsoil layers. With twice the root depth, winter wheat left 81 kg N_{inorg} ha^{-1} less in the 1 - 2.5 m layer than spring wheat (Thorup-Kristensen et al. 2009). Using deep-rooted species, e.g., dyer's woad (*Isatis tinctoria* L.) and chicory as catch crops, the depletion of soil NO_3^- from deep layers was significantly enhanced (Thorup-Kristensen and Rasmussen 2015). In addition, increased root length density at deeper layers was positively related to total aboveground N uptake of winter wheat, as well as the grain yield (Dai et al. 2014).

Crop plants with deep roots enhance soil carbon input in the subsoil as they both improve the soil structure and create a root C pool when roots grow deeper (Kell 2011; Dietzel et al. 2017). Soils store more carbon than the atmosphere and plant biomass, and more than 50% of soil organic carbon (SOC) is located in the first meter (Jobbágy and Jackson 2000). However, deeper soils may also contribute significantly to the sequestration of SOC, as with the increase of depth, the turnover time and resilience of SOC and the carbon age increase (Lorenz and Lal 2005; Balesdent et al. 2018). Based on estimations, Kell (2011) claimed that more carbon could be sequestrated if deep-rooted plants are cultivated. This can be debatable as low nutrient availability, which is common in deep soil layers, can lead to decomposition of recalcitrant SOC (Wang et al. 2014; Liang et al. 2018; Shahzad et al. 2018), thus inhibit the process of SOC stabilization.

To sum up, facing with a number of challenges, many crops still have the potential to develop deep roots in the agroecosystems. Crops with deeper roots have the non-negligible potential for achieving not only higher yield but greater water and N uptake, as well as carbon sequestration. Although several factors constrain deep root growth, breeding for and including more deep-rooted crops in our cropping systems seem to be promising towards a more sustainable agriculture.

3. Deep roots in water and N use

Although it is challenging to attribute most functions to either shallow or deep roots as they are usually studied as a whole system, it is possible to make some distinctions in their contribution to resource uptake and impact on the environment. The contributions and impacts vary a lot with the intrinsic root traits and external conditions. The role of deep roots in water and N use and the intrinsic traits that can affect these functions are discussed in this chapter.

Deep roots and water use

Seasonal water deficits are common in semi-arid dryland farming systems such as those in southern Australia, India, and some areas of China. Climate change will also likely increase the frequency of summer-drought in southern and central Europe (Calanca 2007). Thus, deep-stored water in these areas is valuable to crop production, as it is available to crops and can maintain their productivity when drought occurs and topsoil dries.

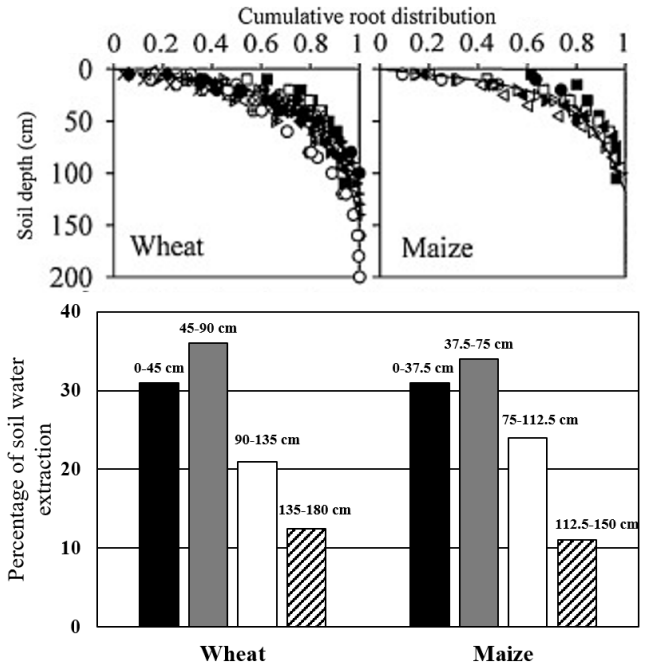

Fig. 6 (top) Cumulative root distribution as a function of soil depth for wheat and maize; and (bottom) average soil water extracted by winter wheat and maize, expressed as a percentage of total water extracted for the active root zone divided into quarters. The active root zone was 0 to 180 cm for winter wheat, and 0 to 150 cm for maize. Redrawn from Fan et al. (2016) and Nielsen and Vigil (2018).

There is convincing evidence showing that deep roots can lead to deeper and greater water exploitation, and increasing water productivity, especially in dry environments (Lilley and Kirkegaard 2016; Rich et al. 2016). In wet soil profiles, deep-rooted crops such as wheat and maize

can also explore water more efficiently than shallow-rooted crops such as millet (*Panicum milliaceum* L.) and pea (*Pisum sativum* L.) (Nielsen and Vigil 2018). Although only a small fraction of roots were found at deep soil layers (> 1 m), more than 10% water was extracted from the bottom quarter of the root zone by wheat and maize (Fig. 6).

The importance of deep roots in water extraction has also been investigated under different crop species, crop sequence, and farming systems (Gaiser et al. 2012; Thorup-Kristensen and Kirkegaard 2016). Besides wheat and maize mentioned above, chicory and oilseed rape are also able to extract considerable water from below 1 m during their late growing stage, although they also have much fewer roots in the subsoil than in topsoil (Fig. 7). Further, deep-rooted crops can improve water uptake when used in crop rotations. Spring wheat developed more roots in the 90 – 105 cm layer and extracted more water from there due to the increased soil biopore density when lucerne, a deep-rooted crop with higher bioporing capacity, was used as a preceding crop (Gaiser et al. 2012). Also, it has been reported that in the Australian oilseed rape-growing system, 33 mm of additional water extracted by roots below 2 m layer increased yield by 1.2 t ha^{-1} under moderate drought stress (Kirkegaard et al. 2021).

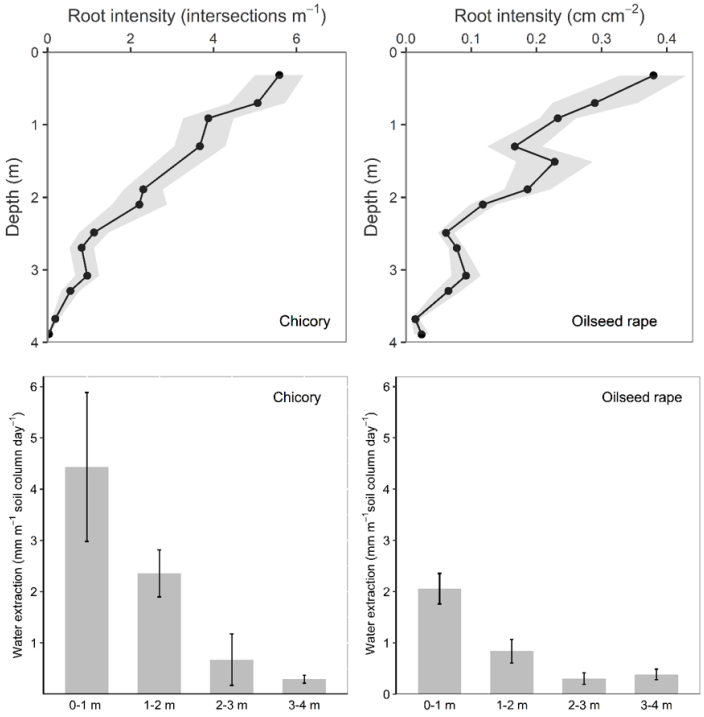

Fig. 7 (top) Root distribution for chicory and oilseed rape at the reproductive stage; and (bottom) soil water extracted by chicory and oilseed rape, as expressed daily water extracted from the soil column divided into quarters. The water extraction was calculated at the same period when root intensity was measured. Note that root intensity for the two species is expressed differently as different methods were used to analyze the intensity. Redrawn from Chen et al. (2021; *Appendix* I) and Chen et al. (*Appendix* II).

In addition to increasing water extraction from deep soil layers, deep roots have been argued to be involved in the process of hydraulic lift, which refers to transferring water from wet to dry layers in the soil (Maeght et al. 2013). Generally speaking, the hydraulic lift is driven by water potential gradients between the soil and roots (Richards and Caldwell 1987). Deep roots absorb and transport water from moist deep soil and release it in the upper drier soil layers during the night. On the following day, shallow roots can take the restored water in the upper soil (Dawson 1993). With hydraulic lift, deep roots enhance total water uptake by supplying water to shallow roots rather than taking the deep-stored water alone (Caldwell and Richards 1989). In summary, there is consistent evidence showing that deep rooting is functioning in plant water uptake, as well as hydraulic redistribution.

Root traits that may benefit deep water uptake

The geometry of the root system determines the volume of soil water extraction and sets a maximum volume of accessible water (Lobet et al. 2014). As water tends to store in deep soil layers over time and is initially depleted in upper soil by crops, especially under drought scenarios, breeding crops with a deeper rooting system has been set as a goal for improving water extraction (Kell 2011; Lynch 2019). Increased rooting depth improves water uptake from the deep soil layer, while other root traits can also be necessary for efficient water extraction. Recent data indicate that some architectural and anatomical traits of roots, e.g., increased suberisation and lignification of endodermis and exodermis, and the larger diameter of xylem vessels, could benefit the water uptake via regulating root radial or axial hydraulic conductance (Lynch et al. 2014). Pate et al. (1995) examined the hydraulic architecture and xylem structure of the root system of *Banksia prionotes* and found that its roots at 2 m depth had larger and longer xylem conduits and higher area-specific hydraulic conductance than the shallow roots, which resulted in more efficient water flow in deeper roots.

Further, steep root growth angles and thicker roots, which help penetrate the hard deep soil layers, are also related to deep rooting and enhancement of water acquisition (Lynch 2013). Steep root angles determine the rooting depth of monocots such as maize and wheat, and benefit root penetration in dicots, in which the new roots originate from primary roots at depth. Still, there are some metabolic costs of deep root development and water acquisition, and it is essential to balance topsoil and subsoil foraging.

Mechanical impedance in deep soil layers creates additional resistance for root penetration and resource exploration. Therefore, the root tip traits that improve root penetration are considered

important for subsoil exploration. The relevant traits may include narrowly pointed root tips, which are more efficient in soil deformation. Increased production of soil mucilage and exudates also benefit deep root penetration since they reduce the soil-root friction and may change the hydraulic properties of the rhizosphere (Bengough et al. 2011). Furthermore, in the compacted subsoil, more than 85% of roots were found within cracks and biopores (White and Kirkegaard 2010). Thus, root traits that allow better exploration of cracks and pores, for example, the production of root hairs for enhanced root-soil contact, plastic root growth behaviour inside the soil voids (Athmann et al. 2013; Huang et al. 2020) can also be advantageous for resource acquisition for the subsoil.

Constraints to deep water uptake

Although deep roots can contribute considerably to total water uptake, their ability to extract water is often limited by constraints such as poor root growth and contact with soil and intrinsic low root hydraulic conductivities (Doussan et al. 2006; Lobet et al. 2014). In addition, in soils where deep root growth is not limited, roots reach the deep soil layers in the late growing period, and the density is generally lower than roots in upper layers, leading to delayed and reduced water uptake from the subsoil. This lag of deep water absorption has been proven by Chen et al. (2021; *Appendix* I). In the late growing stage of chicory, the root intensities at 2.3 and 3.5 m were less than half of that at 1.1 m, leading to less and slower ^2H_2O uptake (Fig. 8).

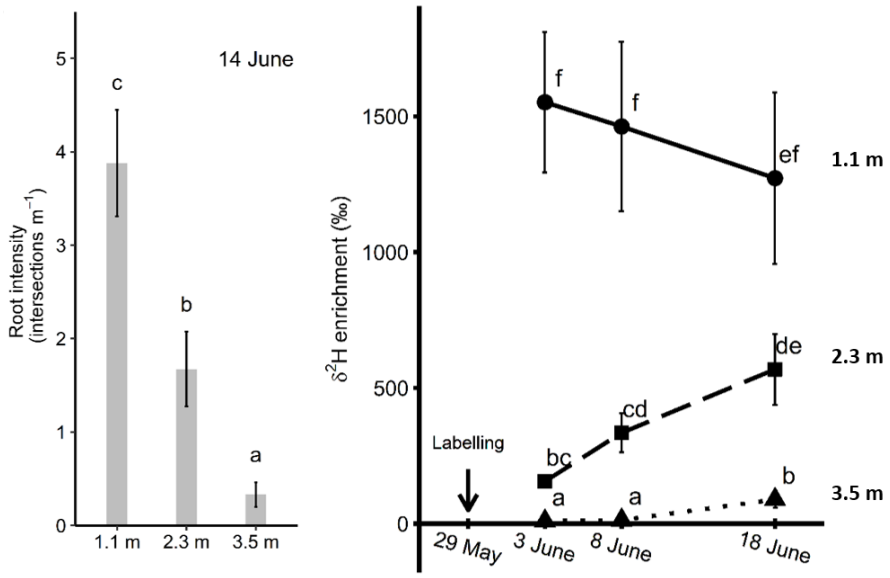

Fig. 8 (left) Root intensity of chicory at three ^2H labelled depths, and (right) the time course of ^2H enrichment in transpiration water during this period. Redrawn from Chen et al. (2021; *Appendix* I).

The contribution of roots to total water uptake also varies with time and their position within the root system (Garrigues et al. 2006). Pierret et al. (2006) pointed out that the major constraint to deep water uptake and transport could arise from significant axial resistance in deep, immature roots. Besides, given that the axial fluxes in roots in the upper soil layers are higher than in the deeper ones and a higher radial flux in the proximal root segments (Zarebanadkouki et al. 2013), the lower water uptake and transport from deep soil layers is expected.

Deep roots and N use

The occurrence of deep-rooted crops in semi-arid and arid cropping systems has traditionally been explained as enhancing the water uptake from deep soil layers (see above). However, McCulley et al. (2004) suggested that deep-rooted plants did not extract water as efficiently as reported in arid systems. Instead, they proposed that the deep-rooted plants in (semi-) arid ecosystems accounted for deep nutrient uptake (McCulley et al. 2004). They also found that some nutrients, such as P, have considerable resource pools in deep soil layers (McCulley et al. 2004). Unlike the less mobile nutrient such as P and K, N is more abundantly available in deep soil layers due to its mobility. Extra N is brought by N fertilizers, which are applied in agricultural systems to obtain higher yields, is usually not used completely by plants, and can be left in the soil (Ascott et al. 2017). For instance, in the east coastal region of the U.S., the mean content of remained NO_3^- N in regularly fertilized soil after cash crops is 115 kg N ha^{-1}, with 55% remained at 0.9 – 2.1 m deep (Hirsh and Weil 2019). As it is highly mobile, the unused soil NO_3^- will further be leached out of the root zone and enter the water bodies (Mills and Jones 1979). N leaching not only wastes valuable N resources but pollutes the groundwater and surface water as well (United Nations Environment Programme and Woods Hole Research Center 2007).

The quantity and depth of N retained in the soil vary with total N input, soil type, and intensity of precipitation and irrigation. Under conventional high N application practices in the wheat-maize rotation system in China, the amount of leached N is 56 – 136 kg N ha^{-1} yr^{-1}; while under the optimum N management, the loss was reduced to 23 kg N ha^{-1} yr^{-1} (Ju and Zhang 2017). Compared with arid, semi-arid, and semi-humid regions, in wet climatic conditions such as northern Europe, where the winter is mild with heavy precipitation, the risk of N leaching is relatively higher (Pedersen et al. 2009). Pedersen et al. (2009) simulated soil NO_3^- retention covering typical precipitation regimes of northern Europe and found that a significant fraction of NO_3^- was retained in soil layers below 0.75 m (Fig. 9). Also, they found that the texture of soil affected the extend of N leaching. Coarse-textured soils, e.g., sandy soil, have a lower water-

holding capacity and lead to more N loss by leaching than fine-textured soils such as clay loam and silt loam soils.

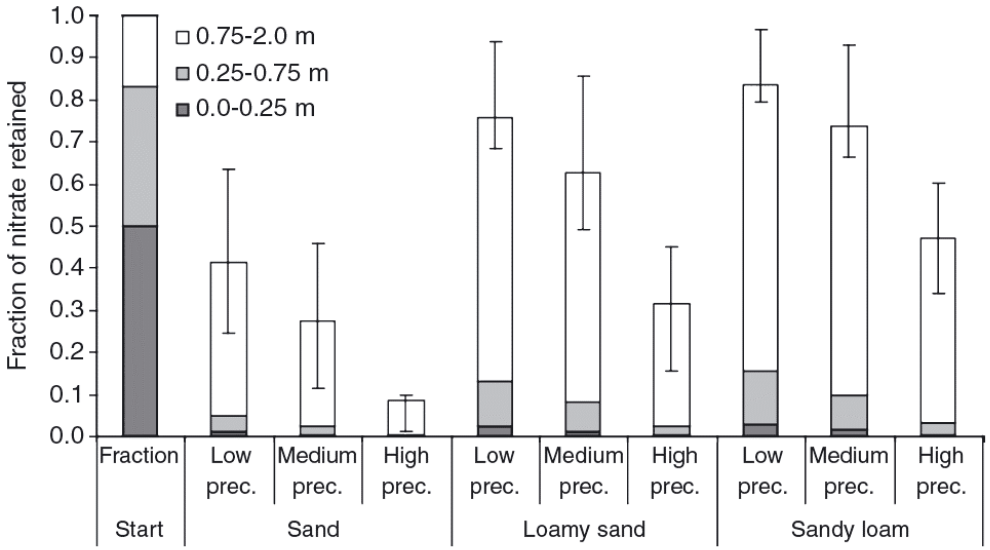

Fig. 9 Start distribution of NO_3^- in August and average fractions of mineral N retained at different depths in the following May. No crops were assumed to be planted in the simulation, from Pedersen et al. (2009).

Utilizing the leached N is expected to provide extra N supply to crops, as well as reduce the risk of pollution. Since that residual N moves deeper into the soil, the use of deep-rooted crops is important for N use. Available evidence from model simulations and experimental studies has shown that deep-rooted crops can efficiently deplete soil NO_3^- and reduce N leaching (Kristensen and Thorup-Kristensen 2004b; Thorup-Kristensen et al. 2009; Pedersen et al. 2009; Dai et al. 2014). For example, winter wheat left 81 kg ha^{-1} less inorganic N in the 1- 2.5 m layer than spring wheat, as the winter wheat had developed deeper roots that reached 2.2 m during the longer growing period (Thorup-Kristensen et al. 2009). Also, with a rooting depth of 2 m, white cabbage (*Brassica oleracea* L.) could reduce up to 113 kg N ha^{-1} in soil layers below 1 m, depending on the sequences of the crop rotation (Thorup-Kristensen 2006).

Due to its potential on depleting deep soil N, deep-rooted crops are also widely used as cover crops (Thorup-Kristensen and Rasmussen 2015; Rosolem et al. 2017). As a cover crop, the deep rooting fodder radish (*Raphanus sativus* L.) had higher root intensity in soil layers below 0.5 m than ryegrass (*Lolium multiflorum* Lam.) and winter rye (*Secale cereale* L.), and depleted the soil NO_3^- down to 2.5 m depth, leaving less than 18 kg N ha^{-1} in 0 -2.5 m soil (Fig. 10).

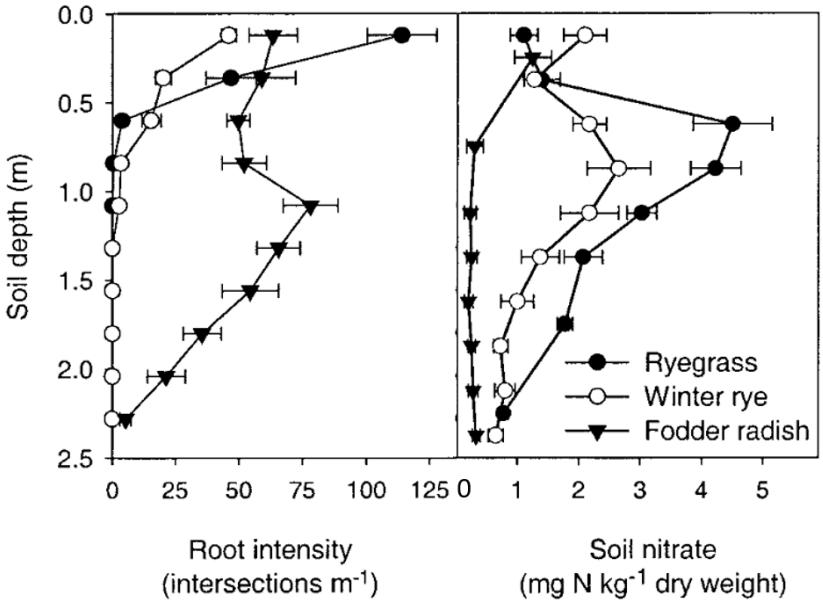

Fig. 10 Root intensity of three different catch crops, and soil NO_3^- content measured at the end of the catch crop growing period. Modified from Kristensen and Thorup-Kristensen (2004b).

Root traits that benefit deep N uptake

Soil resource acquisition is related to resource availability and root foraging. In soils containing a high concentration of available N, for instance, the subsoils mentioned before, the N uptake mainly depends on both the density of roots and their capacity to absorb N (Robinson 1986). In other cases where the soil N is not at a high concentration, the uptake capacity of root system to absorb N, rather than the number of roots at depth, might be a more relevant N absorption trait (Chen et al. 2021; *Appendix I*). Unlike less mobile nutrients such as P, N uptake is assumed to be less affected by rooting density. Despite the lower rooting density in the subsoil, deep root species show greater N uptake as their deep rooting system allows them to explore larger soil volumes and access a larger amount of N from the subsoil (Thorup-Kristensen and Rasmussen 2015; Rosolem et al. 2017). Thus, as with water, root traits that benefit deep root penetration will also enhance deep N uptake. Furthermore, the majority of studies agreed that more rapid and earlier establishment of root systems in deeper soil layers was helpful for N uptake due to the extended exposure of plants to subsoil N, as roots reach the unexploited subsoil earlier and have a longer active period (Liao et al. 2004; Pedersen et al. 2010; Andresen et al. 2016).

Constraints to deep N uptake

Unlike water uptake, there is no direct hydraulic constraint to deep N uptake. However, as N can be supplied to the roots through mass flow, the root hydraulic properties which restrict deep water uptake also indirectly restrict N uptake from the subsoil. As with water, the short growing period and active time of deep roots delay and limit the deep N depletion. A ^{15}N labelling experiment showed that chicory roots at 1.1 and 2.3 m led to rapid and comparable ^{15}N accumulation in leaves after labelling, while for roots at 3.5 m, the accumulated ^{15}N in leaf samples was lower and can only be seen after 20 days of labelling (Fig. 11).

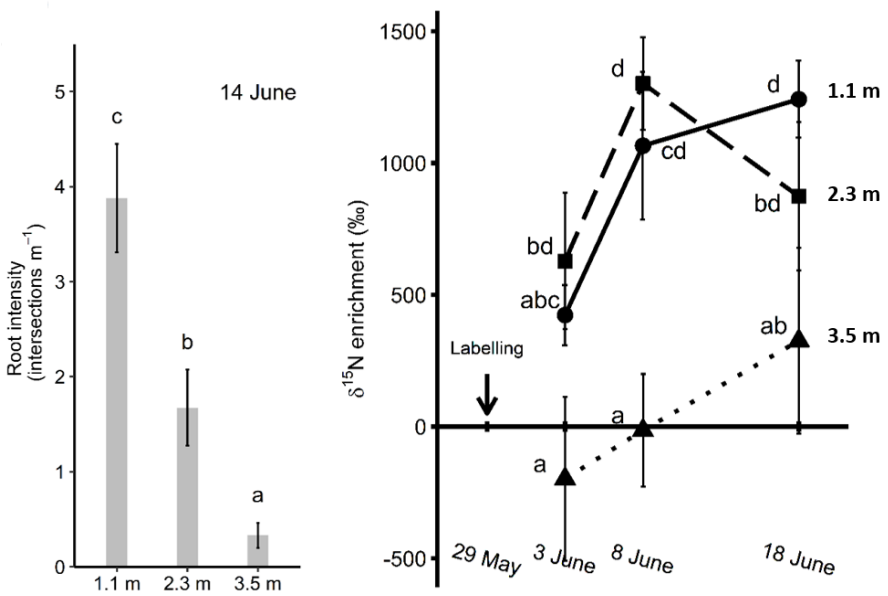

Fig. 11 (left) Root intensity of chicory at three ^{2}H labelled depths, and (right) the time course of ^{15}N enrichment in leaf samples during this period. Redrawn from Chen et al. (2021; *Appendix* I).

While the benefits of deep roots on water and N acquisition are striking and promising, it is essential to remember that these effects strongly interact with environmental and management factors, as they may change resource distribution and root growth (Dresbøll and Thorup-Kristensen 2014; Lobet et al. 2014). In the next chapter, the effects of extrinsic factors, especially water and N supply, on deep water and N acquisition are discussed.

4. Interactions with genotype, environment, and management

Breeding and cultivating deep-rooted crops in agricultural systems can potentially increase the water and N acquisition potential from the subsoil. This helps maintain productivity, especially under suboptimal conditions such as drought and N deficiency. However, it should be kept in mind that the effects of deep-rooted crops on water and N acquisition are the results of interactions between genotype (G), environment (E), and management (M), and cannot be isolated (Dresbøll and Thorup-Kristensen 2014). Besides the investment in plant genetics, optimal environment and efficient management are also crucial for enhancing water and N use.

Various environmental and management factors impact crop water and N use, while the two most important and manageable ones are obviously water and N supply. Applying water and N to the crops affect crop growth and soil resource distribution, hence affect crop resource use and yield. Besides, as strong and complex interactions exist between water and N use, the variation of one resource may lead to co-variation of the other one (Quemada and Gabriel 2016; Plett et al. 2020). Therefore, this chapter focuses on the effects of genotype, environment, and management, especially water and N availability, on water and N use.

The effects of genotype

Plant genotypes with more extensive and deeper root systems have been highlighted to improve water and N acquisition (Wasson et al. 2012; Lynch 2013; Carvalho et al. 2014). Other root traits such as root hair development and larger xylem vessels that have been discussed earlier, have also been demonstrated to improve water and N acquisition by increasing the capacity of resource transport and uptake (Bengough et al. 2011; Comas et al. 2013; Lynch 2013). Thus, breeding new deep-rooted genotypes with these beneficial traits has drawn much attention.

The genetic control on root traits is difficult to isolate as it differs between monocots and dicots, plant species, and its effect depends on the environment (Draper et al. 2001; Osmont et al. 2007; Watt et al. 2009; Zimmermann et al. 2010). However, evidence was found that some genes had stable effects on specific root traits. For instance, the *DRO1* gene in rice (*Oryza sativa*), which was identified by Uga et al. (2013), showed the potential in increasing the rooting depth by controlling root growth angle. Indeed, identifying and introducing deep-rooting related genes into the elite cultivars are urgently needed in current breeding programs, while the G ×E ×M interactions should also be considered. Optimal genotypes for resource extraction will be different under various environmental scenarios and crop management.

The effects of the environment

Although root traits can be genetically determined, root growth and resource uptake are highly responsive to environmental changes, e.g., climate condition, soil type, and temperature (McMichael and Burke 1998; Noulas et al. 2010; Botwright Acuña and Wade 2012; Carvalho et al. 2014; Dresbøll and Thorup-Kristensen 2014; Rich et al. 2016). For instance, Gonzalez-Dugo et al. (2010) summarized that rice's root growth and penetration were increased under drying conditions. Similarly, drought stimulated root growth of maize and rape in the subsoil (Engels et al. 1994). Among all the environmental factors that affect crop root growth and productivity, the most frequently mentioned and widely studied one is water.

Soil water availability sets the limitation for water uptake and influences the availability of mobile nutrients for crop uptake (Sadras et al. 2016). The change in soil water availability stimulates or inhibits shoot and root growth, leading to the variation of resource demand and uptake (Vandoorne et al. 2012; Lobet et al. 2014). Indeed, soil water status affects overall crop growth and resource uptake, but as deep roots can contribute considerably to resource uptake, it is also essential to clarify their development and function under different soil water availability.

Waterlogging

Too much (waterlogging) as well as too little (drought) water restrain crop growth and productivity (Maryam and Nasreen 2012; Iqbal et al. 2020), depending on the extent of the stress. The root growth of most main crops, e.g., wheat, maize, and sorghum (*Sorghum bicolor* L. Moench), is restricted by the lack of oxygen under waterlogged conditions. One exception is rice, which adapts well to waterlogging by forming higher ratio of aerenchyma in root tissues (Maryam and Nasreen 2012). Under such anaerobic environments, roots tend to grow near the soil surface and do not extend as they would under aerated soils (Sairam et al. 2008). Since most crop species have poor plant growth, lower root activity, and conductance under waterlogged conditions, their water uptake decreases, although soil water content is high (Kaur et al. 2020).

In dryland agricultural systems, waterlogging promotes soil N leaching, runoff and denitrification, reduces the rates of soil N mineralization, and causes reductions in soil N concentration and increment in N losses (Kaur et al. 2020). Oxygen deficiency under waterlogged conditions inhibits respiration, which causes the reduction of ATP synthesis in roots, leading to a reduction of N uptake (Steffens et al. 2005). Subsequent poor plant growth caused by nutrient deficiency further diminishes crop N demand, following by decreased N uptake.

Water deficit

Though the plant responses to waterlogging are of great importance, the primary focus of this section is the plant responses to water deficit, especially their responses regarding root growth and resource uptake. The role that deep roots play under such circumstances is of particular interest.

Root growth

Soil water availability is the main factor affecting plant growth. Plant root growth may also be affected directly or indirectly by water deficit through its effects on photosynthesis, carbohydrate availability, soil oxygen content and soil impedance (Lynch et al. 2012). Under field conditions, water deficit gradually dries the soil profile, and its occurrence in topsoil layers was assumed to be a stimulator of deep root development in the wetter and deeper soil layers (Bloom et al. 1985; Skinner 2008). The increased rooting depth may also be the response to increased impedance of the drying topsoil. The dry and hard topsoil inhibits the elongation, while in the wet subsoil roots may keep elongating, leading to deeper root architecture. Notably, the effect of water deficit on triggering the deeper root growth was not consistent in existing studies, which may relate to differences in the severity of drought stress and the timing of drought stress occurrence (Vandoorne et al. 2012; Rasmussen et al. 2020a).

Iqbal et al. (2020) concluded that a general effect of drought stress on root growth was reducing cell turgor pressure. The turgor pressure provides the force for cell wall extension, thus drought stress will result in a reduction of root elongation. Besides, the concurrent mechanical impedance in drying soil was also reported to limit root elongation of maize seedlings (Mirreh and Ketcheson 1973; Veen and Boone 1990). However, more recent study showed that if the effect of soil impedance was negligible, maize seedlings could make more extensible cell walls in the apical part of roots to adapt to lower water potential (Wu and Cosgrove 2000). Sharp et al. (2004) also summarized that the effect of water deficits on root elongation rate was not as strong as on shoot growth (Fig. 12). Therefore, roots may keep elongating under drought stress. The maintenance of root elongation under water stresses was assumed to benefit continuous expansion of the root system and maintain adequate water supply.

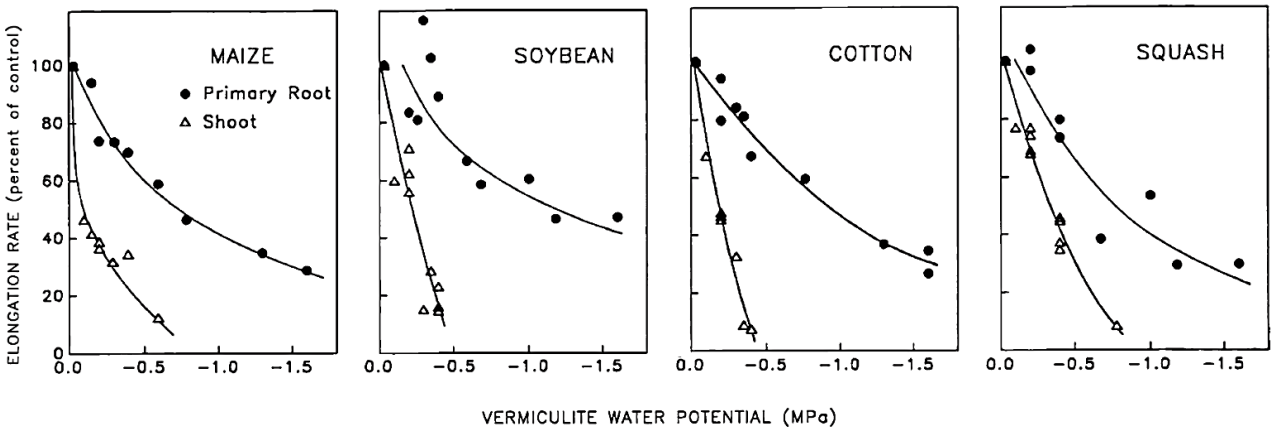

Fig. 12 Shoot and root elongation rates of maize, soybean (*Glycine max*), cotton, and squash (*Cucurbita pepo*) seedlings at different water potentials. Modified from Sharp et al. (2004).

Water use

Water use depends on soil water availability, plant demand, and root uptake capacity. Soil water availability sets the maximum amount of water uptake and regulates root growth, which further regulates root water uptake. Water stress reduces plant shoot growth via restraining cell growth and photosynthetic carbon assimilation and therefore reduces the overall demand of water uptake. However, as roots tend to grow deeper under water stress, the distribution and amount of water uptake within the root system may also be shifted, in which deep roots play an essential role.

The water uptake pattern of a given root system is predictable from the root hydraulic architecture, which encapsulates the radial and axial hydraulic conductivities of individual root segments. Meanwhile, it is also affected by the distribution and availability of soil water. At places where soil is dry and has low soil hydraulic conductivity, water flow towards the root surface will be restricted, triggering compensated water uptake from wet zones to maintain the transpiration demand (Lobet et al. 2014). This compensation has been observed several times in deep soil layers when drought stress occurred in topsoil. For instance, Vandoorne et al. (2012) found that the chicory roots below 0.6 m compensated for the shortage of water in the top horizons. Likewise, Chen et al. (*Appendix* II) observed increased daily water uptake from soil layers below 1 m when oilseed rape plants were exposed to water deficit (Fig. 13). However, contradictory results were obtained by Rasmussen et al. (2020a), where chicory's ability for deep water uptake did not vary a lot under different water availabilities (Fig. 13). This might be the result from the reduction of plant transpiration, which is triggered by the production of ABA and stomatal closure (Tardieu et al. 1992; Dodd et al. 2008).

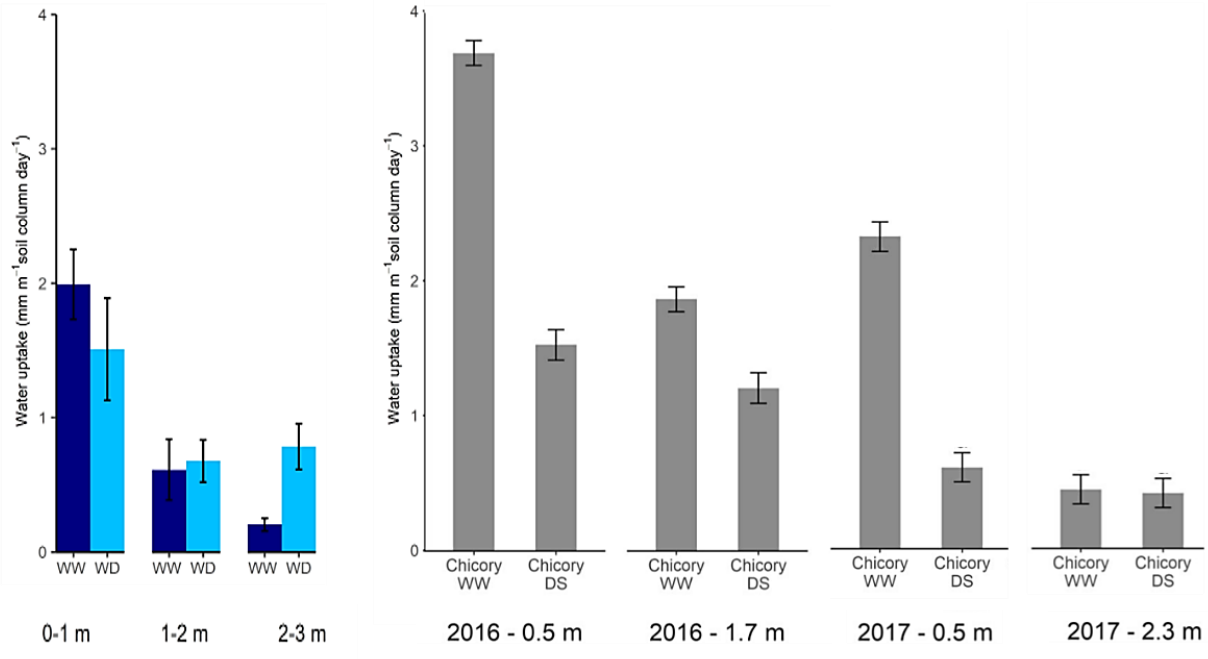

Fig. 13 Daily water uptake from various depths of oilseed rape (left, Chen et al. *Appendix* II) and chicory (right, Rasmussen et al. 2020). Plants were grown under different water statuses, and the maximum rooting depths were observed below 2 m in both experiments. WD = water deficit, WW = well-watered, DS = drought-stressed. Note that the severity of water deficit was not the same in the two experiments. See Chen et al. (*Appendix* II) and Rasmussen et al. (2020) for details of the experimental setups.

In addition to its effects on water absorption, water deficit often impairs transpiration and photosynthesis, for example, by reducing stomatal conductivity and mesophyll conductance (Chaves et al. 2002; Urban et al. 2017). The decrease of photosynthesis further leads to decreased biomass and potential reduction of WUE (Li et al. 2009). However, at the global level, crop WUE may be slightly improved, other than impaired by water deficit (Yu et al. 2020). The improvement of WUE is not equal to the enhancement of yield under water deficit, but rather indicate that compared with transpiration, photosynthesis is less affected by water deficit.

N use

Soil water availability directly influences soil N availability and indirectly affects N uptake via regulating plant growth and root development. In general, water deficit directly reduces plant transpiration, photosynthesis, and carbon assimilation, which leads to poor growth of plants and less demand for N (Fig. 14). Thus, the overall N uptake under water deficit is reduced correspondingly. However, this does not necessarily mean that N uptake from all soil depths will be reduced. Indeed, when water deficit occurs in upper horizons, the N that was concentrated in these horizons would be unavailable to the plants (Garwood and Williams 1967), as water flow

inhibited mass flow transport to the roots, and low water content reduced diffusion transport to the roots. While in deeper and wet soil horizons, the availability of N and the transport processes are less frequently reduced by soil drying, and crops could remove N from there. This hypothesis was supported by Chen et al. (*Appendix* II), who found that oilseed rape plants still depleted comparable $^{15}NO_3^-$ from subsoil when they were exposed to topsoil water deficit. Liu et al. (2018) reached a similar conclusion in wheat, where they found both root length density and N depletion in soil layers below 1 m were increased by reduced water supply, while the effects varied with the rate of N application in their study.

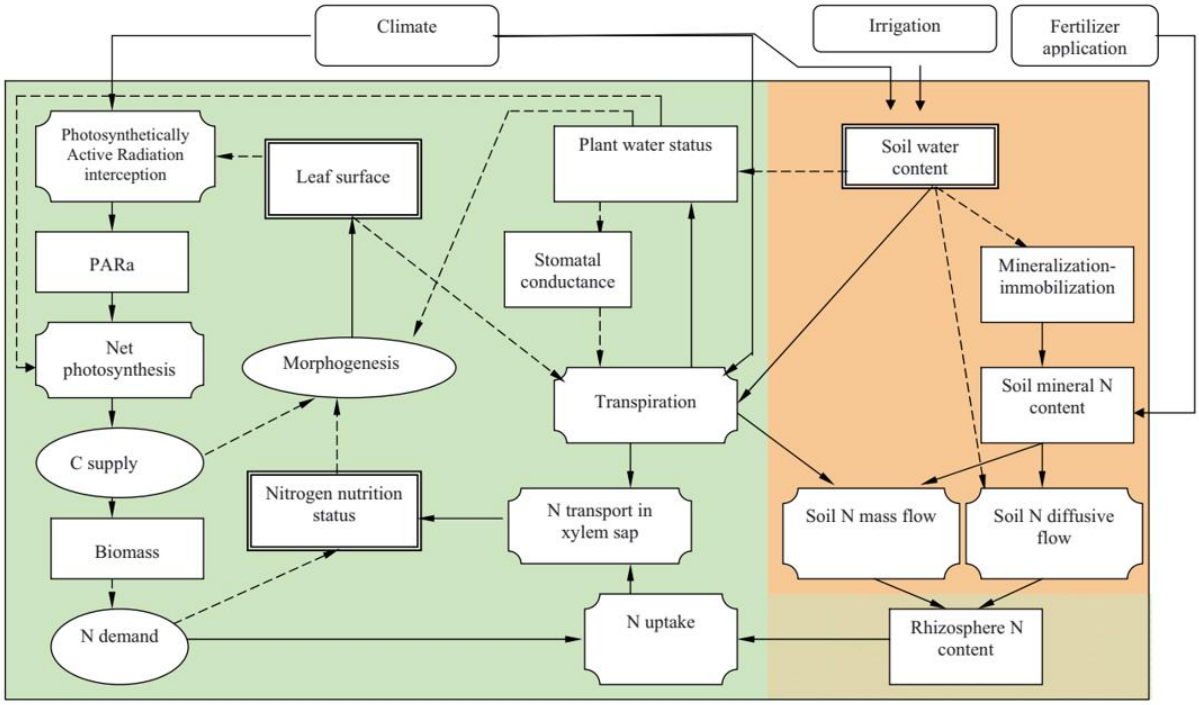

Fig. 14 Principal processes involved in the response of crop N status to water deficit in aboveground (green) and belowground (orange). Solid arrows indicate mass or energy fluxes, and dotted arrows indicate direct causalities. From Gonzalez-Dugo et al. (2010).

NUE is also expected to change with water inputs, and the maximum NUE is observed when water inputs match crop water demand (Quemada and Gabriel 2016). Either sub- or over-optimal input of water will lead to a reduction of NUE. Coupling N input with water supply is therefore crucial for increasing crop NUE.

The effects of management

In addition to genetic improvements, proper management practices (i.e., irrigation, fertilization, crop rotations, weed control, soil mulching…) are also reported to increase water and N uptake

(Li et al. 2009; Waraich et al. 2011; Quemada and Gabriel 2016). N is essential for plant growth and development as it is required in a large amount and easily becomes limiting in crop production (Lal and B.A. 2018). Plant available N is supplied by sources such as soil mineral N available before planting and mineralized N from organic sources, e.g., soil organic matter, crop residues, and soil amendments (De Pascale et al. 2018), while the most modifiable and common source is the application as mineral fertilizer. Besides the implications on yield, N supply is reported to affect plant root growth and distribution (Fageria and Moreira 2011), which strongly influences soil water and N consumption.

N supply

Root growth

Increasing soil N availability with external N supply enhances both shoot and root growth, but more shoot growth than root growth, which results in a decreasing root/shoot ratio (Lynch et al. 2012). Root density and surface area were found to be increased by increasing N supply (Lynch et al. 2012; Rasmussen et al. 2015), while the rate of supplied N does not show a uniform effect on other root traits. Fan et al. (2010) and Chen et al. (2020) found increased N supply enhanced root length and biomass of rice and cotton. In contrast, in other species such as wheat, maize, and oilseed rape, a high rate of N fertilization was found to inhibit the root elongation and rooting depth (Comfort et al. 1988; Tian et al. 2008; Gaudin et al. 2011; Louvieaux et al. 2020).

Likewise, other root morphological traits, e.g., root hair density and root hair length, were also reported to decrease with increasing N supply (Bhat et al. 1979; Robinson and Rorison 1987). Furthermore, it is reported that the timing and placement of N application also influence root growth. Delayed N supply and deep placement of fertilizer promoted root growth (Ennik and Hofman 1983; Jarvis and Macduff 1989; Kristensen and Thorup-Kristensen 2007), possibly reflecting N limitation from the rest of the root zone.

Water use

As discussed earlier, soil water directly affects the availability of soil N for plant use as well as plant N demand. On the other hand, N supply also influences plant water uptake, mainly by improving plant demand via regulating canopy size and transpiration (Sadras et al. 2016). Field studies confirmed that fertilization could improve the depth and amount of soil water extraction. Fertilized winter wheat extracted water from 0- 183 cm depth, while the water extraction of unfertilized wheat was limited to the upper 90 cm (Brown 1971). In oilseed rape, Chen et al.

(*Appendix* II) observed that plants fertilized with 240 kg N ha^{-1} tended to extract more water from all soil depths than those fertilized with 80 kg N ha^{-1}, although the root growth was not significantly changed. Besides the possible enhancement of root growth under additional N supply, recent studies showed that root hydraulic conductivity and aquaporin expression increased with high NO_3^-/ NH_4^+ supply (Gorska et al. 2008; Ding et al. 2016; Wang et al. 2016), which offers another explanation for the higher root water uptake in plants supplied with more N.

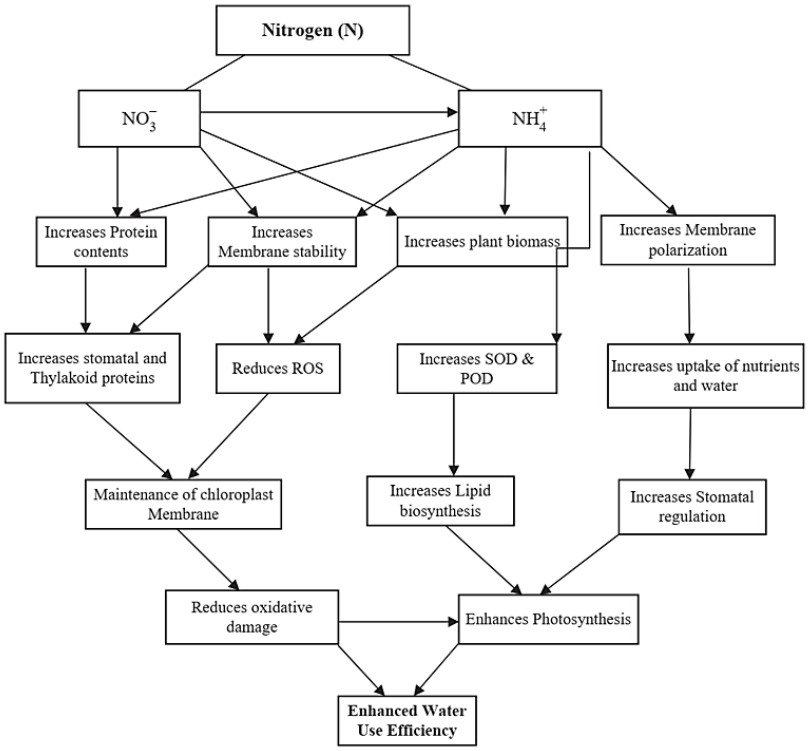

Fig. 15 Principal processes involved in the response of N supply to crop water use efficiency. ROS: reactive oxygen species; SOD: superoxide dismutase; POD: peroxidase. From Waraich et al. (2011).

N supply not only alters the amount and distribution of water uptake, but also regulates biomass production and the overall water use efficiency. Waraich et al. (2011) summarized the possible mechanisms through which N supply may enhance water use efficiency in plants (Fig. 15), focusing on its regulation on aboveground processes. In a word, additional N supply reduces oxidative damage, and enhances plant photosynthesis, therefore improves water use efficiency.

N use

No doubt that with additional N supply, there will be more N available in the soil. This may further lead to the enhanced aboveground plant growth and the possible improvements of root growth, which allow plants to deplete a larger amount of soil N. Increased N supply is often accompanied by an increase in both biomass and N content, and sometimes by an increase of root growth

(Rasmussen et al. 2015), while the uptake efficiency of the applied fertilizer may decline. Khan et al. (2017) observed that in oilseed rape, increasing N fertilization from 60 to 120 and 180 kg N^{-1} decreased the uptake efficiency of fertilizer by more than 5%. Similarly, Kristensen and Stavridou (2017) found significant improvement in NupE when less N fertilizer was given to the rocket (*Diplotaxis tenuifolia* L.).

Both model simulations and field data showed plant took N from the whole rooted zone (Pedersen et al. 2010), thus the change of N supply may also affect root N uptake at all depths. It is clearly that the rate of N supply has significant effect on root N uptake in topsoil, as soil N availability is changed. It also influences root N uptake in subsoil, although it may not alter the availability of subsoil N directly. Oilseed rape fertilized with 80 kg N ha^{-1} at the flowering stage exhibited much higher efficiency in taking $^{15}NO_3^-$ at both 0.5 m and 1.7 m than the oilseed rape fertilized with 240 kg N ha^{-1} (Chen et al. *Appendix* II). However, reduced NupE does not show that the capacity of roots for depleting the soil is insufficient, but rather that the root capacity for N uptake is higher than crop demand (Robinson et al. 1994; Thorup-Kristensen and Sørensen 1999).

Additional N supply increased root density and surface area in upper soil layers, leading to fast and enhanced N uptake from there. These responses are beneficial during early growth, where a large amount of N is required for maintaining plant growth and development. However, during late growth stages, excessive N application beyond the crop demand reduces NupE, and the leaching of unused N leads to environmental problems. Lower N application compromises the root growth in upper soil layers, but promotes subsoil root growth. Deep roots have considerable N uptake capacity as shallow ones and can continuously contribute to plant N uptake during late stages (Chen et al. 2021; *Appendix* I). Therefore, the overall NupE and N uptake under low N application are not necessarily reduced.

Stimulating deep root growth by reducing N supply seems to be a promising strategy for building efficient crop systems, especially in regions that are facing the risk of N leaching. Nevertheless, deep roots develop during the late growing stages, thus deep N uptake always occurs later. It is still important to develop a certain amount of roots in topsoil to maintain plant growth in earlier stages. Further studies on optimizing the time and amount of N supply, to comply with the growth of deep roots and crop N demand are required. In addition, one should always keep in mind that the variations of environmental and genetic factors are important to be included in N uptake studies.

Overall, plant shoot and root growth respond to the variation of the individual water or N supply in various ways. The variation of shoot growth determines the demand, and the response of root system determines resources uptake. Since strong interactions exist between water and N uptake, it is common to see the variation of individual water or N supply results in the concurrent variations of water and N uptake. Understanding the variations may provide ideas for developing breeding strategies and agronomical practices towards efficient crop system under water- and N-limited conditions.

Some plant responses are common in water- or N-limited conditions, such as developing deep roots and reducing aboveground biomass. The occurrence of deep rooting is valuable for maintaining resource uptake under such stressed conditions. However, due to the limitation of measuring and analytical techniques and methods, there are still plenty of gaps in studying deep roots and their activities. In the last chapter of this review, current and potential methodological options for root observation and measurement will be discussed.

5. Methodological considerations

Many studies highlighted the potentials of deeper rooting, as well as the complexities of root resource uptake. Nevertheless, our knowledge of deep roots and their functions is still limited, primarily due to the lack of methods making studies feasible. Since the existing techniques are costly and labour-consuming, renewed and less expensive technologies, which allow direct and detailed observations of root growth and activities will be of great value (Thorup-Kristensen et al. 2020a). In the last chapter of the review, both the traditional and novel methods to access and study roots are highlighted. Their advantages and shortcomings are also discussed briefly.

Root access, observation and sampling

Field

Maeght et al. (2013) described the most important methods to study roots directly and visually under field conditions, especially for those located in deeper soil layers. In the field, the commonly used ones include excavations, soil coring, trenches, minirhizotrons, access shafts, caves, and mines (Fig. 16). These approaches allow either 2D or 3D investigations of roots (see Maeght et al. 2013 and references within).

Excavation, trenching and coring methods (Fig. 16a) usually refer to digging or sampling soil and roots manually or with the help of machines/steel augers. These methods are simple but effective to determine maximum rooting depth and full biomass per individual plant. In addition to the possibility of sampling soil and roots, trenching approach (Fig. 16a) exposes the soil profile and gives horizontal and vertical information of roots. However, these methods have shortcomings to give repeatable measurements, and the applicable depths are often restricted to the upper soil layers (Maeght et al. 2013).

A similar method to the soil coring is the ingrowth core, where the basic concept is to replace a specific volume of soil with root-free soil in a core before the root occurrence (Steingrobe et al. 2001). Compared to traditional coring methods, the ingrowth core technology provides information on root growth in given periods. The sampled root biomass can be quantified, and those root samples can be further analyzed.

Compared with the above-mentioned approaches, access shafts (Fig. 16b) allow non-destructive, repeated, and continuous root observations and are flexible with installing additional devices. Together with imaging or scanning devices, minirhizotrons (Fig. 16c) can also be used for root

observation at depths. The last two methods, mines and caves (Fig. 16d), were less reported in root studies of agronomic ecosystems, as they required the existence of natural caves or mines.

More or less, these methods have some inevitable shortcomings in deep root study. Firstly, almost all methods mentioned above are destructive and labour intensive. Limited time and high labour costs make it hard to dig into deeper soil layers and get enough replicates. Secondly, high soil bulk densities and unexpected rocks in subsoil may prevent the excavation works and the installation of minirhizotrons. Furthermore, in the minirhizotron technique, it is difficult to obtain good contact between the tube and the soil, which leaves gaps for artificial root growth. Soil disturbance caused by the installation is another serious problem, which restricts steady and continuous observation of roots (Maeght et al. 2013).

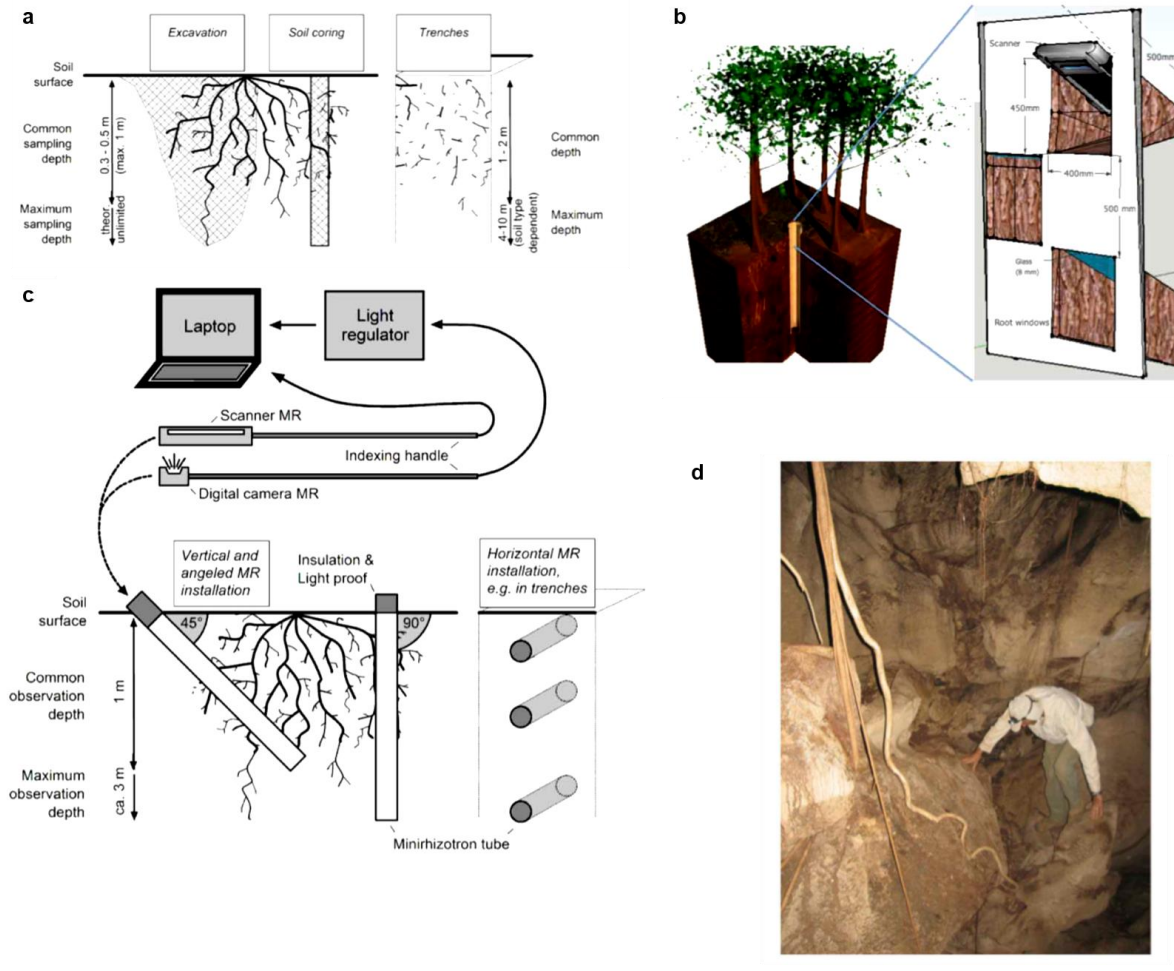

Fig. 16 Schematic view of main direct field methods for (deep) root observation. (a) Excavation, soil coring, and soil trenching techniques. (b) Access shafts. (c) Minirhizotron technique. (d) Cave prospection. Modified from Maeght et al. (2013).

Semi-field

Many researchers chose to grow the targeted plants in artificial/semi-field facilities (Fig. 17), where soil and climatic factors are controllable and easy for sampling. These facilities include pots, rhizotrons of different materials and sizes, and large-scale semi-field infrastructures (Zegada-Lizarazu and Iijima 2004; Svane et al. 2019; Thorup-Kristensen et al. 2020b). Compared with indoor pots or tubes, large outdoor rhizotrons or facilities provide more realistic scale and growth conditions for root studies. The artificial facilities save much more time on root sampling and phenotyping, and offer the possibility to conduct experiments on single roots, but they still have deficiencies. One example of this is the rhizobox facility mentioned in the two manuscripts in the appendix of this thesis (Fig. 17d). Combining indirect methods such as imaging, tracer labelling, and soil moisture measurement, this rhizobox facility gave inspiring information on deep resource uptake of single roots (Chen et al. *Appendix* II). However, the soil temperature, drainage, and evapotranspiration of this facility are different from field conditions, which may lead to different patterns of root growth. Therefore it cannot be seen as a replacement for field study.

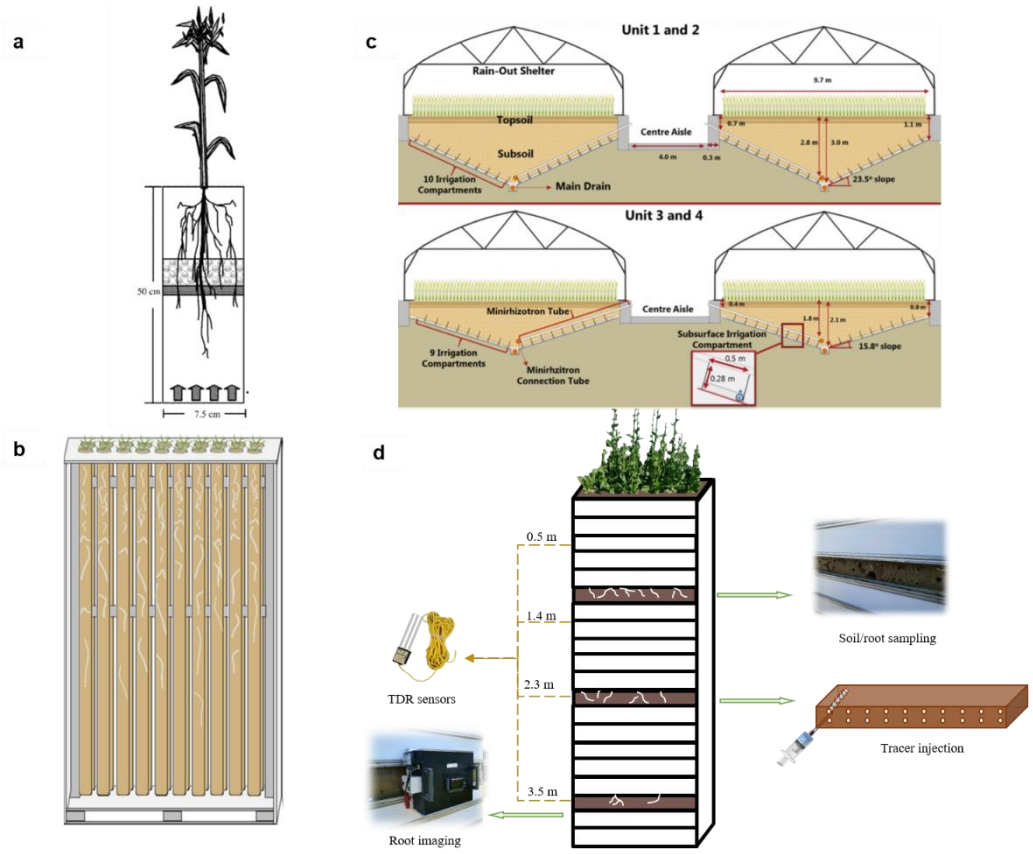

Fig. 17 Schematic view of semi-field facilities for (deep) root observation. (a) Pot. (b) Rhizotrons. (c) A large-scale semi-field facility. (d) Rhizobox. Modified from Zegada-Lizarazu and Iijima (2004), Ytting et al. (2014), Svane et al. (2019), and Chen et al. (2021; *Appendix* I).

Root activity measurements

The methods mentioned above allow direct observation and sampling of soil and roots. Indirect methods including tracer labelling (e.g., Göransson et al. 2006; Beyer et al. 2016), and the use of sensors that measuring soil moisture, temperature and electrical conductivities (Krishnapillai and Sri Ranjan 2009; Illston and Fiebrich 2017; Chen et al. 2021, *Appendix* I), provide the possibility to track and quantify root activities and the dynamics of resource uptake. Image-analysis-based phenotyping techniques, which refer to non-destructive optical analyses of plant traits (Walter et al. 2015), have also been adopted in root studies. An overview of some widely used indirect methods is given in the following.

Tracers

Isotopic tracers have been used as a tool to define rooting depth, investigate soil resource availability, nutrient cycling, and microbial community structure (Kahmen et al. 2008; Beyer et al. 2016; Guo et al. 2016; Heijboer et al. 2016). The commonly used tracer elements in plant studies can be divided into stable isotopes, radioactive isotopes, and nutrient analogous trace elements. The stable isotopes do not decay into other elements, while unstable radioactive isotopes have relatively short half-lives and will decay into other elements. Stable isotopes such as ^{2}H, ^{18}O, and ^{15}N have been widely used in water and N related studies (Bakhshandeh et al. 2016; Beyer et al. 2016; Kulmatiski et al. 2017), and radioactive isotopes such as ^{33}P have been used to estimate plant phosphorus accumulation (Foyjunnessa et al. 2014). The use of radioactive isotopes in plant-soil studies should be handled carefully, as they will bring radiation hazards to human beings and environment. In addition, the short half-lives of radioactive isotopes make repeated injection and prompt analyses necessary in long-term experiments (Pinkerton and Simpson 1979).

Nutrient analogues can be absorbed by plants similarly as the aimed elements, and are thus widely adopted in tracer studies. Some of the most commonly used analogues include strontium (Sr) for calcium (Ca), lithium (Li), cesium (Cs) and rubidium (Rb) for potassium (K), and selenium (Se) for sulphur (S) (Collander 1941; Pinkerton and Simpson 1979; Martin et al. 1982; Terry et al. 2000; White and Broadley 2000).

Measuring the variation of natural abundance of a given tracer in soil-plant pool, or injecting the tracer at different depths then calculating the subsequent recovery in shoot biomass can both reflect the root water or nutrient uptake from depths. The utilization of stable isotopes in plant-water research is based on the observation that the isotopic composition in water remains the same during

water uptake from the soil to the root, and transport within the xylem (Ehleringer and Dawson 1992). When isotopic fractions of the water source are known, analyzing the isotopic fraction of water in xylem water will reflect the currently active rooting zone for water uptake. Isotope fractionation occurs during transpiration, thus water in transpired water and other aboveground tissues near the leaves could be enriched in ^{2}H and ^{18}O (Thorburn and Mensforth 1993). Therefore, water from these tissues may not indicate the water sources. However, the isotopic fraction in transpired water is considered valid for reflecting source water when ^{2}H or ^{18}O is highly enriched in the soil by labelling, since the fractionation can be seen as negligible when the abundance of the isotope is very high. The collection of transpiration water by using plastic bags have been used in labelling studies for tracking water transport and uptake (Beyer et al. 2016; Chen et al. 2021, *Appendix* I).

Yoneyama and Kaneko (1989) claimed that N isotopic composition ($^{15}N/^{14}N$) remained the same during NO_3^- uptake and transport, indicating no fractionation between NO_3^- in the plant and supplied NO_3^-. Thus the isotopic fraction in plant N can be seen as an indicator of N uptake from different depths when soil N isotopic fractions are known. The variations of natural abundances with depth can be relatively small, thus active labelling is often applied since it is easier to distinguish the root uptake from the aimed sources. Using $^{2}H/^{18}O$-labelled water and ^{15}N-labelled N, root uptake of water and N have been related to soil resource availability, rooting depth, and distribution (Andersen et al. 2014; Kulmatiski et al. 2017). Additionally, by using the ^{2}H and ^{15}N dual-labelling technique, Chen et al. (2021; *Appendix* I) showed that water uptake was more sensitive to increased depth than NO_3^- uptake, which revealed the different uptake pattern between these two resources.

Tracer techniques could also be applied together with other direct/indirect methods. For instance, by combining ingrowth cores with tracer labelling, Han et al. (2020) and Rasmussen et al. (2020b) successfully identified root growth and activities to 4.2 and 3.5 m of soil depth. In addition, they claimed that tracer labelling with ingrowth cores helped prevent the tracer leakage and soil contamination, which was advantageous over the more common method tracer injection into the bulk soil, especially for immobile tracers. The core-labelling technique has drawbacks, such as the potential risk of soil-collapse of the ingrowth core soil (Han et al. 2020). Overall, cautions need to be taken when tracer techniques are adopted, mainly because 1) isotopes might be diluted in the soil; thus, predictable dilution effects are required, 2) the soil properties that may affect tracer

uptake, and soil biochemical processes that may compete with tracer uptake should be known (Maeght et al. 2013).

Soil moisture measurement

The changes of soil moisture over time can be seen as a sign of root water uptake (Maeght et al. 2013). Traditionally soil water content is determined by the gravimetric method, which refers to measuring weight loss of soil samples after drying. This method is considered as the standard although the sampling and drying processes are time-consuming. In the past few decades, more *in situ* technologies have been developed for automatic, continuous and non-destructive measurement of soil moisture, including time domain reflectometry (TDR) and frequency domain reflectometry (FDR) (Brocca et al. 2017). It is worth noting that other water movements such as infiltration and drainage also lead to the fluctuations of readings, thus the estimation of water uptake from water sensors should be interpreted with caution.

Image-analysis-based phenotyping techniques

The term phenotyping can be interpreted in diverse ways. Walter et al. (2015) defined plant phenotyping as a quantitative description of a wide range of plant traits, while Fiorani and Schurr (2013) described it as a set of accurate, precise protocols and methodologies for plant growth and architecture measurements. Following the latter definition, the above-mentioned techniques can all be regarded as root phenotyping. Today, image-analysis-based phenotyping techniques are of great interest since they allow non-invasive and high-throughput characterizations of plant traits.

Compared to shoot imaging, imaging root systems *in situ* remains challenging as soil is opaque. The acquisition of root images is traditionally accomplished by separating roots from soil then imaging by cameras or scanners. Non-destructive visible light imaging techniques are often used together with rhizotrons or minirhizotrons (Nagel et al. 2012; Louvieaux et al. 2020). The outputs are usually two-dimensional (2D) images, and information of the overall root system is missing as most of the root system is buried in the soil. Other 2D imaging techniques have also been widely used in determining root activities. Using light transmission imaging, Garrigues et al. (2006) investigated root water extraction from a single root to the whole root system. More recently, Zarebanadkouki et al. (2012) used neutron radiography to measure local water fluxes from soil into lupin (*Lupinus albus* L.) roots.

3D imaging techniques such as X-ray computed tomography (CT), magnetic resonance imaging (MRI), and positron emission tomography (PET) allow non-invasive investigations of root

systems of laboratory grown plants. PET is sensitive to radioactive tracers such as ^{11}C, and works well in visualizing carbon allocation within the root systems (Jahnke et al. 2009), while its resolution is relatively coarse (~ 1.4 mm) compared with the other two methods (Atkinson et al. 2019). Both X-ray CT and MRI techniques performed well in detecting root architecture in soil-based laboratory experiments, while MRI is more expensive and dependent on soil properties (Metzner et al. 2015).

So far, all of these image-analysis-based techniques still require specially designed containers, transparent artificial growth media, or are sensitive to soil characteristics. Indeed, transparent artificial growth media allows the extraction of the root system architecture, but it fails to reflect the actual plant root growth in soil (Atkinson et al. 2019). Further, these techniques have limited potential use in deep root studies and are theoretically impossible to be applied in field experiments.

Image processing

The labour and time costs for device installation and image capturing are high already, but processing the images could be more time-consuming and exhausting. Generally, the excavated roots are washed or separated from the soil and stored to be analyzed later. In the 1960s, the line-intersect method was developed by Newman (1966) for annotating washed roots, where the total root length was estimated by counting the number of roots crossing randomly located lines. The basic idea behind this method is the longer the root is, the more crosses it will make with the lines. This method was further modified and tested by Marsh (1971) and Tennant (1975), who improved the precision by increasing intercept counts. Later, this method was applied in rhizotron /minirhizotron studies by superimposing grids on the surface of rhizotrons or captured root images (Thorup-Kristensen 2001; Rasmussen et al. 2020a). Root intensity in the given area was calculated as root intersections per meter line (Fig. 18b). Root intensity obtained in this way was positively and linearly correlated with root length density measured by washing and analyzing roots using the software (Ytting et al. 2019), which proved the efficacy of the line-intersect method.

Although the line-intersect method provides estimated root length or root intensity in a sample, the process is arduous and does not extract other information on other root traits such as diameter, branching, and angle. These missing root traits can be essential indicators for resource acquisition. Several programs have been developed to process root images acquired from a digital camera or a flatbed scanner. These include WinRhizo, SmartRoot, RhizoVision Explorer, RootPainter, and

so on (Arsenault et al. 1995; Lobet et al. 2011; Seethepalli et al. 2021; Han et al. 2021; more software can be found on https://www.quantitative-plant.org/software). These programs could make morphological, topological, architectural, and color analyses. Taking WinRhizo as an example, it analyzes root traits such as total length, average diameter, total area, numbers of tips, crossings of the objected image, which covers a broad range of root measurements. However, these measurements become less reliable when root samples become more complex, for example, with crossovers, overlaps, and debris. Considering many roots to be analyzed in root studies, it takes lots of time to prepare satisfying root scans or images before the software can efficiently analyze them.

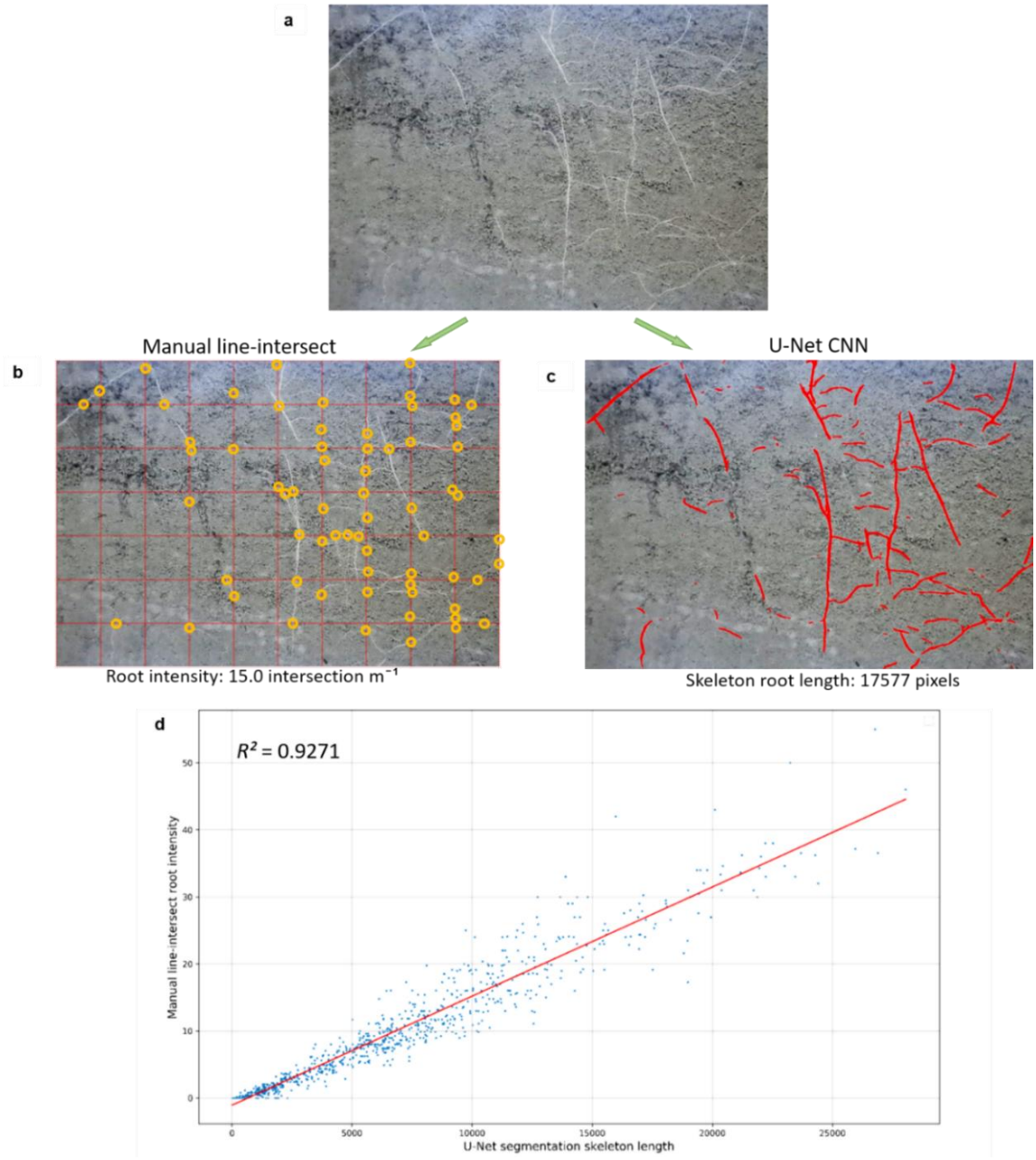

Fig. 18 Two root annotation methods used in the following manuscripts in the appendix of this thesis. (a) Soil and roots as seen from the surface of one of the rhizotrons mentioned in **Fig.** 17d in 2018. (b) The corresponding annotation using the line-intersect method. Yellow circles are marked where roots hit the auxiliary lines. (c) The corresponding annotation showing root pixels in red. The photo was segmented by U-Net convolutional neural network (CNN). (d) The correlation between manually counted root intensity and software segmented root length. The dataset includes 867 images taken from the rhizotrons in 2016. Fig. 18d was modified from Smith et al. (2020b).

Software that can provide quick, automatic, and reliable root segmentation and reduce the time of imaging preparation is of great interest. Convolutional Neural Networks (CNN) based systems have been developed which are effective for root segmentations in both 2D photos and 3D images (Smith et al. 2020b; Soltaninejad et al. 2020). Using a CNN-based software called RootPainter, accurate and satisfying root segmentation was obtained after 3-4 hours of model training (Fig. 18c). The pixels labelled over the detected root segments are extracted and further transformed to actual root length (cm). Within the same dataset, the extracted pixels were linearly correlated to root intensities that were calculated using the line-intersect method (Fig. 18d), which shows the potential of such AI-based software in root studies.

Modelling

Modelling of root water and nutrient uptake was an early and successful application of computers in plant sciences. To some extent, the application of the models is an alternative to studies that are difficult to conduct, such as deep root studies. An example for this is the Daisy model, which simulate water and N balance and crop production using environmental and management factors (Hansen et al. 2012). By simulating root density and N uptake from deep soil layers, Pedersen et al. (2010) found that the main parameters affecting deep N uptake were the distribution of root density at depth and the penetration rate. Their result was validated by field data for red beet (*Beta vulgaris var. vulgaris* L.) and leek (*Allium ampeloprasum*); however, it failed to simulate white cabbage, whose root density increased with depth (Pedersen et al. 2010). So far most of the models are developed for only one or a few species, while with increase of input data it is possible to simulate plant growth and development of more crops. Through model simulations, the effects of altered parameters on crop growth, production, water, and N uptake, and soil resource cycling can be easily obtained. However, one should keep in mind that the validation of the model outputs is always necessary before any general conclusions are drawn. Without efficient validations against the measured data, the simulations may deviate a lot from reality.

The addressed direct and indirect techniques allow deeper rooting observation and activity assessment. However, methods such as excavation, trenching, soil coring and the installation of minirhizotrons are labour-consuming; construction of semi-field facilities are costly; methods such as the application of tracer, sensors, and imaging techniques are also not easy to conduct, and should be followed by subsequent analyses. Models offer simulation of deep root development and activities, while still needing validation using the experimental data. A smart combination of these techniques may lead to a reduction of the time and labour cost, which allows the conduction of experiments focusing on deep root growth and functioning.

Although there are not as many studies on deep roots as there are on shallow roots, the available evidence has clearly shown that deep roots are widely observed. The existence of deep roots leads to significant water and N uptake from subsoil, which will otherwise remain unexploited and may cause environmental problems. Deep roots are also been involved in soil processes such as hydraulic redistribution, and carbon sequestration. Still, it remains difficult to assess the performance of deep roots, due to the lack of suitable sampling and analyzing technologies. Novel techniques and their combinations make it possible to access the deep roots and look into their functions. In the near future, the study of deep roots may still be laborious and costly, while their obvious potential on exploiting the subsoil resource and the pressing demands towards sustainable crop production make it crucial to explore the deeper rooting.

Appendix I*:* Dual labelling by ^{2}H and ^{15}N revealed differences in uptake potential by deep roots of chicory

This manuscript has been published on ***Rhizosphere***.

Picture: Chicory flowers, from Guanying Chen

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Dual labelling by ^{2}H and ^{15}N revealed differences in uptake potential by deep roots of chicory

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ABSTRACT

Aims: Deep-rooted crops have been widely used in agricultural systems to access deep resources such as water and nitrogen (N). However, the potential of deep roots to take water and N at various depths have not been well studied. Here we used chicory (*Cichorium intybus* L.) to study the potential and dynamics of water and nitrogen uptake in deep soil layers (below 1 m).

Methods: Chicory plants grown in outdoor rhizotrons were labelled by injecting a $^{2}H_2O$ and $Ca(^{15}NO_3)_2$ mixture into the soil column at 1.1, 2.3 and 3.5 m depth. Five, ten and twenty days after injection, ^{2}H and ^{15}N were traced in transpiration water and leaves.

Results: We found enriched ^{2}H and ^{15}N in water and plant samples, and both water and N uptake were observed down to 3.5 m. The ^{2}H enrichment after injection at 1.1 m depth was 1552‰, almost 10 times higher than after injection at 2.3 m depth, which was 156‰. In contrast, injection at 1.1 and 2.3 m depth resulted in similar ^{15}N enrichment of leaf samples.

Conclusion: Deep water uptake was found to be more sensitive to increased depth and reduced root intensity than N uptake, and labelled N was used more rapidly than labelled water. We propose several possible explanations for the discrepancies between deep water and N uptake, and further discuss the challenges of using isotopes and models in deep root studies.

1. Introduction

Excessive application of nitrogen (N) leads to an accumulation of N in soils and a risk of leaching, which can cause subsequent pollution of groundwater (Cameron et al., 2013; Ju and Zhang 2017). Effective use of deep-stored water and nutrients in the soil profile by crops is therefore crucial to obtain high yields and minimize nutrient losses to the environment. Several strategies have been proposed to improve deep rooting and subsoil water and N use, in both genetic and agronomic ways (Gregory 2007; Kell 2011; Thorup-Kristensen and Kirkegaard, 2016).

Deep rooting has been highlighted for its potential use of unexploited soil water and nutrients (Thorup-Kristensen 2006a; White and Kirkegaard 2010; Lynch 2013), yet few studies have adequately investigated details of deep resource uptake. Thorup-Kristensen et al. (2020a) suggested that the main limitation of deep root research is that current methods for deep root research are costly and labour-consuming with insufficient throughput. Deep root growth is restricted in various ways such as soil acidity, soil compaction, hypoxia and suboptimal temperature (Lynch and Wojciechowski 2015), and the resource uptake is often constrained by plant demands as well as soil nutrient availability. These limitations make it even harder to isolate the value of deep roots in resource uptake.

Compared with topsoil, typically there are fewer unevenly distributed roots in deep soil layers (Fan et al., 2016). As crops usually do not reach maximum rooting depths until the end of their lifecycle, roots in deep soil exist for a shorter time. This leads to lower exploitation of deep soil resources. However, deep roots can contribute notably to crop water and nitrogen supply (Kristensen and Thorup-Kristensen, 2004; Lilley and Kirkegaard 2016). Nielsen and Vigil (2018) found that wheat and corn extracted water from 0 to 1.8 m in the soil profile, with more than 20% coming from the 0.9–1.8 m soil profile, despite the fact that more than 95% of the root biomass could be found within the top 1.04 m for wheat, and 0.9 m for corn. White cabbage, with a rooting depth of 2.5 m, remarkably reduced N_{inorg} by as much as 113 kg N ha^{-1} below 1 m soil depth (Thorup-Kristensen 2006a). Deep-stored water is important to dryland crops (e.g. wheat) as such water can be the only available source, and is particularly crucial during the grain filling period when water deficit may lead to great yield losses. During seasonal drought

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periods, a small number of deep roots that can take up the growth-limiting water could also be highly valuable to grain yield (Kirkegaard et al., 2007).

Water and N are the two resources with the greatest impact on crop productivity and have been widely studied. Despite the similarities, such as high mobility in soil, there are differences in transport and uptake processes of water and N. Water and dissolved nutrients are brought to the root surface from the soil by mass flow, while N also moves to the root surface independently of water movement via diffusion (Comerford 2005; Chapman et al., 2012). Given that water and N transport and uptake are interrelated (Plett et al., 2020), it is necessary to consider both when relevant studies are made. Being highly mobile, water and N can be acquired from the subsoil by deep roots. Unlike upper soil layers, where soil water and N availability are usually the limiting factors for root resource acquisition, the low root density and the short active period of the deep roots make root uptake potential and dynamics particularly important for efficient root uptake. Therefore, studies on deep root uptake capacity and dynamics are urgently needed.

Stable isotope labelling is a widely used tool in studying soil water and N uptake (Calder 1992; Kahmen et al., 2008; Rasmussen et al., 2020a). Dual labelling with ^{15}N and $^{2}H/^{18}O$ has been successfully used in water and N uptake studies in the top 1.5 m soil over 1–3 days (Bakhshandeh et al., 2016; Kulmatiski et al., 2017). These studies showed that water and nitrogen uptake occur in various depth of root zones, suggesting that root systems have independent uptake strategies for different resources. Similarly, we expect that water and N uptake from deep soil layers (>1.5 m) differ. In general, when there are fewer roots, water and N uptake decreases. However, with increased depth, deep water uptake occurs against gravity and hydraulic resistance (Lobet et al., 2014), while deep N uptake is not affected by these factors. As a result, root water uptake tends to be more influenced by increasing depth than nitrogen uptake. Further, water and N uptake are mainly driven by plant demand. Plant N uptake peaks during the early reproductive stage and then declines (Imsande and Touraine 1994), while plants maintain a high water demand also after the canopy has been built. Here, we may infer that deep water and N uptake will vary during the growing season, especially during the early reproductive stage, depending on the different plant demands.

Models provide us with an alternative way to study root water and N uptake (Wang and Smith 2004; Pedersen et al., 2010; Lilley and Kirkegaard 2016). Model simulations can be used to generalize the results and simulate the dynamics of uptake during the season, but experimental validation of such simulation results are required. Characteristics of the soil and root system, such as soil water and N availability, root distribution and soil and root hydraulic conductivities are used to evaluate water and N uptake (King et al., 2003; Pedersen et al., 2010). Although studies indicate that deep roots have great potential for water and N uptake (Kell 2011; Rosolem et al., 2017), to our knowledge, indirect comparisons against plant N uptake from subsoil have only been made down to 2 m (Pedersen et al., 2009). Further information on deep water and N uptake may provide inputs to evaluate and validate resource uptake modules in the model and get more detailed and precise predictions of root resource uptake.

In this study, we used ^{15}N and ^{2}H dual labelling to investigate temporal and spatial water and N uptake dynamics by deep roots. The following hypotheses were put forward 1) the uptake potential for water is more sensitive to increased depth and reduced root density than the uptake potential for N, 2) the dynamics of deep water and N uptake differ, labelled N being used more rapidly after injection than labelled water. With this study, it is also our aim to show that dual labelling with ^{15}N and ^{2}H labelled water can be used for the study of short term dynamics of water and N uptake from deep soil layers. Chicory (*Cichorium intybus* L.), which is known as a deep-rooted forb (Vandoorne et al., 2012; Thorup-Kristensen and Rasmussen, 2015), was used as a model plant.

2. Material and methods

2.1. Experimental site

The research was conducted using the rhizobox facility (Thorup-Kristensen et al., 2020b) in Taastrup, Denmark (55° 40′ 90.3$\tilde{5}$ N and 12° 18′ 24.8$\tilde{4}$ E, 23 m above sea level). The rhizobox facility is built for investigation of deep root growth and function. The experiment was performed in the spring/summer 2018 with chicory (cv. "Chicoree Zoom F1″) grown in the facility.

2.2. Experimental design

The rhizoboxes are 4 m tall, 1.2 m wide, 0.6 m thick, and fixed on the concrete ground. They are filled with subsoil to 0.25 m from the top taken from below the plough layer at Store Havelse, Denmark, while the top 0.25 m is topsoil collected from fields nearby the facility (Table 1). The average soil bulk density in the facility was 1.6 g cm^{-3}, with little variations among depths.

Each rhizobox is split into two 4 × 1.2 × 0.3 m chambers (Fig. 1), facing two opposite directions. The front of each chamber is divided into 20 panels being either 0.21 m or 0.17 m (every third) tall. Each panel is covered by an acrylic window, which is fixed by a metal frame. A white PVC board that can slide in the metal frame is placed outside the acrylic window to block solar radiation. The PVC boards can be removed to allow root imaging with a camera via the transparent acrylic windows. The acrylic windows can be removed temporarily for tracer injection. The rhizoboxes are outside and receive precipitation, with the option to supply additional with a drip irrigation system that is installed on top of the rhizoboxes with an irrigation rate of 14 mm h^{-1}.

Chicory plants were sown in a greenhouse on 11 April and transplanted to the rhizoboxes on May 3, 2017. Six chicory plants were planted in each chamber, corresponding to a density of 17 plants m^{-2}. All chambers were fertilized with a nutrient solution equivalent to 50 kg N ha^{-1}, 8 kg P ha^{-1}, 40 kg K ha^{-1} on April 12, 2018. On May 28, 2018, all chicory plants were cut down to 0.5 m, and on 14 June the plants started flowering. The main measurements of the experiment were initiated from 28 May to 29 June, after which the biomass was harvested. The weather data was obtained from a meteorological station on site. The mean temperature during this period was 18.0 °C and the total precipitation was 5.03 mm. To prevent drought stress, all chambers were irrigated for four, three, and 3 h on 4, 16 and 25 of June, respectively.

2.3. ^{2}H and ^{15}N labelling

Chicory's uptake of water and nitrate were studied by injecting an enriched $^{2}H_2O$ and $Ca(^{15}NO_3)_2$ solution into the soil volume at three different depths (1.1, 2.3 and 3.5 m), repeated in four chambers for each depth. 4.35 g $Ca(^{15}NO_3)_2$ (>98 at% ^{15}N) was mixed with 600 ml $^{2}H_2O$ (^{2}H content = 99.94%) and 600 ml distilled water. The following assumptions were made to determine the amount of tracer added: 1) the soil volumetric water content is no less than 15% and soil contains 50 kg N ha^{-1}; 2) the abundance of ^{2}H and ^{15}N in the pre-labelled soil pool is natural; 3) 10% of the ^{2}H and ^{15}N injected at a specific soil depth is taken

Table 1
Characteristics of soil used in the rhizoboxes (Rasmussen et al., 2020b).

Depth (m)	Organic matter (%)	Clay(%) <0.002 mm	Silt(%) 0.002–0.02 mm	Fine sand (%) 0.02–0.2 mm	Coarse sand (%) 0.2-2 mm	pH
0–0.25	2.0	8.7	8.6	46.0	35.0	6.8
0.25–4.00	0.2	10.3	9.0	47.7	33.0	7.5

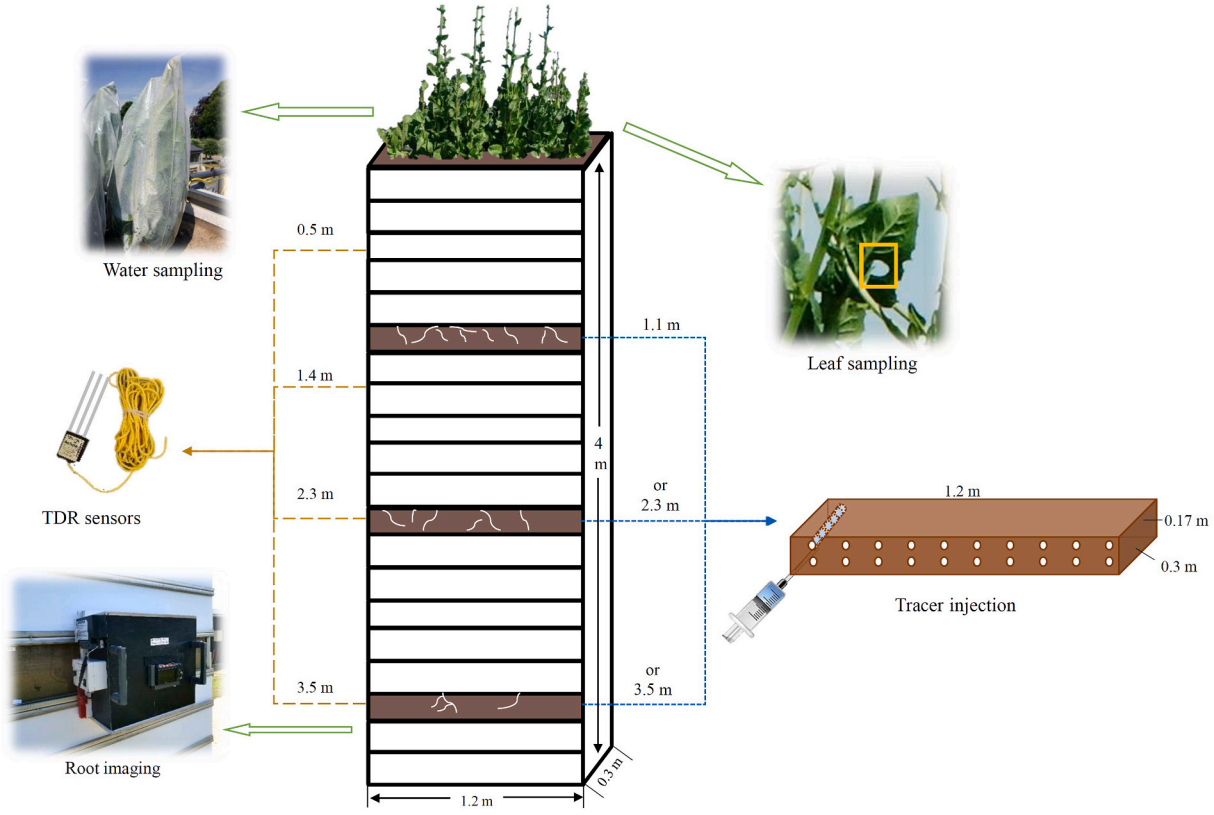

Fig. 1. Schematic drawing of a single chamber from the rhizobox facility and main activities conducted in this experiment. The yellow dashed lines indicated depths where the TDR sensors were installed. Tracer injection was conducted at 1.1, 2.3 or 3.5 m, as blue dashed lines indicated.

up, and 4) tracer distributes evenly at the targeted depth, with a labelled soil volume of 61.2 L. A 100 ml mixture of $^{2}H_2O$ and $Ca(^{15}NO_3)_2$ was injected per injection layer. At the selected soil layer, two parallel rows of ten injection points distributed 10 cm apart were made using a steel rod, resulting in a total of 20 holes. In each hole, the tracer solution was injected at five different points, giving an even distribution of the tracer mixture in 100 injection points in total for each injected depth (Fig. 1). Each point received 1 ml of the mixture. The injection was conducted between 13:00 and 16:00 on May 29, 2018. The injection of ^{15}N and ^{2}H was proven to significantly increase the fraction of ^{15}N and ^{2}H in the targeted depths (Table 2).

Collection of transpiration water and leaf samples to capture tracer uptake signals was conducted in the morning right before the injection as a control, and five, ten, twenty days after the injection. The transpiration water collection method has been validated previously (Calder 1992; Lambs and Saenger 2011; Beyer et al., 2016). From 9 to 11 a.m. on a sampling day, all plant biomass of each target plant was covered with a plastic bag and tightened by rubber bands at the bottom (Fig. 1). Transpiration water was collected 2 h later as droplets of condensed water gathered inside the bags. The water was transferred from bags to sealed plastic bottles. At the end of the experiment, all transpiration water samples were filtered with 2 μm filter paper to remove any leaf fractions, pollen and dust. Filtered water from all plants grown in the same chamber was pooled into one sample.

For ^{15}N analysis, leaf samples were collected by using a puncher with a diameter of 9 mm on the third to fifth leaves from the top on the same day as the transpiration water sample collections (Fig. 1). Two to three pieces of leaf samples were collected from each plant. Leaf samples from the same chamber were mixed and dried at 70 °C over 48 h to constant weight.

2.4. Soil water content and water uptake

Time-domain reflectometry sensors (TDR-315/TDR-315 L, Acclima Inc., Meridian, Idaho) were installed at four depths (0.5, 1.4, 2.3 and 3.5 m) and soil volumetric water content (VWC; %) was recorded every 10 min on a datalogger (CR6, Campbell Scientific Inc., Logan, Utah).

In November, the amount of precipitation fully saturated the soil column. Field capacity (FC) was estimated in each 1 m layer as the mean of VWC, three days after the highest VWC occurred. Assuming there is little water movement when the soil water content is below FC, the measured changes in VWC were used as an approximation of plant water uptake. As irrigation events triggered water movement in the soil (Fig. 2), only VWC data from the periods between irrigation were used,

Table 2
Soil volumetric water content, soil NO_3–N content, original and estimated δ^2H and $\delta^{15}N$ of targeted depths. Soil water content, NO_3–N content and $\delta^{15}N$ in non-enriched soil were measured right before injection.

Depth (m)	Soil volumetric water content (%)	Soil NO_3–N content (mg N kg^{-1} dry soil)	Estimated δ^2H in non-enriched soil water (‰)	Estimated δ^2H in enriched soil water (‰)	$\delta^{15}N$ in non-enriched soil (‰)	Estimated $\delta^{15}N$ in enriched soil (‰)
1.1	16.7	0.4	−36.9	31356.7	55.0	405548.8
2.3	16.5	0.4	−36.9	31737.2	137.4	405548.8
3.5	17.9	0.5	−36.9	29289.0	162.0	326425.6

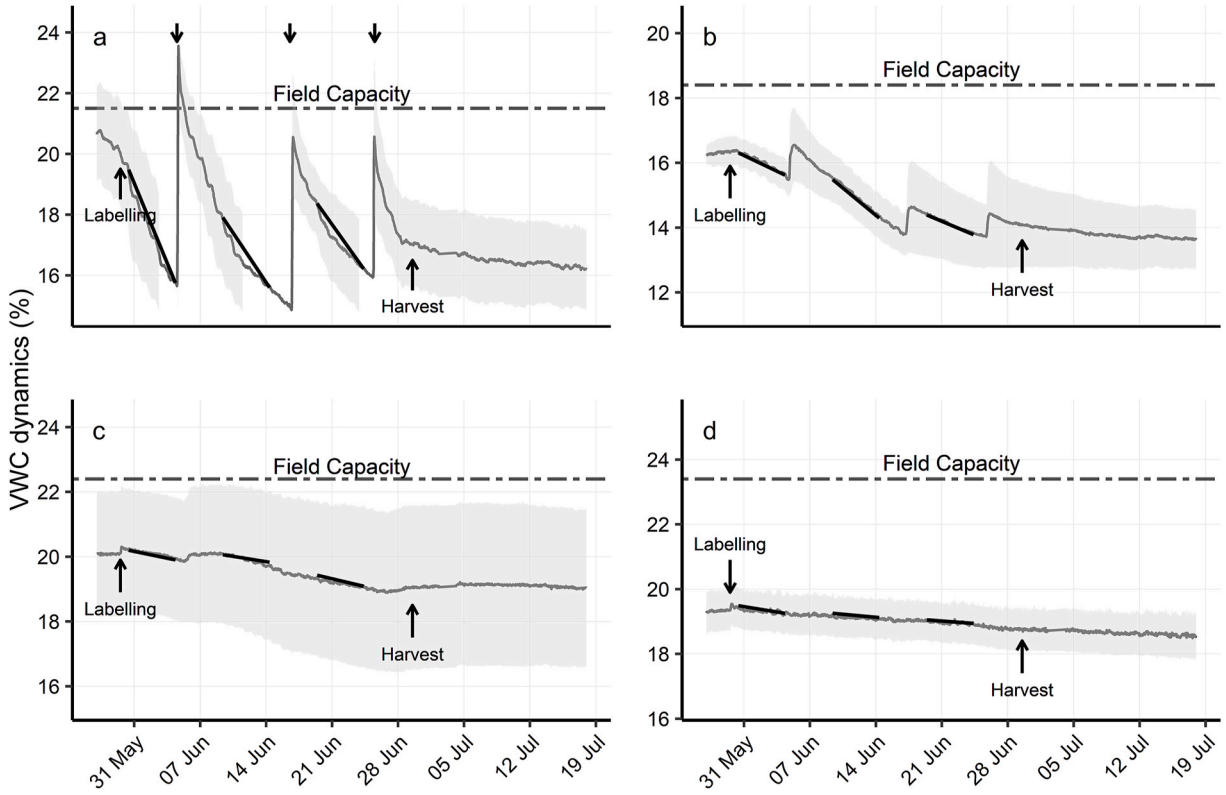

Fig. 2. Soil volumetric water content (VWC; %) dynamics at (a) 0.5, (b) 1.4, (c) 2.3 and (d) 3.5 m depths from 27 May to 18 July in 2018. Data was collected from TDR sensors at the corresponding depths. Data from 0.5 to 1.4 m depths were used to estimate VWC changes at 1.1 m depth. Irrigation events can be seen as peaks most clearly in (a). The segments represented periods that were selected to calculate daily water uptake in Fig. 6. Field capacity data were obtained subsequently, using data measured three days after soil columns were fully irrigated during November in the same year. Bands around the lines denote standard errors (n = 4).

avoiding any data in the first three days after irrigation and waiting until the sensor data indicated that irrigation triggered water movement had stopped. Three five-day intervals, 30 May to 4 June, 9 to 14 June, and 19 to 24 June, were chosen to estimate the average daily water uptake during the labelling period. For calculating daily plant water uptake estimates, each of the four sensors was taken to represent water content in a 1 m soil layer, thereby dividing the whole 4 m soil column into four 1 m sub-columns. Total water amount (W_a; mm m^{-1} soil column) in each sub-column was converted from VWC (Wv; %),

$$W_a = \frac{W_v \times V}{S} \times 1000 \quad (1)$$

where V and S are the volumes and bottom surface area of the sub-column. The daily average decrease of water in each sub-column in the five-day period was interpreted as daily water uptake (mm m^{-1} soil column day^{-1}). The simplification of getting water uptake from changes of VWC has been used in previous studies (Gaiser et al., 2012; Rasmussen et al., 2020a).

2.5. Root measurements

A digital camera (Olympus Tough TG 860) was used to record root growth on the surface of rhizoboxes via transparent acrylic windows. The camera was placed on a half-closed plywood box, with internal LED light strips as a light source (Fig. 1). It was designed to slide along the metal frames of each panel when the PVC boards were removed. With this camera box, four photos that covered the full area of the panel were taken on all 20 panels of each rhizobox chamber. During the experimental period, root imaging was done three times in total.

Root intensity (root intersections m^{-1} line) at each depth was calculated by using the line intersect method. The method was developed by Newman (1966), then modified by Marsh (1971) and Tennant (1975). It has been successfully used in minirhizotron and rhizobox studies previously (Kristensen and Thorup-Kristensen, 2004; Rasmussen et al., 2020b). In this experiment, the root images were covered from wide panels with 20 × 20 mm grids, and the total length of lines per panel was 3.97 m. Images from the first panels were excluded, as the upper part of the panels were exposed to sunlight due to the sinking of the soil, which gave us low-quality images. Narrow-panel images were also excluded as the soil there was disturbed a lot by injection, soil sampling, etc. In the rest panels, root intensity was recorded by counting the total number of roots intersecting the lines at each panel.

2.6. Soil and isotopic analyses

Soil samples from the injection layers were collected twice to compare soil mineral N and ^{15}N enrichment before tracer injection and at the end of the experiment. 20 g sub-samples of soil from each sample was mixed with 100 ml 2 M KCl solution. The solution was shaken for 1 h and filtered through 2 μm filter paper. After filtering, the samples were frozen.

At the end of the experiment, all collected biomass samples were weighed, milled, and encapsulated. ^{15}N concentration in solid and soil solution samples was analyzed using a continuous-flow isotope ratio mass spectrometer (IRMS). Mineral N content in the frozen soil samples was analyzed as well. δ^2H in transpiration water samples was analyzed using a Laser Water Isotope Analyzer V2 (Los Gatos Research, Inc., Mountain View, CA, USA). All analyses mentioned above were done at the UC Davis Stable Isotope Facility.

^2H and ^{15}N values were assumed to be present in samples with delta notation (δ). Definition of δ has been given by Coplen (2011):

$$\delta = \frac{R_{sample}}{R_{standard}} - 1 \quad (2)$$

In eq. (2), for δ^2H calculation, R_{sample} is $^2H/^1H$ ratio in samples and $R_{standard}$ here is Vienna standard mean ocean water (≈1/6412); for $\delta^{15}N$, R_{sample} is $^{15}N/^{14}N$ ratio in samples and $R_{standard}$ here is 0.003676467.

In this paper, ^2H and ^{15}N enrichment (‰) were calculated as the increase of $\delta\ ^2H$ and $\delta\ ^{15}N$ from pre-tracer sampling to post-tracer sampling.

2.7. Statistics

Data were collated and plotted using R (Version 3.5.3, R Core team 2019). The combined effect of sampling date and depth on root intensity was tested in a two-way ANOVA. To test the differences in root intensity among injected depths during the experimental period, a linear mixed model was used, where the depth was the fixed factor and the chamber was a random factor. Analyses of covariance (ANCOVA) was conducted to test depth and date mixed effects on ^{15}N enrichment in leaf and ^2H enrichment in transpiration water, with $\delta^{15}N/^2H$ in samples before labelling as a covariate.

Linear mixed models were used to test the main effects of soil mineral N concentration and ^{15}N and soil, with sampling date and injection depth as fixed effects, and the chamber was included as a random effect. One soil sample, which was sampled at 3.5 m depth on 16 July, was removed due to an unexpected high ^{15}N value compared to the others (atom% was 30% while other replicates were lower than 5%). The main effects of time and depth on daily water uptake was tested using a linear mixed model. Chamber was included as a random factor.

For ^2H enrichment and soil $\delta^{15}N$ analysis, data were log-transformed to fulfil assumptions of normality and homogeneity. Multiple comparisons (Tukey HSD; $P \leq 0.05$) were done based on values derived from linear mixed models, ANOVA or ANCOVA.

3. Results

3.1. Root intensity

Roots were present in the entire soil profile of the 4 m deep rhizoboxes under the one year old chicory plants before the time of injection (Fig. 3a). Root intensity declined with depth, with only few roots observed below 3.5 m. Additionally, there was a tendency towards a decline in root intensity during the experimental period in the upper 3 m of the soil (Fig. 3a), but the decline was not significant at any specific depth. To make sure labelling would not affect root growth, we tested differences of root intensity between labelled and non-labelled soil layers at the same depths, and no significant differences were found.

During the labelling period, the highest root density among all three injection depths was 3.9 intersections m^{-1} at 1.1 m depth, and the lowest was 0.3 intersections m^{-1} at 3.7 m depth (Fig. 3b), while root density was intermediate at 2.1 m depth with 1.7 intersections m^{-1}. Root intensity at 1.1 m was significantly higher than at the other two labelled depths.

3.2. ^2H and ^{15}N enrichment

On the first two sampling days, as well as five and ten days after injection, ^2H enrichment of transpiration water was significantly lower when the tracer was injected deeper (Fig. 4a). The enrichment after

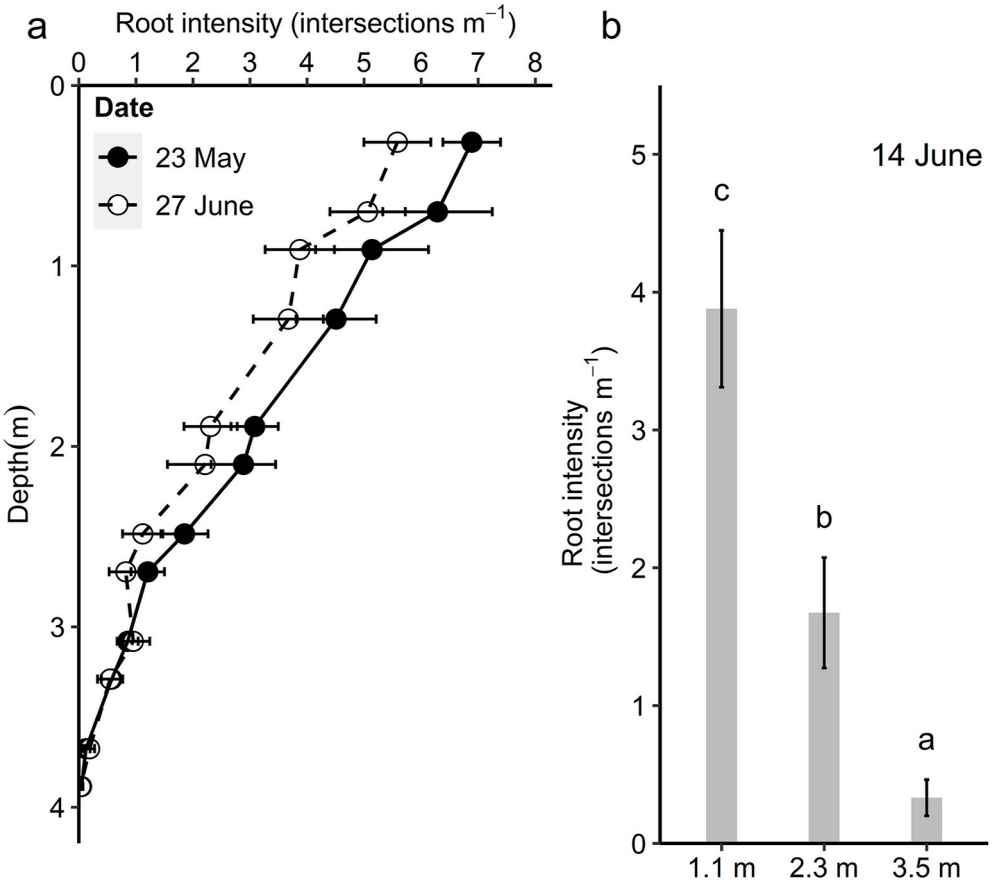

Fig. 3. (a) Root intensity measured on 23 May (six days before tracer injection) and 27 June (two days before harvest) in 2018. (b) Root intensity at three injected depths on 14 June. Root intensity at the injected depth was estimated based upon averages of root intensity at soil layers 0.2 m below and above injected depths. Error bars denote standard errors among all chambers where we injected tracers at different depths (n = 12). Mean values are shown here (±SE).

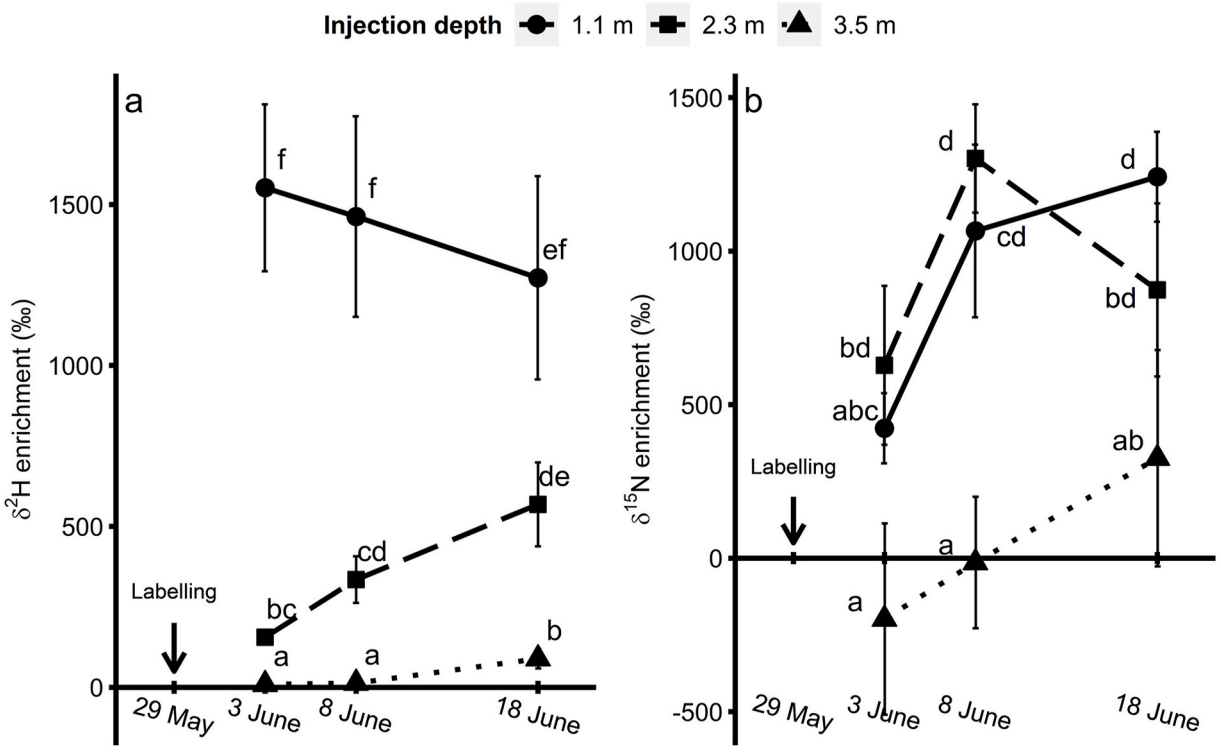

Fig. 4. (a) ^{2}H enrichment in transpiration water and (b) ^{15}N enrichment in leaf samples measured five, ten, twenty days after tracer injection at 1.1, 2.3, 3.5 m of soil depth. Mean values are shown here (±SE). Error bars denote standard errors (n = 4), and letters indicate significant differences across all the treatments ($p < 0.05$).

injection at 1.1 m depth was 1552‰, nearly 10 times higher than after injection at 2.3 m depth, which was 156‰. Almost no enrichment was observed after injection at 3.5 m depth. Furthermore, the time course of ^{2}H enrichment of transpiration water was affected by the injection depth, as the increase in enrichment was delayed by deeper injection. After injection at 1.1 m, maximum enrichment was observed already at the first sampling date, followed by a non-significant tendency to a decline later. After injection at 2.3 m, a significant increase over the sampling times was observed, and after injection at 3.5 m, no effect was observed until the last sampling date when a slight but significant

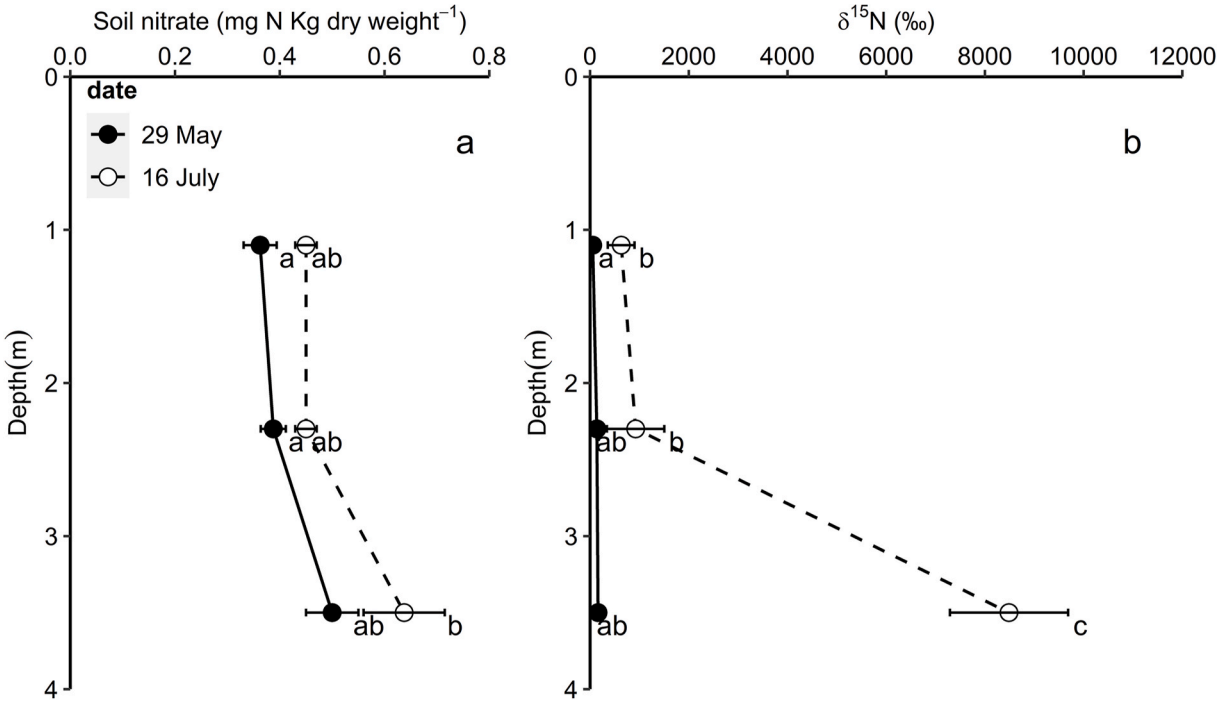

Fig. 5. (a) Soil nitrate concentration and (b) $\delta^{15}N$ at the three injection depths right before injection (29 May), and after final sampling (16 July). Mean values are shown here (±SE). Error bars denote standard errors (n = 4; in Fig. 5b and 3.5 m, 16 July, n = 3), letters indicate significant differences across all the treatments ($p < 0.05$).

increase in enrichment was seen (Fig. 4a).

The ^{15}N enrichment of leaf samples showed a somewhat different result, and the differences observed were smaller, leaving fewer significant differences (Fig. 4b). The ^{15}N enrichment of leaves after injection at the two upper layers showed similar results. Unlike the ^{2}H results, enrichment from 1.1 m to 2.3 m injections were high already at the first measurement date. As with ^{2}H, no enrichment was observed on the first two sampling dates from injection at 3.5 m depth, and only a non-significant increase was seen at the last date. Ten days after injection, ^{15}N enrichment from 1.1 to 2.3 m were significantly higher than from 3.5 m. However, twenty days after injection, there was no significant difference between ^{15}N enrichment from 2.3 m to 3.5 m.

3.3. Soil nitrate concentration and $\delta^{15}N$

As ^{15}N labelling provided an extra nitrate source, the soil nitrate concentrations tended to be higher at all depths after the experiment than before injection (Fig. 5a). Soil nitrate concentration measured before and after the injection showed smaller net increases of 0.09, 0.06 and 0.15 mg kg^{-1} at the three depths, respectively, but the effect was not significant.

The soil ^{15}N values also tended to be higher after ^{15}N injection at all depths (Fig. 5b), but the increase was much stronger at 3.5 m depth than at 1.1 and 2.3 m depth, and only significant there. Before the injection, soil δ ^{15}N values were relatively low and there were no differences among the depths.

3.4. Water uptake

The soil dried out gradually during the experiment at all four depths where water sensors were placed. While infiltrated water from irrigation events (Fig. 2) reached 2.3 m depth and caused an increase of soil water content right after irrigation events, soil water at all depths decreased continuously due to plant water uptake. At all depths, the water uptake after harvest was negligible, indicating that plants stopped extracting water from these depths.

During the selected five-day intervals, the volume of absorbed water decreased with increasing depth (Fig. 6). These intervals started at least two days after irrigation to avoid over-estimation of soil water content. Among all three periods, plants absorbed the most water from the uppermost 1 m of the soil. Within 1–6 days after labelling, the average daily water uptake by plants from 0 to 1 and 1–2 m soil column was 7.2 mm and 1.4 mm. At the same time, plants took less than 1 mm of water per day from the 2–3 and 3–4 m soil layers. Daily water uptake from 0 to 1 m decreased to 4.4 mm at 11–16 days after labelling, and after 21 days, plants still acquired more than 4 mm water from 0 to 1 m soil per day. Daily water uptake from 1 to 2 and 2–3 m tended to be higher in the middle of the labelling period and decreased thereafter, although none of these changes was significant.

4. Discussion

Fewer chicory roots were observed in deeper layers than were found in previous studies (Sapkota et al., 2012; Thorup-Kristensen and Rasmussen, 2015). However, despite the fewer roots, considerable water and N were taken from the soil below 1 m by chicory. Using a dual labelling technique, the work presented here successfully showed the short term uptake potential and dynamics of deep water and N. In this experiment, the root water uptake was found to be more reduced with increased depth and declined root density compared with N uptake. In addition, in the labelling period, ^{15}N tended to be exploited more rapidly than labelled water. The discrepancies between water and N uptake may be caused by various factors, which will be further discussed below.

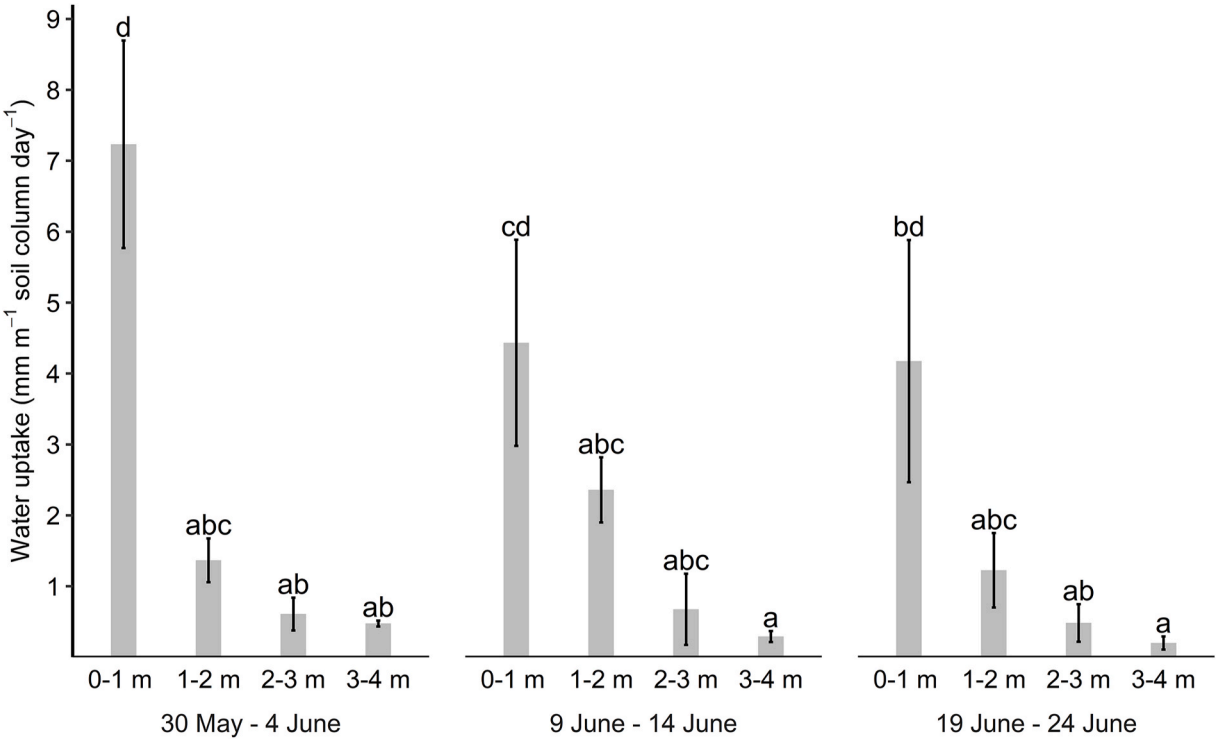

Fig. 6. Mean daily water uptake from 0 to 1, 1–2, 2–3 and 3–4 m depths after labelling. The daily decrease in soil volumetric water content per meter soil column was interpreted as daily water uptake. Soil volumetric water content per meter soil column was recorded by the TDR sensor located in the column. After isotopic labelling at given depths, the soil water content data of corresponded soil columns was collected. Data from 0 to 1 m and 1–2 m helped estimate water uptake from the depth where tracers were injected at 1.1 m. To avoid the effect of irrigation, daily water uptake from each depth was calculated as averages of three five-day periods (30 May to 4 June, 9 to 14 June, and 19 to 24 June), respectively. Error bars denote standard errors (n = 4), letters indicate significant differences across all the treatments ($p < 0.05$).

4.1. Deep water uptake

Water in soil moves with various processes, e.g. infiltration, redistribution, evaporation, plant uptake and drainage (Hillel 1980). Although the isolation of plant uptake from other processes is complicated, we are trying to simplify the processes with the current experimental setup to get an idea of deep water uptake. To avoid the effect of irrigation on water movement, we only selected the periods at least three days after irrigation for water use observations. Assuming there is little water movement caused by evaporation and drainage in wet, deep soil layers in rhizoboxes, details of deep water uptake from different depths can be obtained. ^{2}H enrichment of transpiration water decreased significantly with decreasing root intensity and increasing soil depth. Based on our estimated calculations, daily water uptake from the top 1 m soil reached 7 mm m^{-1} d^{-1} while less than 1 mm m^{-1} d^{-1} water was taken from 2 to 3 m soil during the experimental period. Although several studies have shown that deeper roots allow water acquisition from subsoil (White and Kirkegaard 2010; Gaiser et al., 2012; Cutforth et al., 2013), their limited ability to take up water can be a general feature, as indicated in the present results. Compared with topsoil, subsoil is hard for roots to penetrate, thus there are fewer roots in deep soil layers. The roots that can keep elongating in these conditions also prefer to grow in pores and cracks, which would lead to poor root-soil contact, making it harder to obtain water (White and Kirkegaard 2010). Further, due to higher proportions of immature young roots, roots in deep soil layers generally have higher axial resistance and therefore do not extract water as efficiently as old, shallower roots (Garrigues et al., 2006; Pierret et al., 2006).

4.2. Deep N uptake

^{15}N enrichment in leaves together with ^{15}N left in the soil after harvest indicated little N uptake from 3.5 m, probably as a consequence of the low root intensity. ^{15}N signals were seen in leaves five days after tracer was injected at 1.1 and 2.3 m, and no significant differences were seen for nitrate uptake from 1.1 to 2.3 m. Plant N uptake is affected by soil N availability (Kulmatiski et al., 2017) and plant uptake capacity (Robinson 1986). The plant uptake capacity is further determined by the interactions of plant N demand and root uptake capacity. At the two upper layers, where more roots were found than in the deepest layer, ^{15}N absorption occurred at high rates shortly after the injection. Even a relatively low root intensity at 2.3 m in our experiment was as efficient for N uptake as the higher root intensity at 1.1 m. Similarly, efficient deep N uptake was also found previously, e.g. Thorup-Kristensen (2006a, 2006b). Twenty days after injection, soil ^{15}N had been depleted in upper layers, leading to a decrease of ^{15}N in the young leaves sampled for ^{15}N analysis. We also noticed a gradual increment of ^{15}N enrichment in plants injected at 3.5 m at the same time. In the past few years, while root N uptake is often studied at the level of transporters and root systems (Rowe et al., 2001; Nacry et al., 2013; Kulmatiski et al., 2017), the intrinsic variation of N uptake among root segments has rarely been studied. Our results showed that N uptake may differ within a root system with time and N availability. When N is available in the soil, as it was in the first few days of labelling in our experiment, root length and uptake rate are limiting factors for N uptake. When N is gradually moved by plants from the soil, the availability rather than root length becomes the limiting factor. This explained the decreasing uptake from the top two layers and the lagging absorption from the deepest layer.

4.3. The disparity in the uptake of water and N

Due to the different sampling methods, the isotopic results of water and N were not directly comparable. By sampling young leaf material for ^{15}N analysis, the results include the effect of accumulation of ^{15}N in the plant material over time, contrary to the real-time isotope enrichment as ^{2}H enrichment in transpiration water. Also, the water and N content of injected depths are only partly comparable in the results, as there were no TDR sensors at 1.1 m. Nevertheless, we still conclude that there are discrepancies in water and nitrogen uptake. Soil $\delta^{15}N$ and plant enrichment indicated more rapid uptake of labelled nitrogen than water from the subsoil, especially from 2.3 m depth, which supported our hypothesis (2) that the dynamics of deep water and N differ. We observed low but increasing content of ^{2}H water in transpiration water from 2.3 to 3.5 m 20 days after injection, showing that labelled water remained in the soil, and was taken up at gradually increasing rates. ^{15}N enrichment in leaves showed insignificant changes 10 days after injection. Considering that little labelled N was left at 1.1 and 2.3 m, we concluded that a large proportion of labelled N was taken from these two depths during the first 10 days.

Our results are consistent with McCulley et al. (2004), who suggested nutrient uptake as a contributing explanation for the occurrence of deep roots. Instead of taking water directly from deep soil layers, deep roots played a more important role in altering water and nutrient distribution in the soil profile via hydraulic redistribution (McCulley et al., 2004). Here, we further examined the extent of water and N uptake at different depths and proposed several explanations on their uptake disparities. Firstly, the radial and axial resistances mentioned above may inhibit root water uptake from subsoil but no evidence has been shown for similar inhibition of nitrate uptake. Secondly, the water supply from the topsoil may have been sufficient to supply most of the water demand by the plants with repeated irrigation to the topsoil, while the N demand by the plants exceeded the topsoil supply, leading them to deplete all available soil layers. In deep soil layers where the transpirational force is absent, N may still move to the roots by diffusion (Comerford 2005; Plett et al., 2020). Moreover, from the molecular aspect, when plants are exposed to N limitation, the capacity of high-affinity transport systems (HATS) would be upregulated to improve the N uptake efficiency (Nacry et al., 2013). These mechanisms allow continuous N uptake from the deep soil layers, even when little water is taken up from there. Thirdly, within the same root system, the root water uptake potential of different segments are non-uniform. Upper roots near the soil surface were found to have higher radial and axial fluxes, which benefit both root water uptake and transport (Zarebanadkouki et al., 2013). Conversely, although nitrate uptake kinetics may also vary within root systems, the maximum influx rate of nitrate of root segments was most affected by plant age and nitrate deprivation time, rather than their position (York et al., 2016). This could explain why we observed less and slower water uptake from the lower layers, while the uptake rate of N in the top two layers did not differ significantly.

4.4. Methodological considerations in deep root studies

^{2}H and ^{15}N labelling is a promising way to study the dynamics of water and nitrate uptake (Calder 1992; Kahmen et al., 2008; Bakhshandeh et al., 2016; Kulmatiski et al., 2017). However, there are some inevitable problems when the technique is used in deep root studies. As ^{2}H and ^{15}N are highly mobile in the soil, they can move freely with water movement. In previous studies, ^{2}H moved 0.1 m up along the soil profile in a mesic savanna after one week of tracer injection at 1.2 m (Kulmatiski et al., 2010), while capillary rise transported ^{2}H at distances between +0.1 m and −0.05 m from 1 m in 35 days (Grunberger et al., 2011). Furthermore, there was a clear sign that a small amount of injected ^{2}H can move with the transport of water vapour in a longer period (Beyer et al., 2016). Thus, for labelling with these mobile resources, short time intervals between labelling and uptake measurements are preferable, to be certain that the tracer was taken up at approximately the same depth where it was injected. In addition to the inconsistencies in sampling methods mentioned above, the short active period of the deepest roots makes it even harder to choose the right labelling time. To study nitrate uptake from the deepest roots, ^{15}N labelling has to be done when the roots reach the deepest layers, which usually is at the late growth stages. Since nitrate uptake decreases after

flowering (Fischer 1993; Imsande and Touraine 1994), labelled ^{15}N accumulation in leaves can be relatively low if the isotope is applied after flowering. To obtain more precise results, destructive sampling is preferable, so actual ^{15}N uptake, rather than just ^{15}N enrichment can be determined, but this will require a higher number of treated plots, generally not possible in deep root studies.

Considering the complexities of root studies, several models have been developed and used in simulating resource uptake from soil (Ma et al., 2008; Kumar et al., 2015). However, deep root resource uptake has rarely been considered in soil-crop models. As we observed significant water and nitrogen uptake below 2 m, this should be included in future soil-crop models. Heterogeneous uptake among different parts of roots is often not well accounted for in the models (Rengel 1993; Javaux et al., 2013), and uniform estimations for the whole root system may lead to over- or under-estimation of uptake, especially for water uptake. We expect that our results can be used to better characterize the parameters in future simulations.

Previous studies already showed the substantial value of deep roots for resource uptake (Kristensen and Thorup-Kristensen, 2004; Rasmussen et al., 2020a). Our findings not only confirmed the contribution of deep roots to water uptake but further indicated their potential to uptake N is considerable as well. This potential can be valuable in maintaining crop productivity, especially under drought stress, where water and N uptake in topsoil can be both limited. However, we still lack the understanding of where and when the deep roots are active, and how efficient they can be. This is crucial information needed to improve deep resource use efficiency, as there are few roots in the deepest soil and they are only active within a short period. Here, with the help of isotope labelling, we have successfully looked into detailed uptake dynamics and proved that this method can be used in further studies.

In conclusion, we confirmed that deep-rooted chicory plants can take water and nitrate from the subsoil, and documented uptake to a depth of 3.5 m, but with different efficiency and dynamics. Compared with N, the root water uptake is prone to decrease with increased depth and fewer roots. These findings extend our previous observations on deep water and nitrogen uptake, and are meaningful for model calibration as well.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix II: Dynamics of deep water and nitrate uptake under varied nitrogen and water supply

Photo: Tower soil sampling, from Guanying Chen

Dynamics of deep water and nitrate uptake under varied nitrogen and water supply

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Abstract

Background and Aims: Enhanced nitrogen (N) and water uptake from deep soil layers may increase resource use efficiency whilst maintaining yield under stressed conditions. Winter oilseed rape (*Brassica napus* L.) can develop deep roots and has the potential to access deep-stored resources such as N and water, while this potential has large uncertainties in variable environments. In this study, we aimed to evaluate the effects of reduced N and water supply on deep N and water uptake.

Methods: We examined biomass production, root growth, water, and N uptake of winter oilseed rape in two consecutive years. Oilseed rape plants grown in outdoor rhizotrons were supplied with 240 kg N ha^{-1} or 80 kg N ha^{-1} in 2019 and were treated with a well-watered or a water-deficit treatment in 2020. To track deep water and N uptake dynamics, a mixture of $^{2}H_2O$ and $Ca(^{15}NO_3)_2$ was injected into the soil column at 0.5 and 1.7 m depth in both years. ^{2}H in transpiration water and ^{15}N in leaves were measured four times after injection. At the end of the experiments, $\delta^{15}N$ and $\delta^{2}H$ values in biomass samples were also measured.

Results: Differences in N or water supply showed little effect on root growth. The high N treatment enhanced water uptake throughout the soil profile, and caused significant reductions in ^{15}N uptake efficiency at both 0.5 and 1.7 m. Water deficit in the upper soil layers led to compensatory water uptake from deeper soil layers, while the N uptake was not altered by soil water status.

Conclusion: Our findings demonstrate that root growth is not always tightly connected to N and water supply. We also show that for winter oilseed rape, high N application and water deficiency in shallow layers increases deep water uptake, and that the efficiency of deep N uptake is mainly sensitive to N supply rather than water supply.

Keywords *Brassica napus*, deep root, nitrogen use efficiency, water uptake, dual-labelling

Introduction

Nitrogen (N) and water are main factors that can be modified in agricultural production and have been widely documented for their crucial roles in determining yield (Mueller et al. 2012; Sinclair and Rufty 2012). Inadequate supply of N and water leads to yield loss, while the loss of N from agricultural land also causes environmental problems. Therefore, improving water and N use is crucial for sustainable agricultural production.

Deep-rooted crops have shown great potential in enhancing soil N and water uptake, as well as improving yield (Wasson et al. 2012; Thorup-Kristensen et al. 2020a), however, the development and function of deep roots are highly sensitive to the external environment (Lynch and Wojciechowski 2015). Management and environmental factors such as N or water supply affect plant growth, soil N and water availability, and subsequently affect root uptake. N supply affects overall plant growth and yield (Asare and Scarisbrick 1995; Khan et al. 2017), but the current findings of the effects of N supply on root growth and N uptake seem ambiguous. Increasing N supply stimulates root growth either via more robust shoot growth or as a consequence of increased soil N availability (Hodge et al. 1999). In contrast, Svoboda and Haberle (2006) found that the rooting depth and deep root density of winter wheat (*Triticum aestivum* L.) could be reduced when more N fertilizer was given. Although plant root surface area and total N uptake may be enhanced by a higher fertilization rate (Lynch et al. 2012), it has been reported that nitrogen uptake efficiency in oilseed rape (*Brassica napus* L.) and winter wheat can decline with increasing N fertilizer rate (Rathke et al. 2006; Rasmussen et al. 2015). Riar et al. (2020) found that, independent of irrigation, oilseed rape plants fertilized with 100 kg N ha^{-1} had higher N uptake efficiency than those fertilized with 200 kg N ha^{-1}.

Nitrogen supply is important for improving water use. Increased N application may result in higher water use efficiency of wheat and oilseed rape (Taylor et al. 1991; Waraich et al. 2011), possibly due to improved shoot growth. Compared with no or less fertilization, adequately fertilized crops usually grow more vigorously and have larger leaf areas, increasing transpiration and decreasing soil evaporation. Increasing N supply increases transpiration intensity under normal water supply, thus enhancing water use (Li et al. 2009).

Water availability controls crop growth, especially canopy development, but it also controls root growth. Bloom et al. (1985) hypothesized that plant root growth may be stimulated under drought to enhance or maintain the capacity for acquiring water. Accordingly, Vandoorne et al. (2012) found that although total root length of chicory (*Cichorium intybus* L.) decreased under water deficit

conditions, the overall root profiles developed deeper and triggered compensation from wetter and deeper horizons. Furthermore, both Li et al. (2011) and Álvarez et al. (2011) observed a significant increase in water use efficiency during water deficit.

In addition to altering the root growth and water acquisition, water status affects plant N uptake in various ways. Under the same fertilization rate, irrigation enhanced soil moisture and further improved oilseed rape's N uptake efficiency and N use efficiency by 40% (Riar et al. 2020). Water deficit also affects nitrogen demand, nitrogen availability and nitrogen assimilation and partitioning (Sadras et al. 2016). The reduced shoot growth driven by water deficit reduces plant nitrogen demand and tends to decrease nitrogen use efficiency if N input has not been reduced correspondingly (Quemada and Gabriel 2016). The availability and supply of soil N can be limited by soil dryness due to reduced soil organic N mineralization (Jensen et al. 1997) and restricted nitrate movement by both mass flow and diffusion (Plett et al. 2020). Water deficit could also diminish nitrate reductase activity, hence reducing plant N assimilation (Gonzalez-Dugo et al. 2010). Moreover, assimilates tend to translocate to the roots rather than the shoots in the case of water deficiency (Li et al. 2011). In summary, both N and water supply can affect root growth, water, and N use.

Deep roots are not assumed to be as efficient as shallow roots in N and water uptake, as the roots reach the subsoil layers late in the growing period and are not able to develop as high densities as in the topsoil. Previous studies indicate that water deficiency in topsoil can increase deep water uptake (Kirkegaard et al. 2007) and that decreased N supply in topsoil increased deep nitrogen uptake (Kuhlmann et al. 1989; Haberle et al. 2006). However, it is less clear to what extent deep root growth, as well as deep uptake of N and water, are affected by the total N and water availability for a crop. Studies of deep root growth, water, and N use under different N and water regimes could increase our understanding of the functions of deep roots. In addition, this would increase the understanding of the contribution of deep roots to crop N and water supply and to reduce N leaching losses, and how this is affected by crop management.

Oilseed rape is known for its high capacity for N and water uptake and has the potential to develop roots in soil layers below 2 m (Dresbøll et al. 2016; Kirkegaard et al. 2021). In this study, we used oilseed rape as the model crop and examined how N and water supply affect the root growth, utilization of N and water from deep soil layers, and N and water uptake dynamics in the subsoil. It was hypothesized that (I) N and water deficiency in topsoil stimulate root growth in deeper soil layers; (II) Greater N availability in the upper soil layer improves water uptake, but reduces N uptake from

the subsoil. (III) Higher water availability in the upper soil layer improves the N uptake from the whole soil profile, but decreases water uptake from the subsoil.

Materials and methods

Experimental facility

Two consecutive experiments were conducted in the seasons 2018/2019 and 2019/2020 using the rhizobox facility (Thorup-Kristensen et al. 2020b) at the University of Copenhagen in Taastrup, Zealand, Denmark (55˚40′ N; 12˚18′ E). The facility consists of rhizoboxes that allow observations of root growth and root activity down to 4 m depth. The growth medium was field soil. Both years the topsoil was replaced right before planting (Table 1). The rhizoboxes are rectangular columns of 1.2 × 0.6 m, divided into an east- and a west-facing chamber, each with a surface area of 1.2 × 0.3 m. The front of the chambers is divided into 20 panels by metal frames covered by removable white foamed PVC boards, allowing root observations through transparent acrylic boards. The acrylic boards can be removed for sampling and measurements that require direct soil contact. For further details on the facility, see Rasmussen et al. (2020) and (Thorup-Kristensen et al. 2020b).

Experimental design

Oilseed rape (*Brassica napus* L., cv. "Butterfly") plants were sown in the field on August 16, 2018 (Exp. 1) and in pots on August 13, 2019 (Exp. 2) before being transplanted to the rhizoboxes on October 8, 2018 and August 26, 2019, respectively. Due to a pest infestation *(Delia radicum)* in September 2019, a few plants were replaced by spare ones on September 24, 2019. The re-transplanted plants were smaller than the original ones during the entire growing period. Plant density in both years was five plants per chamber, corresponding to 14 plants m^{-2}.

In Exp. 1, two N treatments were established by fertilizing with a nutrient solution, applying nutrients in a high N treatment (N240) equivalent 240 kg N ha^{-1}, 38 kg P ha^{-1}, 192 kg K ha^{-1} ; and a low N treatment (N80) equivalent to 80 kg N ha^{-1}, 13 kg P ha^{-1}, 65 kg K ha^{-1} respectively on March 27, 2019. During this season, all chambers received water through precipitation and irrigation, which were sufficient to keep them well-watered. In Exp. 2, two irrigation regimes were established. Rainout shelters were mounted on top of all chambers on February 26, 2020, to allow complete control of soil moisture by irrigation. Well-watered (WW) chambers were irrigated with 60 mm water on April 14 and again on April 15, 2020 to establish soil profiles with high initial

water content. No more irrigation was given to the well-watered chambers until May 10, 2020. In the following month, the well-watered chambers were irrigated frequently to keep an adequate water supply. Water deficit (WD) chambers received no irrigation during the whole experimental period. In Exp. 2, all chambers were fertilized with in total of 200 kg N ha^{-1}, 38 kg P ha^{-1}, 192 kg K ha^{-1}. Fertilization was divided into three applications, with N supply of 40, 80, and 80 kg N ha^{-1} on September 5, 2019, March 2, and April 1, 2020. The treatments and timeline of the experiments are shown in Table 2. The two treatments in Exp. 1 and 2 were established in six randomly distributed replicates.

^{2}H and ^{15}N labelling

In both experiments, water and N uptake were traced using isotope labelled water and nitrate injected into the soil at either 0.5 or 1.7 m depths. Tracer application was repeated in three chambers for each depth and treatment. Tracers were injected when the roots had already reached 1.7 m depth. The tracer application rates aimed at ensuring significant enrichment in plants and transpiration water, and were based on estimated N and water availability in the soil, the natural isotope enrichment, and assumed uptake rates of applied tracers. In Exp.1 where two N fertilizer levels were established (240/80 kg ha^{-1}), the ^{15}N application was adjusted similarly and the N240 and N80 treatments received 0.96 g and 0.32 g ^{15}N, respectively. In Exp. 2, each chamber received 0.5 g ^{15}N. Tracer solution was prepared by mixing the specific amount of $Ca(^{15}NO_3)_2$ (>98.9 at% ^{15}N) with 50 ml $^{2}H_2O$ (^{2}H content = 99.94%) and 50 ml distilled water for each chamber.

The tracer was injected into 20 injection holes at each injection depth, which were evenly distributed in two parallel rows. The holes were 25 cm deep, made by a steel stick 0.5 cm in diameter. Inside each of the 20 holes, a 5 ml tracer solution was injected. The syringe needle was pushed 25 cm into the soil, and 1 ml of the solution was released every five centimeters as the syringe was drawn back. In this way, the tracer solution was distributed into 100 individual points in the soil at each injection depth. The injection procedures were conducted between 1:00 – 4:00 pm on April 3, 2019 and April 17, 2020.

Sampling and sample preparation

Transpiration water for ^{2}H tracing and leaf samples for ^{15}N tracing was collected five times in each experiment. The first sampling time was in the morning, right before the injection, and

subsequently four times after the injection (Table 2). The collection of transpiration water was initiated between 10:00 and 11:00 on the sampling day. Each plant was covered with a plastic bag that was tightened by a rubber band at the bottom. After two hours, the condensed droplets of transpired water inside the bags were collected. The water was quickly transferred from the bags to sealed plastic bottles. The collected transpiration water was filtered through 2 μm filter paper to remove dirt and debris. Filtered water from all plants grown in the same chamber was mixed for ^{2}H analysis. Three to five of the latest fully developed leaves were collected on the same days as the transpiration water samplings. Leaf samples were dried, weighed, milled, and then encapsulated for ^{15}N analysis. To determine the extent of water deficit, the leaf samples were also analyzed for ^{13}C in Exp. 2.

The total aboveground biomass was collected on June 5, 2019 and June 18, 2020, in Exp. 1 and 2, respectively. Biomass samples were divided into stems, pods, and leaves. However, in Exp. 2, all leaves had been shed when the total biomass was collected. Biomass samples from all plants in each chamber were mixed, dried at 70°C to constant weight, and weighed and stored until further analysis. In both experiments, biomass samples were analyzed for ^{15}N concentration and in Exp. 2 for ^{2}H concentration in the pods too.

Soil samples from 0.5, 1.1, and 1.7 m soil depths were taken before tracer injection and after the last isotope sampling to determine soil nitrate and ^{15}N concentration. All soil samples were frozen immediately after sampling and stored until further preparation. Subsequently, 20 g soil was taken from each sample and mixed with 100 ml 2M KCl solution. The mixture was shaken for one hour and filtered through 2 μm filter paper. All solution samples were frozen for later analysis.

Isotopic analyses

In Exp. 2, the ^{2}H concentration in pods was measured in Silvatech, INRAE using an isotope ratio mass spectrometer (IRMS). All other isotopic measurements were done by the Stable Isotope Facility, UC Davis. ^{15}N and ^{13}C values in biomass samples were analyzed using IRMS. ^{2}H values in transpiration water samples were analyzed using the Laser Water Isotope Analyzer V2 (Los Gatos Research, Inc., Mountain View, CA, USA). ^{15}N concentration in soil samples was measured using IRMS. Nitrate-N content in the frozen soil solution was measured using the flow injection analyzer method.

^{2}H and ^{15}N enrichment (‰) was calculated as the increase of ^{2}H and ^{15}N values from pre-tracer sampling to post-tracer sampling unless otherwise stated. The ratio (%) of 1.7 m - and 0.5 m - derived ^{2}H enrichment in transpiration water in the same treatment was calculated to investigate the distribution of water uptake. To compare ^{15}N uptake between different treatments more directly, ^{15}N uptake efficiency ($^{15}N_{upe;}$ % g^{-1}) was calculated as:

$$^{15}N_{upe} = \frac{x(^{15}N)_{sample} - x(^{15}N)_{control}}{^{15}N_a} \quad (2)$$

where $x(^{15}N)_{sample}$ and $x(^{15}N)_{control}$ are the atom fraction of ^{15}N in post-tracer samples and pre-tracer samples, respectively. In harvest samples, $x(^{15}N)_{control}$ refers to the natural abundance of ^{15}N in plant organs, which is usually 0.366%. $^{15}N_a$ is the total amount (g) of ^{15}N that was added to the soil.

Soil water measurements

Four time-domain reflectometry sensors (TDR-315/TDR-315L, Acclima Inc., Meridian, Idaho) were installed in every chamber. They were placed at 0.5, 1.4, 2.3, and 3.5 m depth, respectively, recording soil volumetric water content (VWC; %) at least every 30 minutes. The VWC sensor readings were calibrated against VWC in soil samples taken in close proximity to the sensors. The samples were taken using metal rings with a diameter and a height of 5 cm and VWC was calculated based on the fresh and dry weight of the samples. For each sensor at least 3 samples were collected at different times aiming a covering a broad range of water content. Based on the correlations between sensor VWC and sample VWC, the sensor readings were adjusted to obtain an intercept of zero. The correlations did not call for a slope adjustment.

Two periods around the middle of the isotope sampling period were selected for estimating the water uptake during the sampling period. In Exp. 1, it was a 20-day period starting from 4 days after injection and ending four days before the last sampling date. In Exp. 2, a 14-day period was selected, which began four days after injection and ended four days before the last sampling date. Letting each sensor represent a 1 m depth-interval the soil water content in each interval was calculated (mm m^{-1} soil column). No water was added during the selected periods, thus soil water movement was assumed negligible and a decrease in soil water content was interpreted as plant water uptake. In both experiments, the daily water uptake was calculated only for the top 3 m soil columns, where most roots were found.

Root imaging, segmentation, and calculation

During the experimental periods, the growth of oilseed rape roots was recorded every three to four weeks with a digital camera (Olympus Tough TG 860). The camera was in a box excluding daylight but with internal LED light strips as the light source. The box fits the frames of each panel of the rhizobox chambers, and by taking five photos per panel, the total area of each panel was photographed for subsequent image segmentation. RootPainter (Smith et al. 2020a) was used to segment roots from the soil background. A model trained with randomly selected images was used to segment roots on all the images and estimate the root length in each image via skeletonization and pixel counting (Smith et al. 2020b). Root intensity was calculated as cm of root per cm^2 of soil in the images.

Statistics

Data were collated and plotted using R (Version 3.5.3, R Core team 2019). The effect of N/water supply on harvest biomass and N content was tested in t-tests for different treatments in the same experiment within the same organ. T-tests were used for comparing root intensity under different treatments at the specific depths in the same experiment. The main effects of N/water supply and depth on daily water uptake were tested using a linear mixed model. A linear mixed model was used to examine differences in $\delta^{13}C$ in leaf samples that were collected from water treatments during the isotope sampling period in Exp. 2, where dates and water treatments were fixed effects and chamber was a random effect. Linear mixed models were used to examine differences in ^{2}H enrichment in water samples and $^{15}N_{upe}$ in biomass samples among N/water treatments, dates, and injection depths, where the combined factor of N/water level and depth (level-depth combined treatment, e.g., N80 - 0.5 m) and dates were fixed effects and chamber was a random effect. Linear models were used subsequently to test for changes in ^{2}H enrichment and $^{15}N_{upe}$ within the same date or the same level-depth combined treatment. Chamber was included as a random factor. In both experiments, the effect of N/water supply on $^{15}N_{upe}$ in harvest samples within the same organ was tested using linear mixed models with level-depth combined treatment as a fixed factor and chamber direction as a random factor.

For ^{2}H enrichment analysis, data were log-transformed to fulfill assumptions of normality and homogeneity. Multiple comparisons (Tukey HSD; $P \leq 0.05$) were based on values derived from linear mixed models.

Results

Biomass

Oilseed rape plants grew well in both years. In Exp. 1, the effect of N fertilization rate was evident, as the N240 treatment resulted in significantly higher leaf, stem, and pod biomass than N80 (Table 3). The N content in all three organs also increased when more N was given.

No significant differences were found in biomass or N content between the water treatments in Exp. 2. Plants that grew under lower soil water content tended to have a lower stem and pod biomass, while the N content in the pod and stem samples at harvest was slightly higher when less water was supplied, although not significant (Table 3).

Root growth

Root growth was recorded from March to June, covering tracer injection and sampling periods in both years (Fig. 1). Roots were present below 1.7 m already in April in both experiments. In Exp. 1, roots reached just below 2 m depth during the labelling period (Fig. 1a). In Exp. 2 roots were present below 3 m in April (Fig. 1b). At the time of labelling, the average root intensities in the top 2 m soil layers were approximately four times higher in Exp. 2 than in Exp. 1 (0.25 and 0.06 cm cm^{-2}, respectively).

In both years and all treatments, root intensity tended to increase below 0.5 m from fertilization in March to June (Fig. 1c and d). There was a tendency towards more root growth in the N240 than in the N80 treatment in the lower soil layers. No significant differences in root growth were found between the two water regimes. In both experiments, the root intensity in the top 0.5 m decreased from March to June.

Water extraction

VWC at the three recorded depths were similar in the two N treatments during the isotope sampling period in Exp. 1, while the VWC at 0.5 m depth in the WD treatment tended to be lower during the sampling period in Exp. 2 (Fig. 2a and c).

Based on the simplified estimations of daily water uptake, more than 1 mm of water was removed from the 0-1 m soil layer per day during the selected labelling period, while less than 1 mm was removed from the 1-2 and 2-3 m soil layers in Exp. 1 (Fig. 2b). It was clear that with higher N application, water uptake throughout the whole soil profile was increased, though not significant.

The total amount of water taken up in the two water regimes in Exp. 2 was similar. In total, 2.80 and 2.97 mm water per day was removed within the selected period from the top 3 m of the soil column in the WW and WD treatment respectively. However, a shift towards water uptake from deeper soil layers in the WD treatment was observed, as the water deficit in the topsoil increased water uptake in the 2-3 m interval. However, the trend was not significant. Additionally, slight and insignificant increases in $\delta^{13}C$ values were observed in leaves, which further indicated plants under the WD treatment were not drought-stressed during the labelling period in Exp. 2 (Fig. 3).

^{2}H enrichment

A higher enrichment of ^{2}H in transpiration water was found during the whole measurement period, where the tracer was injected at 0.5 m depth instead of 1.7 m at both N treatments (Fig. 4a). Besides, the ^{2}H enrichment of the transpiration water was higher in N240 than in N80 treatments on all dates and both injection depths. The concentration of ^{2}H in transpiration water increased significantly with time when the injection was conducted at 1.7 m. However, when ^{2}H was injected at 0.5 m, no increase in concentration with time was observed.

During the labelling period in Exp. 2, the lowest ^{2}H concentration in the transpiration water was found in the WW treatment when the tracer was injected at 1.7 m. When injected at 0.5 m depth, higher ^{2}H concentrations in transpiration water at the WD treatment than at the WW treatment was seen at the first sampling dates, but in the WD treatment, it fell by c. 60% between April 27 and May 3, while it did not change much over time in the WW treatment (Fig. 4b).

At the same time, the enrichments of ^{2}H derived from 1.7 m were 5 – 35% of the ^{2}H derived from 0.5 m, showing a larger proportion of ^{2}H uptake from 0.5 m than 1.7 m (Table 4). This ratio of deep and shallow derived ^{2}H enrichment was 0 – 10% higher in the N240 than the N80 treatment during the sampling period. In the WW treatment, the ratio of ^{2}H enrichment with tracer injected at 1.7, and 0.5 m was approximately 20% three weeks after injection. While in the WD treatment, the ratio was over 30% and further increased to 67% at the last sampling date (Table 4).

When oilseed rape plants were harvested in Exp. 2, higher ^{2}H concentrations were found in pod samples under the WD treatment than the WW treatment (Fig. 5). The injection depths did not significantly affect ^{2}H concentration in pods.

N depletion and accumulation

In spring both years, soil nitrate concentrations of the top 1.7 m soil were low (Table 5) and did not change much between the first and second sampling dates. In Exp. 1, less ^{15}N was left in the soil at the shallow injection depth of 0.5 m than at 1.7 m at the end of the labelling period (Table 5). There was a high variation in ^{15}N concentration in the soil nitrate at the soil sampling after the isotope sampling period, and no significant differences were found between nitrate and ^{15}N under different N treatments at any of the sampled depths. In Exp. 2, no significant differences were found in nitrate depletion between the WW and WD treatments (Table 5).

Corrected for ^{15}N already in the soil before tracer injection, additional ^{15}N tracer resulted in higher ^{15}N enrichment in the biomass samples (Fig. 6). At the cessation of Exp. 1, plants under the same N treatment, with ^{15}N tracers injected at either 0.5 or 1.7 m, exhibited similar ^{15}N uptake efficiency. No significant differences were observed among the different organs. ^{15}N use efficiency of oilseed rape plants in the N80 treatment was twice as high as in the N240 treatment. In general, an extra gram of ^{15}N led to a 12 -15% increase in biomass ^{15}N atom fraction in the N80 treatment, while in the N240 treatment, the increase in ^{15}N atom fraction per gram ^{15}N added was only around 6% (Fig. 6a). During the labelling period in Exp. 1, the leaf ^{15}N uptake efficiency tended to increase with time (Fig. 6b), which indicated more ^{15}N was accumulated in the leaves. However, this was only significant when ^{15}N was injected at 1.7 m. Independently of ^{15}N injection depths a larger fraction of the applied ^{15}N was found in leaves of oilseed rape plants that had been fertilized with a lower amount of N.

^{15}N uptake efficiency was almost unaffected by the soil water status or injection depth in the harvest samples of Exp. 2 (Fig. 6c). While the fraction of ^{15}N in leaves significantly increased with time, no clear water treatment or injection depth effect was observed at any measurement date (Fig. 6d).

Discussion

Effect of N and water supply on root growth

The difference in root growth along the soil profile between two N treatments was small in the current study. However, we found a non-significant tendency towards deeper and more roots in the subsoil after the high rate of N application. The effects of N supply on root growth were found to be inconsistent in previous studies. Svoboda and Haberle (2006) claimed that a high N fertilization rate led to reduced wheat rooting depth and density in deeper layers, while Hodge et al. (1999) found the increased soil N availability to lead to stronger shoot and root growth. The local soil N availability in subsoil was similar the high and low N treatments. Thus, the observed enhancement of deeper root growth under high N supply was most likely a concurrent effect with better shoot growth.

The relationships between ^{13}C isotopic composition/discrimination and water stress have been widely studied for evaluating the performance of crop under water stress (Farquhar and Richards 1984; Farquhar et al. 1989; Dercon et al. 2006). Plant $\delta^{13}C$ was reported to increase under water-limited conditions (Yousfi et al. 2012). As the foliar $\delta^{13}C$ values showed, oilseed rape plants under the WD treatment were exposed to slight water deficiency at the beginning of labelling. According to previous studies, water deficiency has been proven to be the stimulator of deep root growth and the uptake of deep soil water (Bloom et al. 1985; Vandoorne et al. 2012). Surprisingly, the effect of water supply on root growth was not seen in this study. In both treatments, the increment of root intensity along the soil profile except at 1.5 m depth was less than 0.1 cm cm^{-2}, indicating little and non-preferential root growth under short-term water deficit. One possible explanation for this could be that the water deficiency we observed in the experiment was not severe or long enough to stimulate the deep root growth previously observed (Skinner 2008; Vandoorne et al. 2012). The other explanation is that plants grew under the water deficit treatment were available to obtain adequate water from the subsoil. Therefore their growth was not affected by the water deficit.

Compared with roots in topsoil, the contribution of deep roots to resource uptake is often ignored, as they usually develop late and have a limited active period. However, deeper and denser roots in subsoil do indicate a better capacity for resource acquisition from deep soil layers (Kell 2011; Maeght et al. 2013). Thus, the factors that directly or indirectly affect deep root growth may also affect deep resource uptake.

Effect of N and water supply on water uptake

N supply affected the amount, distribution, and dynamics of water uptake. With a higher N fertilization rate, root water uptake was higher at all depths along the soil profile, with most of the water taken from the top 1 m layer. ^{2}H enrichment in transpiration water further confirmed the effect of N supply on water uptake. During the four weeks after labelling, more ^{2}H-labelled water was found in N240 treatment compared to N80 treatment wherever the tracer was applied. With higher N application, the water uptake is promoted by the increased aboveground growth, transpiration, and photosynthesis (Taylor et al. 1991; Waraich et al. 2011). In addition, high nitrate supply improves radial water fluxes in roots, as it up-regulates the expression of aquaporin and enhances root hydraulic conductivity (Gorska et al. 2008; Wang et al. 2016). Still, the improvement of water uptake was more evident at upper soil layers, where most of the roots were located and directly affected by the N supply.

Topsoil water status did not significantly affect the total amount of water uptake, while we still observed altered water uptake distribution under reduced water supply. More water was taken from deep soil layers in WD treatment than WW treatment, which implied water deficit stimulated water uptake compensation from deep soil layers. This corresponds to previous findings by Vandoorne et al. (2012) and Hashemian et al. (2015), showing that moisture level of different soil layers is a key factor which affect root water uptake distribution. However, with a similar experimental setup, Rasmussen et al. (2020a) concluded that chicory failed in compensating water uptake from deeper soil layers. They suggested that the high hydraulic resistance and drought-induced stomatal closure might reduce root water uptake and plant water demand, leading to the failure of compensation (Rasmussen et al. 2020a).

The pod ^{2}H enrichments were significantly affected by water treatment but not by injection depth at 0.5 or 1.7 m. However, it is important to keep in mind that the overall ^{2}H extraction can be limited by total soil ^{2}H availability rather than root uptake potential over a longer period. When labelled-water is exhausted, the pod ^{2}H enrichment will no longer increase. This might explain why the higher uptake from 0.5 m depth shown in the early ^{2}H enrichment of transpiration water did not lead to a higher ^{2}H enrichment in the pod biomass at the final measurement in our study.

Except in the WW treatment, the ratio of ^{2}H-enrichment derived from 1.7 m, and 0.5 m increased with time, from 5% to 31%, 11% to 35%, and 36% to 67% in N80, N240, and WD treatment, respectively. This distribution shift suggests the rising importance of deep roots in water uptake over the three-week period following injection. The effect of time on deep water uptake may be due to a direct effect on the development and maturity of roots, which has also been demonstrated by Garrigues et al. (2006), or to exhaustion of the ^{2}H label at the 0.5 m depth.

Effect of N and water supply on soil N content and N uptake

Our results showed that increasing N fertilization from 80 to 240 kg N ha^{-1} did not significantly change the content of inorganic N in the top 1.7 m soil, and water deficiency had little effect on the concentration and distribution of soil nitrate. However, these findings should be interpreted with caution as there were only a few weeks between the applications of N or water treatments until the soil measurements. In other studies, such treatments lasted longer, and oilseed rape grown under higher N fertilization rate or less water supply left more soil nitrate in topsoil layers (Smith et al. 1988; Dresbøll et al. 2016).

N uptake efficiency depends on both root uptake capacity and crop demand. Oilseed rape needs high N input during the vegetative growing stage (Rathke et al. 2006). Nevertheless, even with a high capacity to absorb N in autumn and winter, the recovery of fertilized N by oilseed rape is generally found to be poor (Sieling and Kage 2010), and an increasing rate of N supply can further reduce the N recovery (Rathke et al. 2006; Bouchet et al. 2016). This reduction in N recovery was also observed in our study. Despite the fact that the biomass in the N240 treatment was higher than the N80 treatment, ^{15}N recovery in the biomass was higher in the N80 treatment, which indicated more thoroughly soil N depletion under a lower N fertilization rate. Svoboda and Haberle (2006) pointed that the effect of high nitrogen supply in the topsoil on reduction of N depletion in the subsoil can be the result of both less N demand from the subsoil, and reduced root growth in the subsoil. In our case, the latter was not observed.

Effects of water supply on ^{15}N uptake efficiency were not observed in the current study. This contrasts with others' findings, which demonstrated that water deficit would reduce N uptake and use efficiency in various ways (Jensen et al. 1997; Gonzalez-Dugo et al. 2010; Sadras et al. 2016; Riar et al. 2020). In general, topsoil water deficit reduces the availability of soil N (Jensen et al. 1997) and restricts its movement via mass flow and diffusion (Plett et al. 2020), hence reducing N uptake from top layers. To meet crop demand, the N uptake from subsoil would be stimulated. However, it does not seem to be the case in our study. Two possible explanations may account for the absent observation of compensated N uptake; one is that the extent of water deficit was not so severe that it affected soil N availability and subsequent N uptake. The other is that soil dryness directly reduced crop N demand via reducing the shoot growth; therefore, the efficient N uptake from subsoil is no longer needed. As no significant reduction in biomass was found in our case, we assume the second explanation was not the valid reason for the absence of enhanced deep N uptake.

The injection depth did not affect ^{15}N uptake efficiency measured in harvest biomass samples in either experiment, while the continuous leaf sampling after injection showed different patterns of ^{15}N uptake dynamic at 0.5 and 1.7 m. When ^{15}N-labelled nitrate was applied at 0.5 m, the ^{15}N uptake efficiency reached a peak in one to two weeks, while the efficiency kept increasing after injection at 1.7 m, suggesting some ^{15}N-labelled nitrate still remained in the soil. Besides, in Exp. 1, one month after the injection, soil ^{15}N enrichment at 1.7 m was much higher than at 0.5 m, which further confirmed that more ^{15}N had been left at 1.7 m after injection. The continuous and delayed N uptake from subsoil was also observed in winter wheat by Haberle et al. (2006). They suggested the inadequate N supply from topsoil might be the stimulator of subsoil N uptake. Still, ^{15}N uptake was less affected by deep

placement than ^{2}H uptake during the three-week sampling period. This indicates that while we see substantial uptake of both water and N by the deep parts of the root system, uptake of the two resources is not equally limited by the low root density and the short time available for active uptake (Chen et al. 2021).

There is no doubt that supplemental N and water supply also affect biomass production. In general, oilseed rape plants grown with extra N and water supply have been shown to have a higher yield (Taylor et al. 1991; Schjoerring et al. 1995; Dresbøll et al. 2016; Riar et al. 2020). In the current study, higher fertilization rate exhibited positive effects on biomass and plant N content. However, we only observed slight and non-significant increases of biomass under well-watered condition, together with a non-significant decrease in plant N content. This showed that the overall growth and development of oilseed rape plants were not restricted by water deficiency.

Conclusions

Overall, when roots are already well developed in upper soil layers, water and N supply could still alter the water and N uptake via regulating deep water and N acquisition. The effects of increased water and N supply on deep water and N uptake were not always the same. Increased water uptake from deep soil layers was not always accompanied by increased nitrogen uptake. Further research on the optimal water and N management strategies and the corresponding response of deep root functioning will be required to maximize the benefits of deep roots and maintain biomass production.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Tables

Table 1 Characteristics of the soil in rhizoboxes

Depth	Coarse sand (%) 0.2–2.0 mm	Fine sand (%) 0.02–0.2 mm	Silt (%) 0.002–0.02 mm	Clay (%) <0.002 mm	Organic matter (%)
0 - 0.2 m	46.4	39.7	5.5	7.0	1.4
0.2 – 2.0 m	26.8	51.1	4.2	17.6	0.3
2.0 – 4.0 m	21.1	54.1	8.7	16.0	< 0.1

Table 2 Treatments and timeline of the experiments. The oilseed rape plants were fertilized with 240 kg N ha^{-1} (N240) or 80 kg N ha^{-1} (N80) applications in Exp. 1; in Exp. 2 plants were grown under well-watered (WW) or water deficient (WD) conditions at the beginning of the labelling.

	Exp. 1 2018/2019	Exp. 2 2019/2020
Sowing	16 August 2018	13 August 2019
Transplanting	8 October 2018	26 August 2019 [a]
Rate of N application	N240: 240 kg N ha^{-1} N80: 80 kg N ha^{-1}	200 kg N ha^{-1}
Date of N application	27 March	1st: 5 September 2019 2nd: 2 March 3rd: 1 April
Water status	WW: Well-watered	WW: Well-watered WD: Water deficit
Key irrigation events [b]	None	WW 1st: 14 April, 20 mm WW 2nd: 15 April, 40 mm WD: None
Tracer injection	3 April	17 April
Isotope sampling	1st: 2 April 2nd: 10 April 3rd: 17 April 4th: 23 April 5th: 1 May	1st: 16 April 2nd: 22 April 3rd: 27 April 4th: 3 May 5th: 8 May
Final biomass collection	5 June	18 June

[a] Pest infestation (*Delia radicum*) occurred in some of the chambers in September 2019. Thus, a few plants were replaced on September 24, 2019. Replaced plants were sown together with the original ones, but appeared smaller than the original ones throughout the experiment. [b] Irrigation events which might change the soil water status before tracer injection were counted.

Table 3 Mean plant dry matter and N contents for different N or water treatments at final collection (n = 6). In Exp. 1 the oilseed rape plants were grown under 80 kg N ha^{-1} (N80) or 240 kg N ha^{-1} (N240) applications and were all well-watered (WW); in Exp. 2 all plants were fertilized with 200 kg N ha^{-1}, and grew under well-watered or water deficit (WD) conditions. In the same column, different letters indicate significant differences between treatments in Exp. 1 ($p < 0.05$). No significant differences were found between water treatments in Exp. 2. Pods were not fully ripe in either experiments. *There were no leaves left when oilseed rape plants were harvested in 2020.

Experiment	N level	Water status	Plant biomass (g $chamber^{-1}$)			N content (mg g^{-1})		
			Leaf	Stem	Pod	Leaf	Stem	Pod
Exp. 1	N80	WW	31.9b	215.7b	179.4b	17.1b	5.7b	16.3b
	N240	WW	56.2a	299.7a	281.5a	18.3a	7.1a	17.4a
Exp. 2	N200	WW	*	357.7a	445.5a	*	3.7a	11.1a
	N200	WD	*	326.5a	410.9a	*	3.9a	11.8a

Table 4 The ratio of ^{2}H – enrichment in transpiration water with tracer injected at 1.7 m (Enrich $_{1.7}$) and 0.5 m (Enrich $_{0.5}$). The oilseed rape plants were grown under 80 kg N ha^{-1} (N80) or 240 kg N ha^{-1} (N240) applications and were all well-watered (WW) in Exp. 1; in Exp. 2 all plants were fertilized with 200 kg N ha^{-1} fertilizer, and grew under well-watered or water deficit (WD) conditions.

Experiment	Year	N level	Water status	Sampling date	Enrich $_{1.7}$ /Enrich $_{0.5}$ (%)
Exp. 1	2019	N80	WW	10 April	5
				17 April	10
				23 April	24
				1 May	31
		N240	WW	10 April	11
				17 April	23
				23 April	23
				1 May	35
Exp. 2	2020	N200	WW	22 April	22
				27 April	19
				3 May	17
				8 May	25
		N200	WD	22 April	36
				27 April	35
				3 May	50
				8 May	67

Table 5 Means of soil nitrate content and ^{15}N concentration in different soil layers before and at the end of labelling periods (in 0.5 and 1.7 m, n=3; in 1.1 m, n=6). The oilseed rape plants were grown under 80 kg N ha^{-1} (N80) or 240 kg N ha^{-1} (N240) applications and were all well-watered (WW) in Exp. 1; in Exp. 2 all plants were fertilized with 200 kg N ha^{-1} fertilizer and grew under well-watered or water deficient (WD) conditions. In Exp. 1, soil samples were taken on April 3 and May 3, 2019; in Exp. 2, soil samples were taken on April 17 and May 9, 2020. For soil nitrate content analysis, soil samples, which were taken from labelled depths, were excluded to avoid the effects of tracer application. For soil ^{15}N concentration analysis, soil samples of 0.5 and 1.7 m were taken from labelled depths, soil samples of 1.1 m were taken from all depths.

Experiment	N level	Water status	Depth (m)	Soil NO_3^- before (mg N kg^{-1} dry weight)	Soil NO_3^- after (mg N Kg^{-1} dry weight)	Soil ^{15}N before (‰)	Soil ^{15}N after (‰)
Exp. 1	N80	WW	0.5	2.4	1.7	364	1323
			1.1	1.8	2.4	31	38
			1.7	2.9	2.9	70	5109
	N240	WW	0.5	2.6	2.0	102	1116
			1.1	2.4	2.2	19	33
			1.7	2.9	2.2	163	8669
Exp. 2	N200	WW	0.5	0.1	0.1	*	*
			1.1	0.1	0.2	*	*
			1.7	0.1	0.2	*	*
	N200	DS	0.5	0.1	0.1	*	*
			1.1	0.1	0.1	*	*
			1.7	0.1	0.1	*	*

* Soil ^{15}N concentration was too low to detect.

Figures

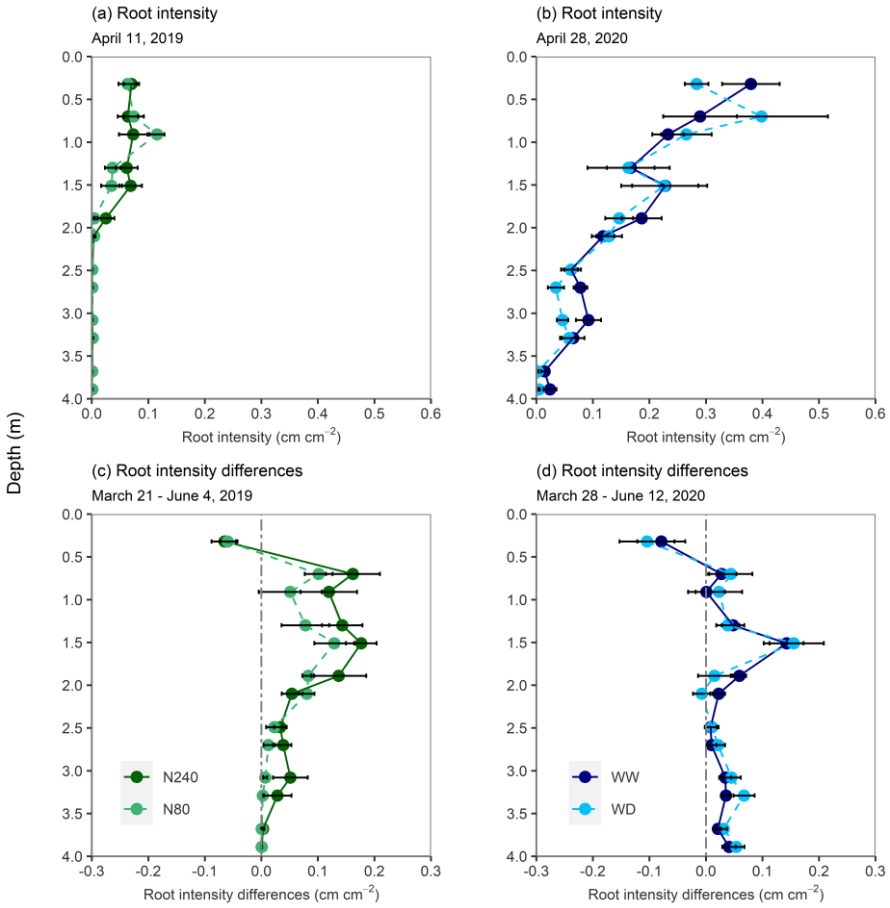

Fig. 1 Root intensity measured on April 11, 2019 (a), 8 days after tracer injection and on April 28, 2020 (b), 11 days after tracer injection. Differences in root intensity from March 21 to June 4, 2019 (c) and from March 28 to June 12, 2020 (d).). N240 = 240 kg N ha^{-1}, N80 = 80 kg N ha^{-1}, WW = well-watered, WD = water deficit. Error bars denote standard errors. No significant differences were found at any depths.

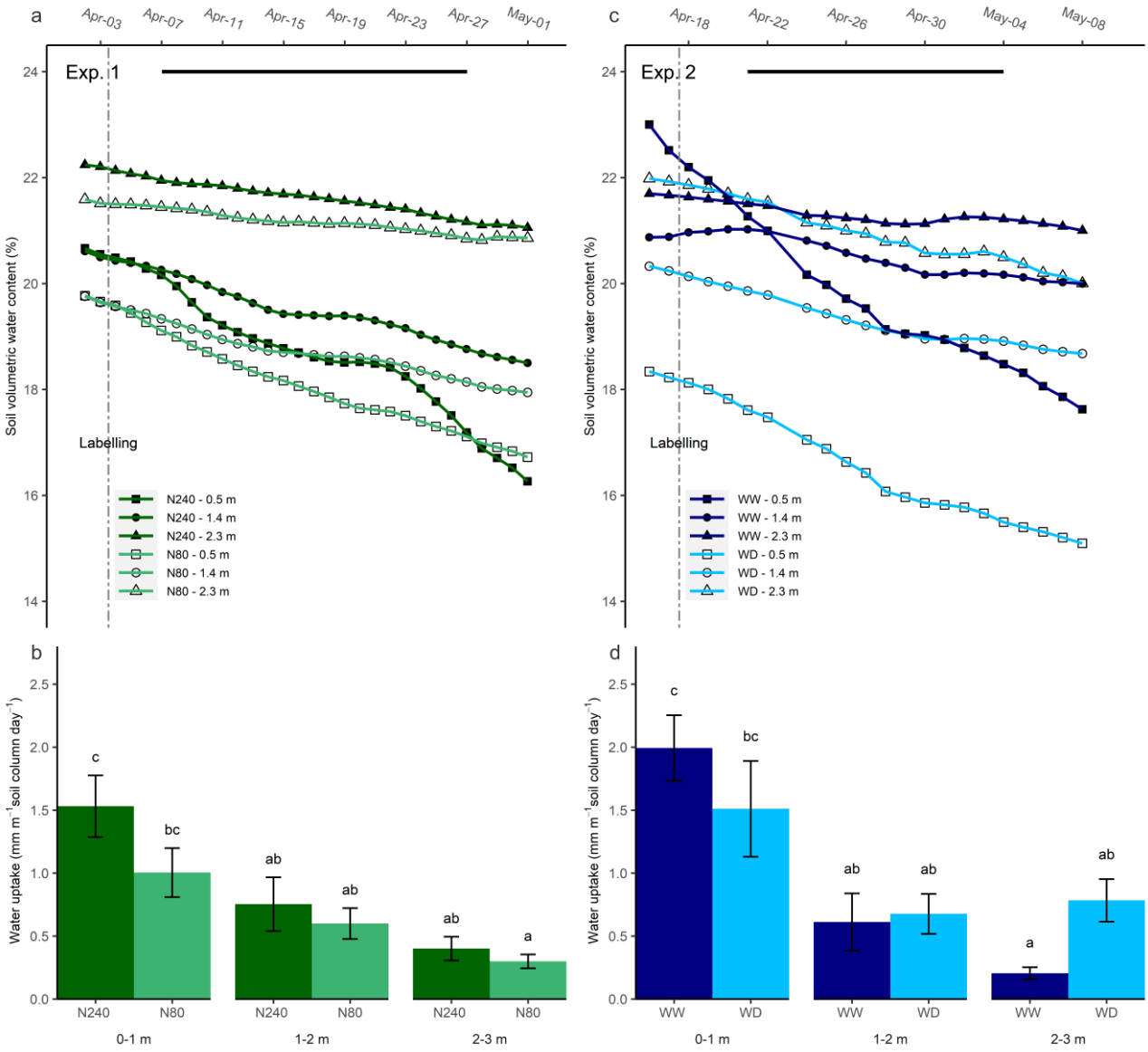

Fig. 2 Soil volumetric water content (VWC; %) and water uptake in Exp. 1 (a, b) and Exp. 2 (c, d). N240 = 240 kg N ha^{-1}, N80 = 80 kg N ha^{-1}, WW = well-watered, WD = water deficit. Data were collected from April 2 to May 1, 2019 in Exp. 1 and from April 16 to May 8, 2020 in Exp. 2. Daily averages of recorded VWC are shown in a and c. The black segments denoted selected periods for daily water decrease estimations in Exp. 1 (b) and Exp. 2 (d). Daily water uptake from each 1 m interval of soil column was estimated as averages of daily water decrease from that column from April 7 to April 27, 2019 in Exp. 1 and from April 21 to May 4, 2020 in Exp. 2. Error bars denote standard errors, letters indicate significant differences across the treatments in the same experiment ($p<0.05$).

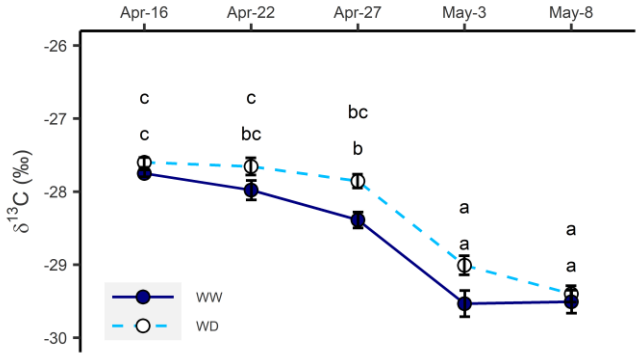

Fig. 3 $\delta^{13}C$ measured in leaf samples collected during the isotope sampling period in Exp. 2 in well-watered (WW) and water deficit (WD) treatments. Error bars denote standard errors, letters indicate significant differences across the treatments ($p<0.05$).

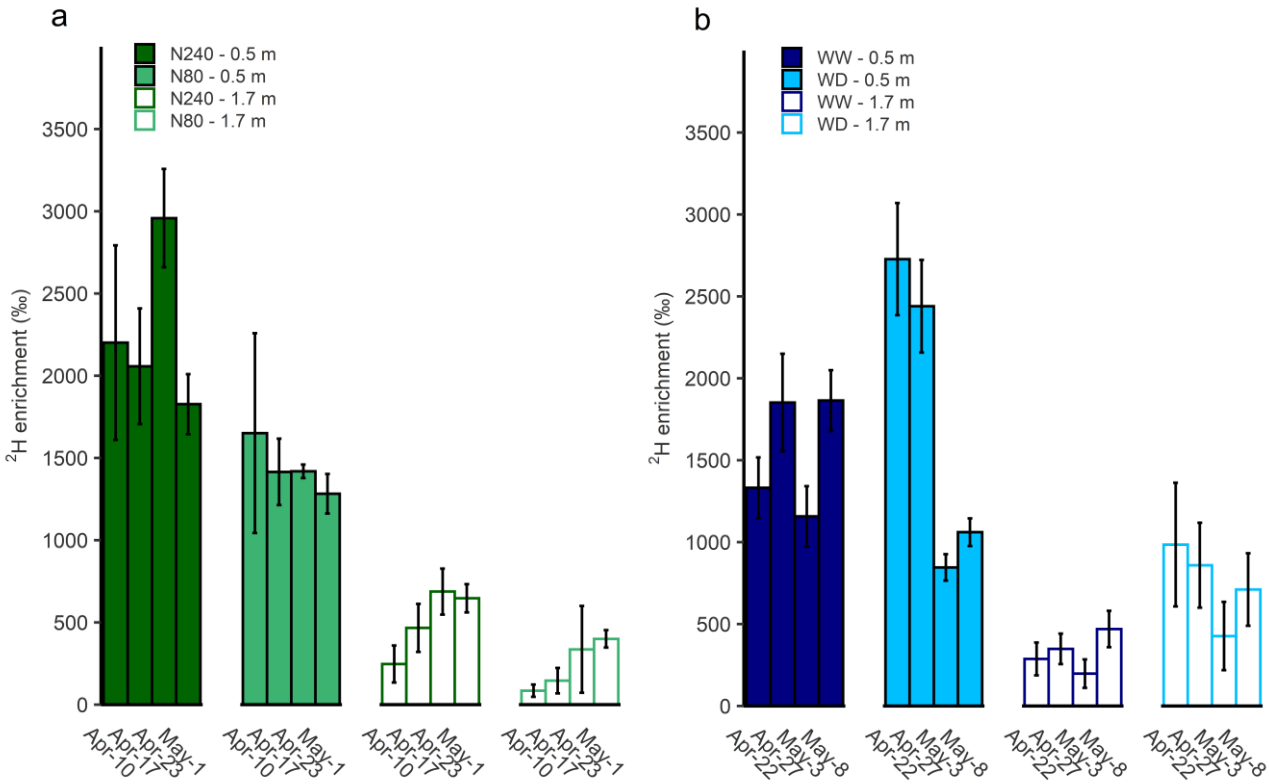

Fig. 4 Time course of ^{2}H enrichment in transpiration water was shown under N (a) or water (b) treatments during isotope sampling periods. N240 = 240 kg N ha^{-1}, N80 = 80 kg N ha^{-1} (a), WW = well-watered, WD = water deficit (b). ^{2}H labelled water was injected at either 0.5 or 1.7 m in each treatment. Error bars denote standard errors (n=3). Mean values are shown here (± SE).

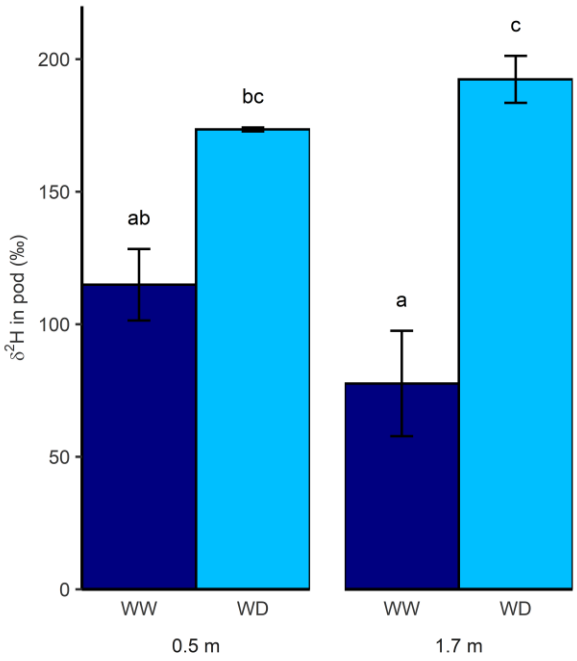

Fig. 5 δ^2H in pods at harvest in Exp. 2. WW = well-watered, WD = water deficit. ^{2}H labelled water was injected at either 0.5 or 1.7 m in each treatment. Error bars denote standard errors (n=3), and letters indicate significant differences between treatments and depths ($p<0.05$).

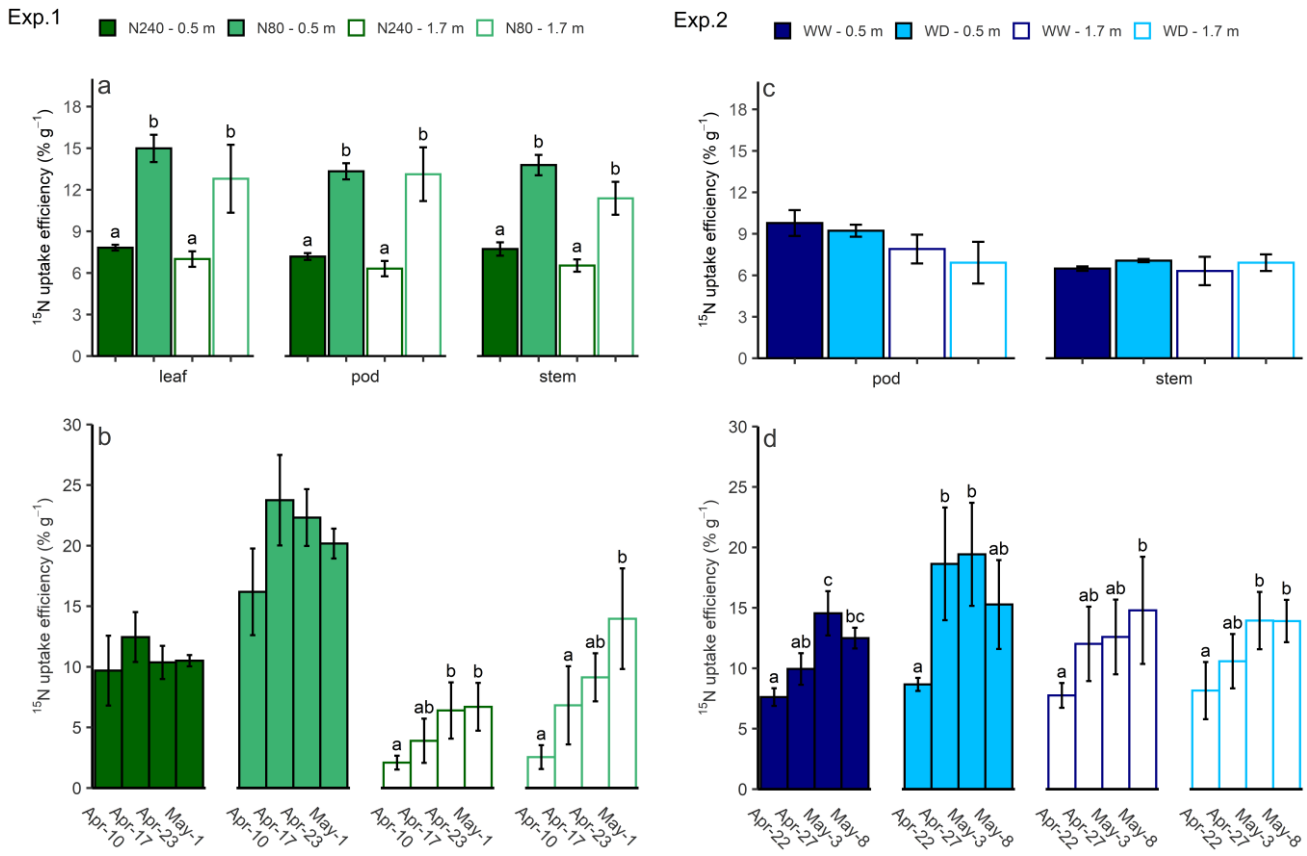

Fig. 6 ^{15}N uptake efficiency (% g^{-1}) that was measured in harvest samples (a, c), and leaf samples (b, d) which were collected during the labelling periods in Exp. 1 (a, b) where, N240 = 240 kg N ha^{-1}, N80 = 80 kg N ha^{-1} and Exp. 2 (c, d) where, WW = well-watered, WD = water deficit. ^{15}N tracer was injected at either 0.5 or 1.7 m in each treatment. Mean values of harvest ^{15}N use efficiency in different organs under different N (a) or water (b) treatments are shown here (± SE). Error bars denote standard errors (n=3), and letters indicate significant differences among treatments within the same organ ($p<0.05$). Time course of leaf ^{15}N use efficiency (% g^{-1}) under different N (c) and water (d) regimes was calculated. Error bars denote standard errors (n=3), and letters indicate significant differences among all sampling dates under the same treatment and injection depth ($p<0.05$). Mean values are shown here (± SE).

Conclusion and Perspectives

The case studies mentioned above demonstrate that deep roots perform differently in water and N uptake. Root water uptake was limited by increasing soil depths and reducing root intensity, while root N uptake at deeper soil layers was comparable to that at shallower layers. The limited hydraulic conductivity of the deeper zone of the root system and low root intensity at deeper layers could be the main limitations to water uptake. Root water uptake below 2 m depth gradually increased with time, indicating a rising hydraulic conductivity in the deeper root system. In contrast, deep roots efficiently exploited subsoil N, and the uptake was not restricted by the depth of soil and reduced root intensity. Unlike water uptake, the drastic increment of foliar ^{15}N accumulation was observed ten days after tracer application. After ten days of tracer application, the accumulation of foliar ^{15}N increased insignificantly, which suggested there was no enhancement of deep N uptake potential with time.

From the studies of water and N supply variations in deep root water and N uptake, it was concluded that deep resource acquisition could be altered by the resource availability in topsoil, while the degree of alteration varied between water and N uptake. When N application was lower in topsoil, deep roots were more efficient at taking $^{15}NO_3^-$ at 0.5 and 1.7 m depths. However, the water uptake at the corresponding depths was insignificantly diminished by reduced N supply, which might cause by a reduction of plant growth. The effects of water supply were also inconsistent with water and N uptake. Deficient water supply in topsoil stimulated deep water uptake while had little effect on N use efficiency. These results confirmed that the primary limitations in deep water and N uptake were different and suggested the potential of using deep-rooted crops for increasing water and N uptake under suboptimal conditions.

The findings extended our understanding of deep root functioning and further raised the following questions, (1) What are the physiological and molecular limitations in deep water and N uptake? (2) What kind of technologies are required to better quantify and investigate the contribution and dynamics of the deeper root system to water and N uptake? (3) How can we maximize the benefits of deep roots on resource acquisition and fit them into the changing climate and currently used management practices? Although a few studies have addressed the importance of root hydraulic conductivity, hormonal response, and aquaporin activity in regulating water uptake, the regulation of deep N uptake was rarely studied. Also, despite the fact that the studies here presented the possibility of using the ^{2}H-^{15}N dual-labelling method in tracking water and N uptake dynamics simultaneously,

it is still hard to quantify and compare the uptakes directly. Further *in situ* techniques and accurate model simulations for measuring and quantifying deep root activities are urgently needed. Last but not least, while deep-rooted crops are advantageous in enhancing water and nutrient uptake, their potential in resource acquisition interacts strongly with the environmental conditions and management practices. These interactions should be taken into consideration when deep-rooted crops are included in agricultural systems.

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