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Naturally coloured roots as a tool for studying root interactions in mixed cropping

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Abstract: The objective of this study was to evaluate the usage of species with coloured roots to study root growth patterns during intercropping. Red beet (*Beta vulgaris* L. cv. Detroit), having clear red roots, was used in a semi-field and field experiment to allow identification and quantification of roots of the individual species in the mixture. In the field experiment, red beet was strip intercropped with lucerne (*Medicago sativa* L. cv. Creno) and kale (*Brassica oleracea* L. var. Sabellica), respectively while the red beet-lucerne intercropping was conducted in large rhizoboxes where root growth distribution and ^{15}N isotope uptake was determined. The study confirmed that the direct visual measurement of root growth using species with coloured roots and indirect tracer uptake measurements contributed to the success of studying root growth dynamics in intercropping systems. Red beet root intensity was not considerably affected by the strip intercropping when the crops were established at the same time, but when established between existing lucerne strips, a reduction in roots at the border row was shown. Lucerne and kale were both observed to be able to exploit the deep soil layers beneath the red beet border row.

Keywords: deep rooting; root competition; ^{15}N tracer; minirhizotron; intraspecific; interspecific

Intercropping, the deliberate growth of two or more crop species simultaneously in the same field, is an ancient practice well-known to increase yields, reduce fertiliser and pesticide inputs, and increase biodiversity. Especially with the focus on yields and resource use, several advantages of intercropping have been identified, e.g. intercropping faba bean and maize increase the legume reliance on N_2 fixation (Li et al. 2006). Although past research on plant competition and the effects of intercropping have focused primarily on the aboveground plant parts, studies on root growth and belowground interactions in intercropping have been conducted. Xie and Kristensen (2017) reported that the deep-rooted dyer's woad was an efficient tool in intercropping to reduce nitrate leaching in organic leek production. In addition, intercropping barley with pea resulted in a faster and deeper root distribution, and intercropping maize with wheat or faba bean produced a greater root length density as compared to sole-cropped maize (Li et al. 2006). Finally, root interactions do not only increase the root length density of intercrops but also increase the efficiency of utilising soil resources in deeper soil layers and beneath the neighbouring crops (Gao et al. 2010, Cardinael et al. 2015).

A specific problem when studying root interactions as opposed to aboveground plant parts is the difficulty in distinguishing between crop species, thus complicating the study of root growth and distribution in intercropping (Maeght et al. 2013). Hence, coloured roots have been used for root distinction when studying root interactions between mixed crops in rhizotrons (Tosti and Thorup-Kristensen 2010, Andersen et al. 2014, Hassan et al. 2021). In

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this study, the methodology was expanded to include semi-field and field studies of intercropping. Thus, the aim was to evaluate the usage of species with coloured roots for studying root interactions using direct visual distinction and, furthermore, to determine if indirect measurements of roots by the use of isotopes in a semi-field intercropping system would confirm the visual distinctions. We hypothesised that neighbouring plants of different species would affect the root depth and distribution differently compared to neighbouring plants of the same species. Furthermore, we hypothesised that deep-rooted species grown in strip intercropping systems were able to distribute roots horizontally, especially in the deep soil layers. Finally, it was hypothesised that interactions between an established perennial root system and a newly planted annual crop would differ from root interactions in two annual crops established simultaneously.

MATERIAL AND METHODS

Experimental site and plant material

Experiment 1. Experiment 1 was conducted in rhizoboxes at the University of Copenhagen in Taastrup, Denmark (55°66'89.4''N, 12°30'53''E) between April and September 2016. The dimensions of the rhizoboxes were 4 m × 1.2 m × 0.60 m (height × length × width), with each rhizobox divided into two root chambers, each with a width of 0.3 m. Each root chamber had 20 transparent acrylic panels, which enabled observation of the roots. The acrylic panels were covered by white PVC foam panels to avoid light penetration and reduce the temperature effect on the soil. The rhizoboxes were filled with two types of sandy loam soil: topsoil (0–0.25 m) and subsoil (0.25–4.0 m) (Table 1). A drip irrigation system was installed at the soil surface of each chamber, with a dripping hose flowing at a rate of 14 mm/h. Each root chamber was equipped with three water sensors (Acclima TDR sensors (Meridian, USA) low power) at 0.5 m, 2.3 m and 3.5 m depth in order to monitor the soil moisture content. The sensors were connected to a data logger collecting data at 5-min intervals throughout the study period (Figure 1). In the bottom of the rhizoboxes, a hanging wick ensured free drainage and avoidance of waterlogged conditions.

Seedlings of four-week-old red beet (*Beta vulgaris* L. cv. Detroit) and lucerne (*Medicago sativa* L. cv. Creno) were transplanted into the three root chambers on 28th April 2016. Three red beets and three lucerne plants were planted alternately in each of the three chambers with a plant-to-plant distance of 0.16 m. Fertilisation corresponding to 50 kg N/ha (NPK 5-1-4) was applied as liquid fertiliser one week after transplantation. On 11th July 2016, the lucerne plants were trimmed at 0.03 m above the soil surface to reduce aboveground competition.

Experiment 2. Experiment 2 was a field experiment also established in April 2016 at the field research facilities of the University of Copenhagen. The soil was an Agrudalf soil that was classified as sandy loam according to the ISSS classification (Table 2). Weather data were obtained from a meteorological station that was located less than 500 m from the experimental site (Figure 2). Two field strip intercropping combinations were used in this experiment, a red beet-lucerne strip intercropping and a red beet-kale (*Brassica oleracea* L. var. Sabellica) strip intercropping (Table 3). The red beets were sown with 50 seeds/m^2 at a 0.5 m row distance in May 2016 in both strip intercropping systems. The lucerne plants had already been sown in October 2015 at 30 kg/ha, whereas kale seeds were sown in the greenhouse four weeks prior to transplanting and was transplanted with a row planting distance of 0.50 m and an interrow plant distance of 0.50 m in May 2016. Red beet and kale were fertilised with 100 kg N/ha, no fertiliser was added to lucerne (Table 3).

One year prior to the experiment, 6.0 m long minirhizotrons were inserted into the soil at an angle of 30° from vertical, reaching 5 m depth. For each strip intercropping system, three replicate plots (10 × 19.5 m)

Table 1. Soil properties of soil used during the experiment in the root tower

Soil	Depth interval (m)	Clay	Silt	Fine sand	Coarse sand	pH_{CaCl_2}	P	K	Mg	SOC
		(%)					(mg/kg)			(%)
Topsoil	0–0.2	9.0	8.9	46	34	7.6	45	95	44	1.9
Subsoil	0.2–4.0	10	9.0	48	33	8.0	4	36	41	0.2

SOC – soil organic carbon

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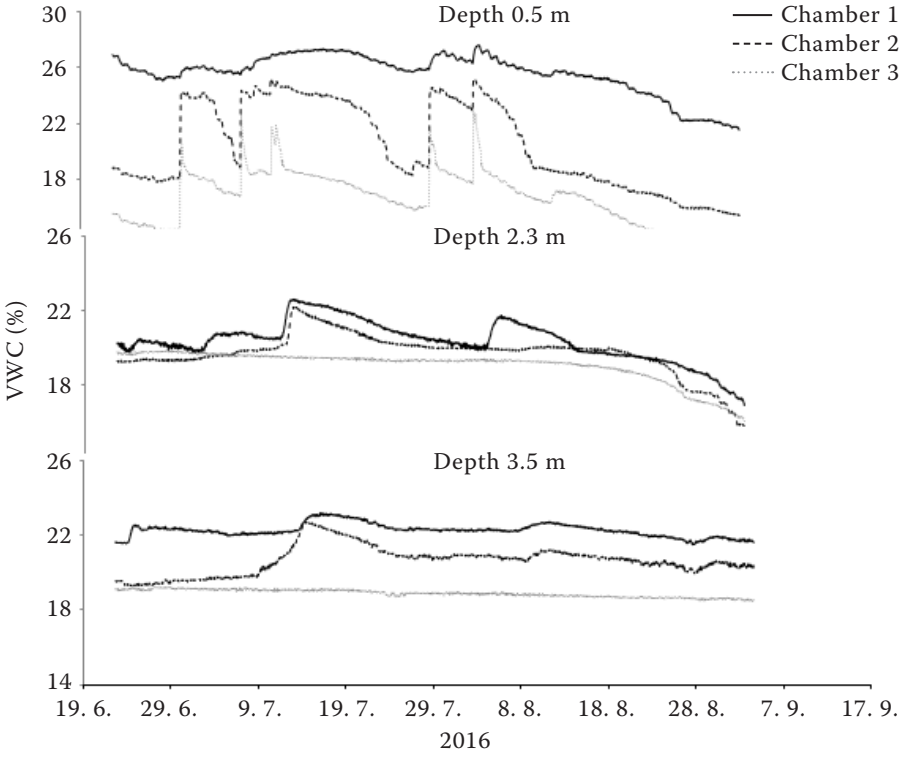

Figure 1. Volumetric water content (VWC) for all root towers at three different depths (0.5, 2.3 and 3.5 m) monitored from 22 June 2016 to 3 September 2016 using Acclima TDR sensors (low power) logging for every 5 min interval throughout the period

with alternating strips were established. Each plot contained four 3.0 m wide strips and five 1.5 m wide strips. Red beet was planted in the 3.0 m wide strips with either lucerne or kale established in the 1.5 m wide strips. A minirhizotron tube was placed in the middle of two wide and two narrow strips in each plot to determine "sole crop" root development. Additional, a minirhizotron tube was placed in the two remaining red beet strips approximately 0.5 m from the edge of a narrow strip to evaluate possible root interactions between the two species. In this way, six minirhizotron tubes were placed in each plot, and with three replicates and two strip intercropping systems, this summed up to a total of 36 minirhizotrons for the studies. The layout of the minirhizotrons is presented in Figure 3.

Root measurements

Experiment 1. Images of roots were taken weekly starting from the seventh week after transplanting over a total period of 12 weeks. A special designed photo box was used for root imaging where a camera was attached to a closed box with an internal LED light source to avoid light reflection from the acrylic panels. Four images (0.31 × 0.20 m) were taken for each panel. After that, a grid with squares of 20 × 20 mm was placed onto the pictures for root counting. The root intersections with the grid lines were counted, and the number of intersections per meter grid line was calculated to assess the root intensity (RI) as described in Thorup-Kristensen (2001). The soil depths were divided into 0.4 m intervals for analysis.

Table 2. Physical and chemical soil properties to a depth of 2.3 m from a field experiment in Taastrup

Depth (m)	Clay	Silt	Sand	pH	P	K	Mg	SOC
	(%)				(mg/kg)			(%)
0–0.5	15.5	13.8	69.0	6.6	26.8	117.5	45.8	1.0
0.5–1.0	20.3	14.8	64.5	6.9	15.3	59.8	57.8	0.5
1.0–1.5	19.5	16.0	64.5	7.3	8.0	48.8	66.8	0.3
1.5–2.0	18.5	18.0	63.3	7.5	5.8	49.3	58.5	0.2
2.0–2.3	19.0	19.8	61.8	7.6	4.8	54.9	54.3	0.1

SOC – soil organic carbon

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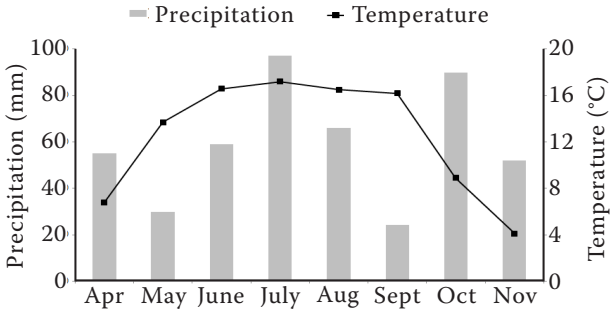

Figure 2. Weather data for the experimental period, experiment 1 from April until September 2016 and experiment 2 from May until November 2016. The figure shows monthly precipitation and temperature

Experiment 2. Images were taken within the minirhizotrons four times from August to November 2016 at 0.05 m intervals with a specially designed camera. Each image covered an area of 50 × 43 mm on the upward-facing side of the sloping minirhizotron. The RI was determined by counting the number of root intersections per meter grid line (10 × 10 mm grid) applied on the images and divided by the total grid length (0.24 m).

^{15}N injection and aboveground biomass

In experiment 1, root activity in deep soil layers was estimated by ^{15}N isotope uptake. The ^{15}N-enriched solution was injected into the root chambers two weeks before the final root measurements. The injection depth was determined based on the deepest roots of lucerne observed, resulting in an injection depth of 2.4, 2.6 and 3.2 m in the three chambers, respectively. The red beet roots were able to colonise the deep soil layers, and red beet dominated the root growth in the layers where the ^{15}N-enriched solution was injected. Ten holes, 25 cm deep and 6 mm in diameter, were made at the specific depth with a steel rod to ensure uniform distribution of the ^{15}N-enriched solution. The ^{15}N solution ($Ca^{15}NO_3$, 99% atom ^{15}N) was injected with 2 mL solution at five points in each hole with a syringe providing 243 mg of ^{15}N in total to each root chamber.

The aboveground plant biomass in each root chamber was harvested two weeks after the injection of the ^{15}N-enriched solution. Lucerne was cut 10 mm

Table 3. Summary of operation during experiments in semi-field and field-scale

Management	Date	Details
Experiment 1 (Root Tower)		
Sowing and transplanting	21 April 2016	red beet (*Beta vulgaris* cv. Detroit) and lucerne (*Medicago sativa* cv. Creno)
Installing sensor	30 May 2016	nine water sensors – Acclima (low power) was installed at depth 0.5, 2.3 and 3.5 m
Irrigation	28, 30 June 2016	2 h of drip irrigation corresponds to 10 L of water (irrigated when precipitation was low or temperatures high)
Fertiliser	02 May, 10 July 2016	applied 30 mL per root chamber corresponds to 50 kg N/ha
Root measurement	15, 23, 29 June, 6, 13, 20, 27 July, 03, 17, 24, 31 Aug 2016	root imaging using the camera
Injection of tracer	18 Aug 2016	applying ^{15}N at 2.4, 2.6 and 3.2 m, after 2 weeks, plant biomass was harvested for ^{15}N analysis
Experiment 2 (DeepRootLab)		
Planting/sowing	10 May 2016, October 2015 25 May 2016	red beet (*Beta vulgaris* cv. Detroit) lucerne (*Medicago sativa* cv. Creno) kale (sown in the greenhouse 4 weeks before transplanted)
Planting density		red beet – 8 plot (50 seeds/m) lucerne – 5 plots (30 kg/ha) kale – 5 plot (0.5 m × 0.5 m), (40 000 plants/ha)
Installing of minirhizotrons	June 2015	R (12), L (6), R-L (6), K (6), R-K (6)
Fertiliser	29 July 2016	100 kg N/ha (except lucerne for the first harvest)
Weeding		manual weeding when needed
Root measurement	25 Aug, 20 Sep, 17 Oct and 17 Nov 2016	imaging minirhizotron (ProInvent Machine)

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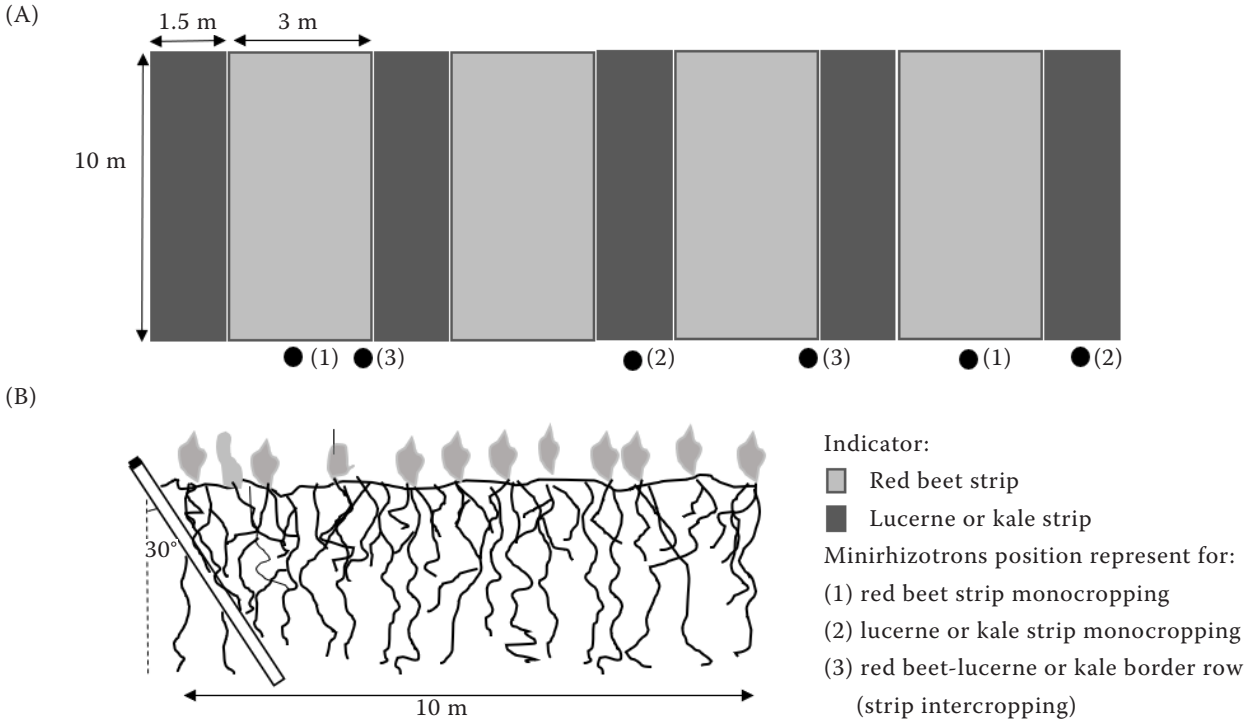

Figure 3. The layout of minirhizotrons from (A) aerial view and (B) side view. Each plot had four 3 m width of red beet strips intercropping and five 1.5 m width of lucerne/kale strips intercropping, and six (6) minirhizotrons which two (2) replicates for each treatment (red beet monocropping, lucerne/kale monocropping, and red beet-lucerne or kale border row)

above the soil surface, whereas red beet plants were divided into two samples, the leaves and the edible taproot. The biomass was oven-dried at 70 °C for 48 h. After drying, the plant material was weighed, milled, and finely ground. The total nitrogen (N) content and $\delta^{15}N$ (%) was determined by combustion with an Elementar Vario Micro Cube elemental analyser (Elementar Analysensysteme GmbH, Hanau, Germany) interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK) at the Stable Isotope Facility of UC Davis. The total ^{15}N uptake per plant was calculated as the product of the N concentration and the biomass. The ^{15}N uptake was obtained by using the ^{15}N atom % provided during analysis and the atmospheric natural ^{15}N abundance (0.3663%) (Sarr et al. 2016).

Statistical analysis

Statistical analyses were conducted using Statistical Package Social Science (IBM SPSS Statistics 22, Armonk, USA). Significant differences in the uptake of ^{15}N from crops and root growth from field intercropping were tested using one-way ANOVA. Root data from experiment 1 were tested using an independent samples *t*-test. In addition, Tukey *HSD*'s (honestly significant difference) test was used to determine the treatment effect. Statistical tests with $P < 0.05$ was considered statistically significant in assessing differences between the results. All data were tested for normality prior to statistical analyses using the Shapiro-Wilk normality test and Levene's test for homogeneity.

RESULTS AND DISCUSSION

Red beet was shown to be an efficient model crop for studying root interactions in intercropping, and the roots of the individual species in the mixtures could be distinguished easily on the images from both semi-field and field experiments, even on the complex soil background. As anticipated, the red coloured red beet roots were different from the white or light brown colour of the roots of the other species examined (Figure 4). Thus, the usage of coloured red beet roots for direct visual distinction allowed the observation of root development of crops grown in different intercropping systems, even in deeper soil layers. However, a major constraint of the method

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is that it is only applicable if one of the crops has coloured roots, which is not found in many crops. To address this predicament, Murakami et al. (2006) developed a method for staining the root systems of neighbouring plants to distinguish them from the plant of interest. The authors developed a method involving pressure injection of dye into the plant roots under dry soil conditions (Murakami et al. 2011). Although this method facilitated the distinction of roots of different plants, it was limited to crops that were grown in a pot or were shallow-rooted and was not feasible in deeper soil layers. Thus, for model studies of root interactions, the usage of crops with natural coloured roots seem more promising.

RI of semi-field intercropping

The root intensities of red beet and lucerne were found to be significantly different at certain soil depths in the semi-field facility 19 weeks after transplanting (WAT) (Figure 5). The growth of red beet roots dominated in the top layer ($P < 0.01$), while lucerne had significantly more roots in the 1.6–2.0 m layers by the end of the experiment. Both crops reached 3.6 m depth, and no significant differences were found between the root intensities from 2.4 m to 3.6 m depth at 19 WAT. Generally, red beet was observed to have a faster root penetration to the deeper soil layers compared to the lucerne, although both of them were found to be deep-rooted crops. In line with previous studies, legumes are usually found to be the weaker competitor when intercropped with non-legumes (Tosti and Thorup-Kristensen 2010).

The root intensity of both red beet and lucerne decreased in the top layers with time, but the root intensity still differed significantly ($P < 0.05$) at 13 WAT. The intensity of lucerne roots increased slightly at a depth of 0.8 m to 1.6 m as the red beet root intensity decreased. The results were similar to that of a study conducted by Zhang et al. (2013), which reported that lucerne roots penetrated deeper soil layers to reduce

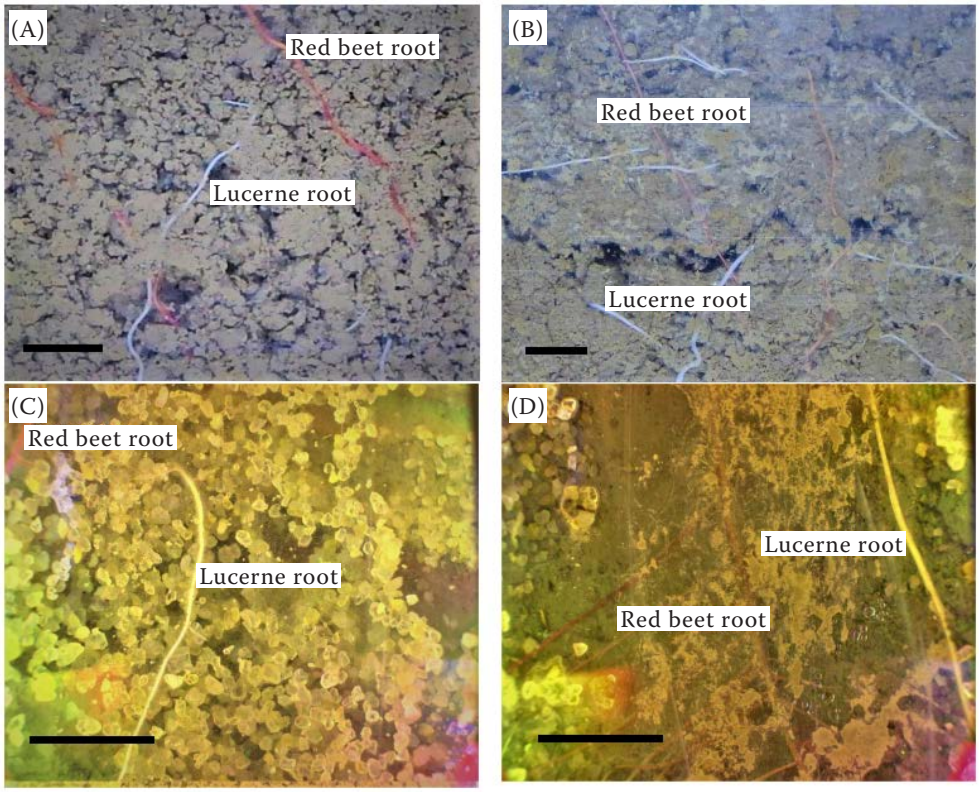

Figure 4. Root images are from experiment 1 (A and B) and experiment 2 (C and D) using the direct visualisation technique. Images A and B are red beet-lucerne roots in rhizoboxes. Image C and D are red beet-lucerne roots from minirhizotron in the field strip intercropping. Image A and C are more clear to visual and count while images B and D are also easily seen, but the colour differences are not so big due to the roots are a bit older but still measurable. Scale bars denote 1 cm

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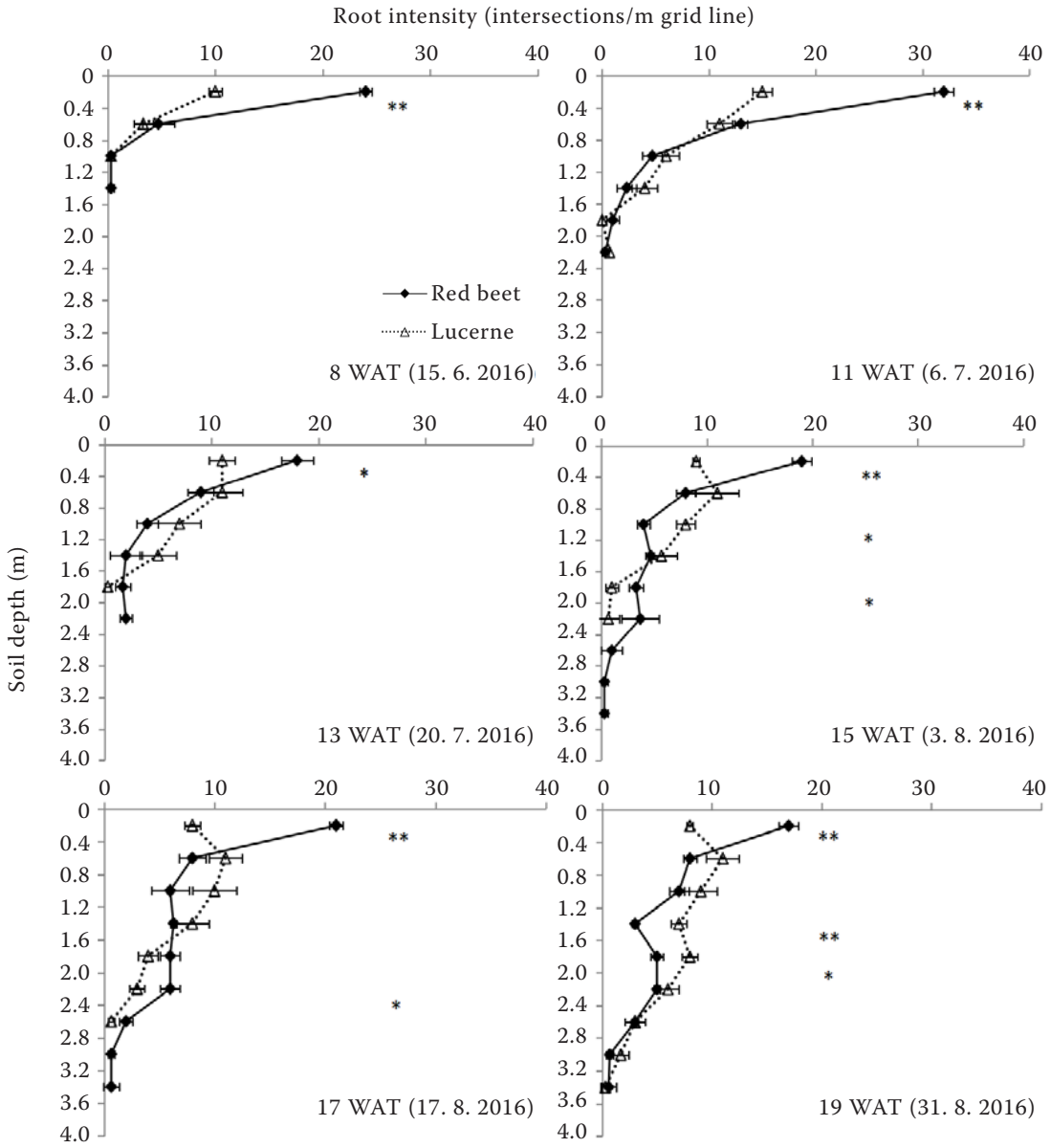

Figure 5. Root intensity (intersections/m grid line) of red beet and lucerne intercropping in the semi-field facility for 19 weeks after transplanting (WAT). Bars represent standard error, $n = 3$. Statistically different values are indicated with $^{*}P < 0.05$ and $^{**}P < 0.01$. The small standard error, especially in the deepest depth, was due to the low of data and only just found in one replicate

the competition with maize for soil mineral nutrients and water in the upper soil layers in an intercropping system. This indicated that root plasticity might often cause crops that are grown in intercropping to grow deeper and access nutrient resources from deeper soil layers in order to avoid exploitation by the other crops in the mixture (Cardinael et al. 2015). During the experimental period, the root intensity of lucerne exceeded the intensity of red beet roots at certain intermediate soil depths, but a significant difference was only found on a few dates.

^{15}N uptake, root activity and dried biomass of semi-field intercropping

Significant differences ($P < 0.05$) in root activity between red beet and lucerne at the deepest parts of the shared root zone were observed based on the ^{15}N uptake (Figure 6). The ^{15}N content in the shoot (56.1 µg ^{15}N/chamber) and taproot (50.4 µg ^{15}N/chamber) were significantly higher in red beet than that in lucerne shoots (19.2 µg ^{15}N/chamber). These results were consistent with the study by Tribouillois

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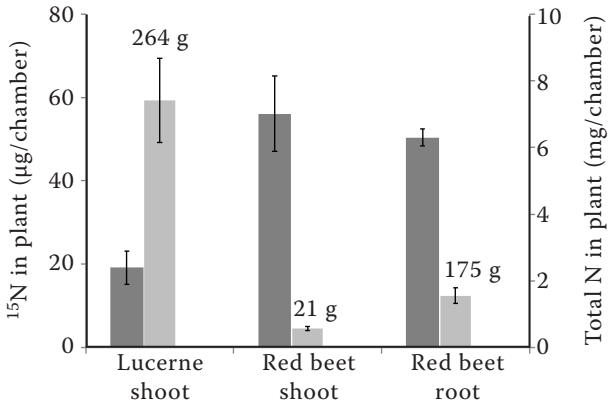

Figure 6. Uptake of ^{15}N (µg/chamber) in red beet and lucerne (dark bar chart), measured two weeks after injection of 243 mg ^{15}N/chamber at depths representing the bottom of the lucerne root system, but where red beet roots were well established. Based on this, the injection was made 2.4, 2.6 and 3.2 m in the three chambers, respectively. Pale bar chart representatives total N in the plant (mg/chamber). Dried biomass (g) of crops is indicated inside the bar chart. Mean values at different letters denote significant differences using Tukey *HSD*'s (honestly significant difference) test ($P < 0.05$), $n = 3$. Error bars denote standard error

et al. (2016), who stated that non-legume species in mixtures tend to be dominant in nitrate capture, as compared to the legume species.

The average RI for both red beet and lucerne at the injected depths was 1.83 intersections/m grid line and 0.5 intersections/m grid line, respectively. However, lucerne showed a significantly higher total N uptake than that of red beet, with the two species showing 7.41 mg/chamber and 2.1 mg/chamber, respectively. This corresponded with their biomass production, where lucerne shoot weight at harvest was 264 g/chamber, which was higher than that of red beet with 175 g tap root and 21 g shoot biomass per chamber. The depth of ^{15}N injection, which was close to the bottom of the root zone, was also validated by the soil water data (Figure 1), as the water uptake from 2.3 m depth started approximately one week before the end of the experiment, whereas no water uptake was indicated at 3.5 m of depth. The ^{15}N enrichment reflected the activity of both species in deep soil layers, in accordance with previous results showing the correlation between root intensity and ^{15}N uptake (Kristensen and Thorup-Kristensen 2004, Chen et al. 2018). However, the differences in ^{15}N uptake were stronger than the differences in root intensity. The more than five times higher ^{15}N uptake by red beet was comparable to the almost four times higher root intensity of red beet than of lucerne at the specific depths and time of ^{15}N injection. However, the difference in root intensity between the two species disappeared in the period from ^{15}N injection until sampling, so over the period of ^{15}N uptake, the difference in root intensity between the species was smaller. One explanation might be that lucerne as a legume crop has a lower affinity for soil N, and it is well known that in legume non-legume intercrops, the non-legumes tend to acquire most of the soil N, leaving the legumes to rely on their biological N fixation for N supply (Hauggaard-Nielsen et al. 2001). For example, in an intercropping system between wheat and soybean, the wheat benefitted in soil N acquisition compared to soybean, but as a whole, the intercropping system did indeed increase the total N acquisition (Li et al. 2001). Thus, in our study, the difference in competitive abilities for soil N of the legume and non-legume species might have increased the N uptake by red beet.

RI from field strip intercropping

In the red beet-lucerne strip intercropping (Figure 7), the lucerne was established in the previous autumn prior to the establishment of red beet in the spring, which was reflected by a higher RI within the lucerne strips. However, the red beet still showed fast root growth, especially in the top layer (Figure 7C, D), and had deeper soil penetration than the lucerne during the experiment despite the earlier establishment of lucerne. Lucerne roots were observed in the minirhizotrons inserted in the red beet strip close to the edge of the lucerne strip, but no red beet roots were found in the minirhizotron placed in the middle of the lucerne strip. Lucerne growth under the red beet crop was lower than that in the middle of the lucerne strip in the topsoil but not in the deeper layers (Figure 7C, D). The red beet roots were significantly affected by the neighbouring plants, and the RI of the border red beet plants was lower throughout most of the soil profile until the depth of 2.0 m during early growth stages compared to plants in the middle of the strip (Figure 7A, B). However, towards the end of the season, the red beet roots were not significantly affected by neighbouring lucerne, only in the deep soil layers below 1.6 m depth (Figure 7C, D).

The rooting depth in the kale strips was deeper than that of the red beet in the kale-red beet strip

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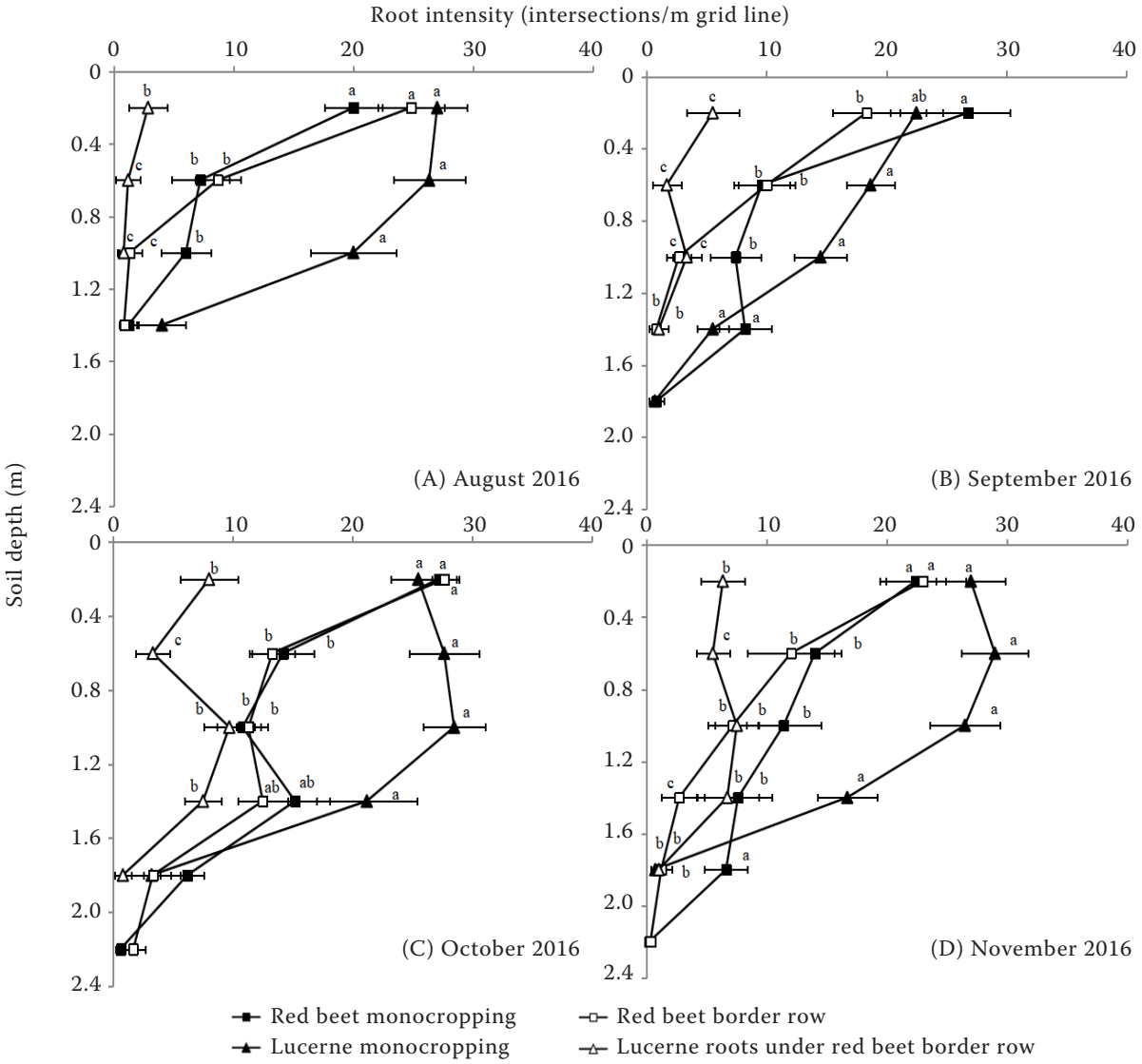

Figure 7. Root intensity (intersections/m grid line) of red beet (*Beta vulgaris*) and lucerne (*Medicago sativa*) in the field strip intercropping experiment. Root growth was measured in the monocrops in the middle of the crop strips and in the red beet border row, c. 0.5 m from the lucerne crop. Border row is the position where the minirhizotorn were placed in the red beet but at the edge close to the lucerne crop, c. 0.5 m from the edge. Statistical tests were using ANOVA followed by Tukey *HSD*'s (honestly significant difference) post hoc test. Bars represent standard error, $n = 6$. Statistically, different values are indicated with different letters where separate tests were made for each depth ($P < 0.05$)

intercropping in the early measurements (Figure 8). Kale roots were observed in the minirhizotrons placed under red beet at the border to the kale strip, but no red beet roots were found in the middle of the kale strips. The RI of kale under the red beet, however, was substantially lower than in the middle of the kale strip (Figure 8). In addition, the red beet roots in the middle of the red beet strips grew faster than the red beet grown next to the kale (Figure 8), which was clearly observed after two months of root measurements (Figure 8B). However, the growth of red beet roots was less affected by the neighbouring kale crop after three months of root measurements (Figure 8C, D). The red beet roots reached 2.4 m depth both in the middle of the strip and with a neighbouring kale crop, while the RI decreased due to seasonal changes at the end of the measurements. The field intercropping study showed that both lucerne and kale roots were able to spread into the neighbouring red beet crop and grow a substantial root intensity

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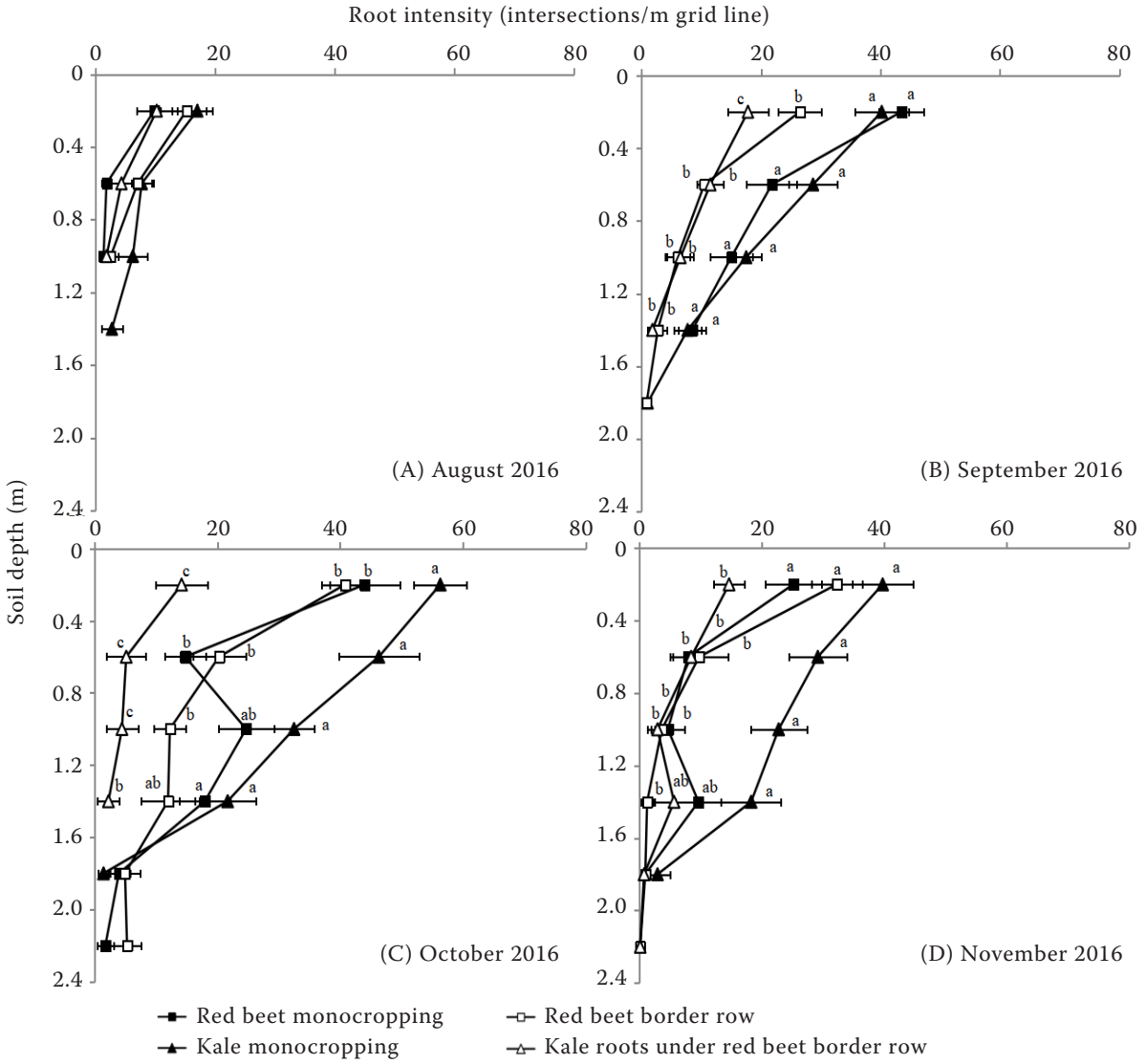

Figure 8. Root intensity (intersections/m grid line) of red beet (*Beta vulgaris*) and kale (*Brassica oleracea*) in the field strip intercropping. Root growth was measured in the monocrops in the middle of the crop strips and in the red beet border row, c. 0.5 m from the kale crop. Border row is the position where the minirhizotorn were placed in the red beet but at the edge close to the kale crop, c. 0.5 m from the edge. Statistical tests were using ANOVA followed by Tukey *HSD*'s (honestly significant difference) post hoc test. Bars represent standard error, $n = 6$. Statistically, different values are indicated with different letters where separate tests were made for each depth ($P < 0.05$)

c. 0.5 m away from the lucerne and kale crops, and that especially lucerne was able to develop a high root intensity at this distance. Several studies have shown that the horizontal spread of roots can vary among crops (Thorup-Kristensen 2006). Despite the time of establishment (delayed or simultaneously), the red beet roots were rather unaffected by intercropping with lucerne or kale. Red beet had a faster root penetration rate than the companion crops, although all of the crops were deep-rooted crops, as also reported in previous studies (Tosti and Thorup-Kristensen 2010, Andersen et al. 2014, Hassan et al. 2021). The lucerne-red beet strip intercropping data corresponded to the results of a previous study, which reported that the non-legume maize roots were able to grow deeper, extending beneath the soybean strips (Gao et al. 2010). Lucerne and kale were both seen to be able to distribute their roots horizontally into the red beet border row.

The study confirmed that the integration of direct visual measurements of root growth using species

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with coloured roots and indirect measurements of root activity by the use of tracers contributed to the success of studying root growth dynamics in different intercropping systems. The development of methods useful for observations of belowground interactions in field intercropping of different crop species is pivotal and would increase the understanding of the mechanisms that are responsible for the yield advantages of intercropping systems, crop design, and optimisation of agronomic choices in intercropping systems.

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