Imaging Rhizosphere CO_2 and O_2 Concentration to Localize Respiration Hotspots Linked to Root Type and Soil Moisture Dynamics

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Research Article

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Abstract

Purpose

Rhizosphere respiration strongly affects CO_2 concentration within vegetated soils and resulting fluxes to the atmosphere. Respiration in the rhizosphere exhibits high spatiotemporal variability that may be linked to root type, but also to small-scale variation of soil water content altering gas transport dynamics in the soil. We address spatiotemporal dynamics of CO_2 and O_2 concentration in the rhizosphere via non-invasive *in-situ* imaging.

Methods

Optodes sensitive to CO_2 and O_2 were applied to non-invasively measure *in-situ* rhizosphere CO_2 and O_2 concentration of white lupine (*Lupinus albus*) grown in slab-shaped glass rhizotrons. We monitored CO_2 concentration over the course of 16 days at constant water content and also performed a drying-rewetting experiment to explore sensitivity of CO_2 and O_2 concentration to soil moisture changes.

Results

Hotspots of respiration formed around cluster roots and CO_2 concentration locally increased to > 20 % pCO_2 (CO_2 partial pressure). After rewetting the soil, cluster roots consumed available O_2 significantly faster compared to non-cluster lateral roots. In wet soil, CO_2 accumulation zones extended up to 9.5 mm from the root surface compared to 0.3-1 mm in dry soil.

Conclusion

Results from this imaging experiment indicate that respiratory activity differs substantially within the root system of a plant individual and that cluster roots are hotspots of respiration. As rhizosphere CO_2 and O_2 concentration was strongly sensitive to soil water content and its variation, we recommend monitoring the soil water content prior and during the measurement of rhizosphere respiration.

Introduction

Respiration by plant roots can seasonally account for the majority of soil CO_2 production (Hopkins et al. 2013; Hanson et al. 2000) and substantially impacts CO_2 concentration within and fluxes from vegetated soils. The rhizosphere, defined as the volume of soil influenced by the activity of roots (Hinsinger et al. 2009), represents a hotspot of respiration: autotrophic respiration of living root tissue combined with the high abundance of microorganisms that decompose root exudates and other rhizosphere deposits leads to increased formation of CO_2 and consumption of O_2 within this region (Kuzyakov and Blagodatskaya, 2015). As both root and microbial respiration occur simultaneously and are difficult to separate, we refer to all respiration processes in the rhizosphere as “rhizosphere respiration” (as proposed by Kuzyakov, 2006). Similar to other physicochemical gradients and biological process rates in the rhizosphere,

respiration and the resulting distribution of CO_2 and O_2 concentration exhibits high spatiotemporal heterogeneity (Kuzyakov and Razavi 2019). Within a plant individuals' root system, respiration rates can differ substantially along with photoassimilate allocation, growth rate or tissue N content (Lambers et al., 2002). Small-scale variation of soil properties, such as porosity and connectivity of the pore space, and particularly soil water content, affect soil aeration and gas transport dynamics, which control the amount of oxygen available for aerobic respiration and the local accumulation of CO_2 (Ben-Noah and Friedman 2018). In our study, we address the spatiotemporal dynamics of CO_2 and O_2 concentration in the rhizosphere of white lupine together with root system development and soil moisture changes via non-invasive imaging using planar optodes.

The application of planar optodes enables visualization and quantification of rhizosphere respiration with an emphasis on capturing spatial and temporal heterogeneity (Freschet et al. 2021). Planar optodes are fluorescent sensor foils sensitive to e.g. pH, CO_2 or O_2 concentration (Blossfeld et al. 2013; Rudolph-Mohr et al. 2013; Rudolph-Mohr et al. 2014) and can be used to measure rhizosphere processes under controlled environmental conditions at the millimeter to decimeter scale (Oburger and Schmidt 2016; Santner et al. 2015). So far, studies applying CO_2 optodes in the rhizosphere were limited to wetland plants grown in saturated or submerged soil (Lenzewski et al. 2018; Koop-Jakobsen et al. 2018) where pCO_2 (CO_2 partial pressure) is higher and thus easier to detect (Blossfeld et al. 2013). Only recently, Holz et al. (2020) tested the application of CO_2 optodes in unsaturated soil and found that volumetric soil water content significantly altered CO_2 concentration measured around maize roots. In contrast, O_2 optodes have been applied in unsaturated soils more frequently. Rudolph-Mohr et al. (2015) visualized O_2 consumption by pesticide treated lupine roots; and Rudolph-Mohr et al. (2017) showed that O_2 concentration gradients in the rhizosphere of maize depend on root type. Moreover, imaging via O_2 optodes has been combined with using pH optodes at the same time (Rudolph-Mohr et al. 2014). However, a systematic investigation of both CO_2 and O_2 concentration in the rhizosphere of non-wetland plants in unsaturated soil *in-situ* over a longer time period (weeks) and under varying soil moisture conditions is not yet available.

Soil water content impacts rhizosphere respiration as it affects the availability of O_2 (Ben-Noah and Friedman 2018). Diffusive gas transport is decelerated considerably at high volumetric soil water content; this strongly restricts O_2 supply from the atmosphere into the soil and towards the plant roots while CO_2 produced by rhizosphere respiration accumulates in the soil. As a result, CO_2 fluxes measured shortly after irrigation or rainfall can lead to a substantial underestimation of soil CO_2 concentration, particularly in fine-textured soils (Bouma and Bryla 2012). Several field studies show that drying-rewetting cycles impact soil CO_2 flux and respiration rates (Morillas et al. 2017; Zhu and Cheng 2013; Min et al. 2020), and such short-term variations in soil moisture often occur under natural conditions. Studies addressing the dynamics of respiration at the rhizosphere scale as a function of soil moisture and its short-term fluctuation are lacking because of methodological difficulties of observing root-soil interaction *in-situ*.

We measured CO_2 and O_2 concentration in the rhizosphere of white lupines over the course of 19 days applying planar optodes and investigated the effect of variations of soil moisture. We chose white lupine (*Lupinus albus*) as it is a well-studied model plant with agricultural relevance (Neumann and Martinoia 2002). *Lupinus albus* features distinct physiological adaptation mechanisms under phosphorous (P) limited conditions. The plants invest a particularly large amount of carbon in the growth of cluster roots (Funayama-Noguchi et al., 2020) which release a high quantity of exudates such as citrate (Dinkelaker et al. 1989) and other organic acids (Watt and Evans 1999) to solubilize otherwise unavailable P resources. Experiments on hydroponically grown white lupines suggest that cluster roots can exhibit increased respiration rates and that root tissue nitrogen (N) content may positively correlate with respiratory activity (Langlade 2002; Funayama-Noguchi et al. 2020; Kania et al. 2003). We quantified effects of rhizosphere respiration of white lupines grown in soil via non-invasive mapping of pCO_2 and pO_2 and hypothesize that 1) rhizosphere respiration of *Lupinus albus* shows distinct spatiotemporal heterogeneity linked to root type, diurnal variation of plant activity and root tissue N content and that 2) the magnitude of measured CO_2 and O_2 concentration in the rhizosphere is highly sensitive to fast changes in water content.

Materials And Methods

To assess the spatiotemporal variability of respiration along with root system development of *Lupinus albus* and changes in soil water content, we conducted three experimental time series. First, we measured rhizosphere pCO_2 daily with soil water content kept constant and statistically located hotspots of respiration activity during 16 days. Second, we quantified the diurnal variation of respiratory activity by repeated measurements of pCO_2 during the photoperiod of selected days within this period. Finally, we conducted a 3-days drying-rewetting experiment to investigate the sensitivity of CO_2 and O_2 concentration to fast changes in soil water content. After these 19 days, we harvested and analyzed the roots from regions where pCO_2 strongly increased or pO_2 strongly decreased after rewetting to correlate root tissue N content and respiratory activity.

Rhizotron preparation and plant growth

We prepared five glass rhizotrons (150 mm x 150 mm x 15 mm) with planar optodes sensitive to CO_2 and two of them additionally with O_2-sensitive optodes. The CO_2 optodes (range: 1-25 % pCO_2, size: 80 mm x 104 mm, product code: SF-CD1R, PreSens GmbH, Regensburg, Germany) were equilibrated in buffer solution (pH = 7.5) over night and then glued to the inner front windows (plants L1 – L5). The O_2 optodes (size: 130 mm x 105 mm, manufactured as described in Rudolph et al., 2012) were attached to the inner back sides of two of the five rhizotrons (plants L4 and L5). Sandy soil (91 % sand, 8 % silt and 1 % clay, calcium acetate lactate (CAL) extractable P 8.6 mg kg^{-1}, total N 0.01 %, total C 0.13 %, and $pH_{(CaCl2)}$ 7.6) was sieved to < 2 mm and filled horizontally into the rhizotrons (mean bulk density: 1.45 g cm^{-3}). Seeds of white lupine (*Lupinus albus*) were sterilized in 70 % ethanol and planted after germination. Each plant was initially watered with 85 ml of a nutrient solution (containing 7% N, 3% P_2O_5, 6% K_2O and

micronutrients, as described in Rudolph-Mohr et al., 2017). Water was added to obtain an initial volumetric water content of 0.30 cm^3 cm^{-3}, which is equivalent to 77 % of saturation water content. After plant emergence, a gravel layer of 10 mm was placed at the soil surface to minimize evaporation. The water content was re-adjusted every morning to 0.30 cm^3 cm^{-3} by irrigating from the top; no further nutrients were supplied throughout this experiment to obtain P-deficient conditions and stimulate cluster root development. The lupines were grown under controlled conditions in a plant growth chamber ($temperature_{day}$ 24 °C, $temperature_{night}$ 19 °C, 14 hours photoperiod with a light intensity of 300 µmol m^{-2} s^{-1}, relative humidity 60 %). Light intensity was increased from 0 % to 100 % between 6 a.m. and 10 a.m. and ramped down again to 0 % between 4 p.m. and 8 p.m.; temperature was changed between 19 °C (night) and 24 °C (day) accordingly. All samples were kept in an upright position, so roots distributed in soil towards both sides of the rhizotrons. The rhizotrons were covered with aluminum foil to protect the optodes from photobleaching.

Imaging of CO_2 and O_2 concentration

CO_2 concentration was monitored with VisiSensTD, a commercial 2D fluorescence imaging and readout system (PreSens Precision Sensing GmbH, Regensburg, Germany). The CO_2 optodes contain two fluorescent dyes (one sensitive to changes in pCO_2, the other acting as a reference dye). A ring light source (built into the camera lens) and two external blue LEDs (wavelength 450 - 550 nm) were used to excite the fluorescent dyes. The fluorescence intensity was captured with an RGB camera (1292 x 964 pixels) at an exposure time of 70 ms and the signal ratio of the red and green channel (red:green ratio) was stored pixelwise. To convert this information into CO_2 concentration (pCO_2 in %), a calibration curve was fitted. For calibration, two pieces of CO_2 optode were equilibrated overnight in a buffer solution (pH = 7.5, ionic strength = 40mM) and then fixed inside a small glass box filled with a similar buffer solution. The solution was flushed with gas mixtures of stepwise increasing CO_2 concentration between 0 % and 25 % pCO_2 and images were captured every 60 seconds at each calibration point until the signal was stable (taking between 15 and 20 minutes per concentration step). The calibration curve was fitted using the software VisiSens AnalytiCal (PreSens Precision Sensing GmbH). Fluorescence images (pixelsize 213 µm) were captured and directly converted to pCO_2 maps in the software VisiSens AnalytiCal via the calibration curve.

The oxygen optodes were prepared according to Rudolph et al. (2012), with platinum (II) 5,10,20,-terakis(2,3,4,5,6-pentafluorophenyl)porphyrin as fluorescent dye incorporated into a polystyrene matrix. The optodes were calibrated in water with O_2-concentration between 0 mg L^{-1} and 10 mg L^{-1} and a calibration curve was fitted based on the measured fluorescence intensities (as described in Rudolph et al., 2012). Fluorescence signals after excitation with UV light (type 215 L, Peqlab, Erlangen, Germany) were captured with a camera (Kappa DX 4C-285 FW) with a 500 nm long-pass filter and a cooled CCD sensor (1392 x 1040 pixels). The gray-value images (pixel size: 219 µm) were converted to O_2-concentration maps based on the fitted calibration curve in MATLAB R2020(a) (The MathWorks).

Time series of rhizosphere pCO_2 at constant water content and diurnal variation of rhizosphere pCO_2 (experiment 1 & 2)

Experiment 1.We monitored pCO_2 in the soil every day until day 16 after planting (DAP 16) to be able to identify hotspots of respiration amongst the growing root systems and the rhizosphere. As the only study to date applying CO_2 optodes in unsaturated soil (Holz et al. 2020) suggests that measured magnitude of rhizosphere CO_2 concentration is strongly influenced by soil moisture, we kept the volumetric soil water content constant at 0.30 $cm^3\ cm^{-3}$ by irrigation every morning at 8:30 a.m. and always conducted the measurements 30 minutes after adjusting the water content to enable comparisons across plant individuals and root replicates.

Experiment 2. Additionally, we explored diurnal variations of respiration by measuring rhizosphere pCO_2 in the morning (9:00 a.m.), at noon time (1:00 p.m.) and in the late afternoon (5:00 p.m.) on DAP 5, 8, 12 and 14 of experiment 1. On these four days watering to 0.30 $cm^3\ cm^{-3}$ took place at 8:30 a.m. as usual, but was re-adjusted also 30 minutes prior to the second and third measurement of the day, if water content varied by more than 0.02 $cm^3\ cm^{-3}$. The first measurement in the morning took place 3 h after start of illumination, the second 3 h after reaching 100% illumination and the last measurement 3 h before the light in the plant growth chamber was turned off for the night.

Changes of pCO_2 and pO_2 after rewetting of dry soil (experiment 3)

Experiment 3. The third section of the experimental time series aimed for quantification of pCO_2 and pO_2 in the rhizosphere following a fast increase in soil moisture. For that we stopped irrigation on DAP 16 and in the following conducted a drying-rewetting experiment. Water content declined to 0.10 $cm^3\ cm^{-3}$ (26 % of saturation water content) on DAP 19 and we measured CO_2 concentration (all five plants L1-L5) and O_2 concentration (plants L4 and L5) in the dry soil. Afterwards, the rhizotrons were rewetted to 0.30 $cm^3\ cm^{-3}$ (77 % of saturation water content) from the bottom. Then CO_2 and O_2 concentrations were measured directly (0.2 h) after rewetting as well as 1 h, 2 h, 3 h, 4 h and 5 h after increasing soil moisture.

To limit stress during our experiments, plants were only briefly taken out of the plant growth chamber to a darkroom for imaging and returned directly afterwards. In the darkroom the rhizotrons were placed in a sample holder mounted on a table to ensure that they were always aligned in the same position relative to the camera.

Measurement of root position and cluster root development

Since the CO_2 optodes include an optical isolation layer, it was not possible to capture optical images of the precise location of roots systematically without removing the optode. To avoid disturbing gas transport dynamics in the soil, we did not open the rhizotrons or remove the optodes until the end of all experiments. However, several cluster and lateral roots or root segments were visible through the optode and we could trace their position with a pen on the glass window. These regions were later used for

quantitative analysis of root zone pCO_2 in experiment 1 and 2. After the drying-rewetting experiment on DAP 19, we opened the rhizotrons, removed the CO_2 optodes and captured images of the exposed root systems (plant age: 21 days) to locate the position of all roots growing along the optodes. Plants L1, L2, L3 and L5 had developed several cluster roots close to the CO_2 optode. Just plant L4 grew only lateral roots without clusters close to the CO_2 optode (**Fig. S1**). The O_2 optodes are semi-transparent and therefore we could trace roots directly from images taken at ambient light conditions. Both L4 and L5 grew cluster roots close to the oxygen optode. After imaging the opened rhizotrons, we washed the root systems carefully to remove soil particles and captured images to determine the extent of cluster root development amongst the entire root system.

Root sampling for nitrogen (N) content analysis

Based on the fluorescence image time series captured during the drying-rewetting experiment (DAP 19), we selected regions of considerably higher and lower respiratory activity (considering both CO_2 and O_2 concentration) and took root samples there. We did not distinguish between cluster and lateral roots during sampling, but only selected roots growing close to the optodes. The sampled roots and root segments were dried at 60 °C for at least 48 h and then ground for analysis. Root carbon (C) and nitrogen (N) contents as well as the C:N ratio were determined in two replicates per region by elemental analysis (Euro EA 3000 Elemental Analyser, HEKAtech GmbH, Wegberg, Germany).

Image analysis

All images were registered with the Plugin “Stackreg” in ImageJ prior to further analysis. CO_2 concentration (in % pCO_2) was directly calculated from the fluorescence images in the VisiSens AnalytiCal software and saved as TIFF images. O_2 concentration was calculated in MATLAB R2020a as described in Rudolph-Mohr et al. (2017) and converted to % pO_2.

We statistically located hotspots of CO_2 concentration in the rhizosphere following the approach suggested by Bilyera et al. (2020). First, we converted the CO_2 image time series of each plant to 8-bit gray value maps of pCO_2 and saved the histogram of gray values of each image (MATLAB R2020a). The gray value distribution was then statistically split into two distributions (package “mixtools” in RStudio, Bengalia et al., 2009) to separate hotspots from background. Pixels were classified as hotspots when the gray value was higher than the mean + 3SD (three times the standard deviation) of the background pixel values (Bilyera et al. 2020). The hotspot area (in mm²) was calculated by multiplying the number of hotspot pixels by the pixel size and was compared to the total area covered by the optode.

Diurnal variation of pCO_2 was compared in selected regions of interest (10 x 10 pixel, approx. 4 mm²) close to roots that were visible through the optode (cluster roots: n = 7, lateral roots: n = 18 on DAP 14) and within the bulk soil (n = 25). To compare rhizosphere respiration during the drying-rewetting experiment on DAP 19, we first segmented roots growing close to the optodes from the images of the exposed root systems captured after opening the rhizotrons (“SmartRoot” Plugin in ImageJ, Lobet et al.,

2011). We then interactively selected a total of 47 non-overlapping roots or root segments from the binary images obtained from segmentation (“drawpolygon” and “poly2mask” function, Image Processing Toolbox, MATLAB R2020a). CO_2 and O_2 concentration as a function of distance to the root surface was calculated using the Euclidean distance transform (via “bwdist” function in Matlab). We graphically estimated the extent of CO_2 accumulation resp. O_2 depletion zones at different volumetric soil water contents by fitting local regression curves (function “loess” in RStudio) to the mean CO_2 resp. O_2 concentration with increasing distance from the roots.

Statistics

Measured CO_2 and O_2 concentration in the root zone and the bulk soil were analyzed for normality and homogeneity of variances applying Shapiro Wilk’s test and Levene’s test, respectively. Differences between cluster and lateral roots as well as the effect of soil water content were tested for statistical significance using Kruskal-Wallis test followed by a Wilcoxon test. C and N content and C:N ratio of roots from regions of high vs. low respiration was compared pairwise also applying a Wilcoxon test. All statistical tests were computed at a significance level of $\alpha < 0.05$ in RStudio (R Core Team, 2020).

Results

Experiment 1: time series of rhizosphere pCO_2 at constant water content

During the first 16 day after planting (until DAP 16), pCO_2 was measured every morning at 9:00 a.m. at constant water content (0.30 $cm^3\ cm^{-3}$). Initially, the tip of the taproot and young parts of the growing lateral roots released most CO_2 (Fig. 1a). Between DAP 10 to 13, all plants with the exception of L4 developed cluster roots close to the CO_2 optode where large, overlapping hotspot areas formed and local CO_2 concentration increased to a maximum of 22.8 % pCO_2 (DAP 16, Fig. 1a). At that stage the CO_2 hotspots ($pCO_2 \geq$ mean background concentration + 3SD) covered 27 % of the optode area (Fig. 1c). Plant L4 grew no cluster roots in direct vicinity to the CO_2 optode (Fig. S1). We measured lower overall CO_2 concentration (Fig. 1b) with a maximum of 8.6 % pCO_2 at the root surface (DAP 16) and smaller hotspot areas (max. 0.7 % of the area covered by the optode, Fig. 1d) in the rhizosphere of this plant. Despite plant L4 formed multiple cluster roots elsewhere in its root system (images of washed root systems of L3 and L4 in Fig. S1), their effect on CO_2 concentration was not measurable because they grew at some distance to the optode. In general, hotspot area increased over time as more roots developed and CO_2 from rhizosphere respiration accumulated in the soil as the high water content decelerated gas exchange with the ambient air.

Experiment 2: Diurnal variation of pCO_2 in the lupine rhizosphere

Rhizosphere CO_2 concentration increased between morning and noon (9 a.m. to 1 p.m., Fig. 2, center panels). In certain regions, pCO_2 continued to rise until the afternoon (5 p.m.), but already decreased in

other parts of the root system (Fig. 2, right panels).

Comparing different individual root regions across all plant individuals (Fig. 3a) shows that pCO_2 around some root segments peaked at noon or increased throughout the afternoon, but other roots did not exhibit a clear diurnal variation in respiration and pCO_2 remained close to constant. Close to several cluster roots near the CO_2 optode, CO_2 concentration strongly increased, but pCO_2 around some lateral roots was within the same order of magnitude (Fig. 3a). The smallest diurnal variation of pCO_2 was measured for plant L4 (no cluster roots close to the CO_2 optode). Average root zone CO_2 concentration (mean ± SD) was 8.7 ± 3.9 % pCO_2 and 5.3 ± 3.3 % for cluster and lateral roots, respectively, in the morning and 11.7 ± 4.5 % pCO_2 vs. 7.1 ± 4.3 % in the afternoon. However, due to the pronounced heterogeneity between the selected root segments and the resulting scatter of the morning and afternoon values it could not be shown with statistical significance that the mean CO_2 concentration was higher in the afternoon than in the morning (Fig. 3b). Nevertheless, CO_2 concentration in the selected root regions individually increased significantly ($p<1.7*10^{-7}$) from morning to afternoon, with an average rate of 0.27 % pCO_2 h^{-1}. Thus, we can conclude that pCO_2 in the rhizosphere of the selected root segments increased statistically significantly from morning to afternoon and that the individual afternoon value in a root segment is significantly larger than its morning value.

Mean bulk soil CO_2 concentration increased significantly ($p=0.0098$) from 1.82 ± 0.75 % pCO_2 in the morning to 2.34 ± 0.84 % pCO_2 in the afternoon (mean ± SD, n= 25, Fig. 3b). This was likely caused by the diffusive spread of CO_2 also released from rhizosphere respiration in parts of the root system located at greater distance to the optode.

Experiment 3: Sensitivity of rhizosphere pCO_2 and pO_2 to changes in soil water content

Hotspots of respiration form after rewetting from dry conditions

After three days without irrigation (DAP 16 to 19), the rhizotrons were rewetted from 0.10 cm^3 cm^{-3} to 0.30 cm^3 cm^{-3} and pCO_2 and pO_2 were measured hourly. In general, CO_2 concentration increased around the roots after rewetting and continued to rise over the course of five hours. Fig. 4a shows the evolution of pCO_2 of plant L2, where the increase of CO_2 around cluster roots after rewetting was most pronounced (images of other plants in Fig. S2). Similar increase and hotspot formation after rewetting was observed for the other plants except plant L4, where no cluster roots grew close to the CO_2 optode. Three hours after rewetting, CO_2 concentration at the surface of cluster roots growing close to the optodes was significantly higher than at the lateral root surface (Fig. 5a, $p<0.05$). Statistically defined hotspot area of the plants with cluster root abundance near the CO_2 optode increased (Fig. S3). However, for plant L3 hotspot area remained < 3 % after rewetting (Fig. S3) despite pronounced cluster root abundance close to the CO_2 optode and high rhizosphere CO_2 concentration observed until DAP 16 (see Fig. 1). This could be due to cluster root maturation and associated decrease of respiration activity, but we could not track the exact age of the root segments in this experiment.

For plants L4 and L5, O_2 concentration was measured by an optode attached to the back side of the rhizotron. Immediately after rewetting, regions of high oxygen consumption formed, and after only one hour, pO_2 measured at the surface of cluster roots was significantly lower than concentrations at the surface of lateral roots (Fig. 5b). Five hours after rewetting, most of the available oxygen in the rhizosphere was consumed and the depletion zones of different roots overlapped in large parts (plant L5: Fig. 4b, center and last panel; this was similar for plant L4 (not shown)). Oxygen consumption around cluster roots was faster and more pronounced compared to lateral roots (Fig. 5b).

Extent of CO_2 accumulation and O_2 depletion zones depend on soil water content and root type

The spatial extent of the CO_2 accumulation zone around roots varied with changes in soil water content and differed between root types (Fig. 6, Tab. S1). In wet soil (daily irrigation to 0.30 $cm^3\ cm^{-3}$, measured on DAP 16), the region of increased CO_2 concentration extended approx. 8 mm from the cluster root surface, but only ≤ 0.3 mm in dry soil (0.10 $cm^3\ cm^{-3}$, three days after irrigation was stopped, DAP 19, Fig. 6). After rewetting the soil to 0.30 $cm^3\ cm^{-3}$, the CO_2 accumulation zone expanded rapidly up to 9.5 mm. At the cluster root surface, CO_2 concentration was significantly higher ($p < 0.001$) in wet and rewetted soil than in dry soil (Tab. S1). Around lateral roots, gradients of pCO_2 extended ~ 1 mm from the root surface in dry soil. Despite similar water content (0.30 $cm^3\ cm^{-3}$), the pCO_2 gradients from the surface of the lateral roots extended twice as far from into the rewetted (4-5 mm) than into the wet soil (~ 2 mm). And though soil water content in wet and rewetted soil was similar, lateral root surface and bulk soil CO_2 concentration 5 h after rewetting exceeded values measured in wet soil ($p<0.05$, Tab. S1).

After rewetting, the O_2 depletion zone extended > 10 mm from the cluster root surface, but only ~ 5 mm from the lateral root surface (Fig. S4). One hour after rewetting, pO_2 at the cluster and lateral root surface was lower than in wet soil prior to drying ($p<0.05$, Tab. S1).

Respiration hotspots and root tissue N content

Roots from the regions with the highest as well as the lowest change in CO_2 or O_2 concentration during the drying-rewetting experiment were sampled and root tissue N and C content was measured. The roots from regions with high rhizosphere respiration contained significantly less N and C (Tab. 1).

Tab. 1: Results of C/N-analysis for regions exhibiting high vs. low respiration after rewetting, n=5 plants. Significant differences between the regions following pairwise Wilcoxon test ($\alpha < 0.05$) are highlighted with * ($p \leq 0.05$) and ** ($p \leq 0.01$).

	High respiration activity after rewetting	Low respiration activity after rewetting
N (mg / g dry weight, mean ± sd)	25.9 ± 6.7	37.0 ± 2.5 **
C (mg / g dry weight, mean ± sd)	218.9 ± 49.2	297.6 ± 19.3 *
C:N ratio	8.6 ± 0.8	8.0 ± 0.2

Discussion

Spatiotemporal variability of rhizosphere respiration is linked to root type

Our experimental results confirm the hypothesis that rhizosphere respiration varies between root types among the root system of the same plant individual. Cluster roots represent a unique form of physiological adaption in the root system of white lupine and form in response to P and iron deficiency (Pueyo et al., 2021) or dry conditions (Dara et al., 2015). We show that around cluster roots of *Lupinus albus* hotspots of elevated CO_2 concentration form. As one of five investigated plants did not develop cluster roots close to the CO_2 optode, the impact of cluster roots on the overall level of detected CO_2 concentrations became obvious. Statistically separating hotspots from background CO_2 concentration as suggested by Bilyera et al. (2020) allowed for a quantitative interpretation of image time-series. Hotspot area strongly increased for all plants (> 27 % of optode area) that developed cluster roots close to the CO_2-optode, but remained small for the plant where cluster roots were not present in the vicinity of the CO_2 sensor (hotspot area < 1 %). Our finding that cluster roots represent hotspots of rhizosphere respiration activity match results from Funayama-Noguchi et al. (2020) who measured strongly increased O_2 consumption of cluster compared to lateral roots of white lupine. Furthermore, Langlade (2002), Neumann et al. (2000) and Kania et al. (2003) measured higher respiration rates of (young) cluster roots of *Lupinus albus* (measured as O_2 consumption of excised roots) compared to the apical part of non-cluster roots.

Differences of rhizosphere pCO_2 and pO_2 between root types were most pronounced after rewetting the soil from dry conditions: around cluster roots, oxygen consumption was significantly faster and CO_2 release significantly higher compared to lateral root segments without clusters. In contrast to the mentioned studies where (autotrophic) root respiration rates were quantified for soil-free roots, we refer to rhizosphere respiration only as the sum of CO_2 released respectively O_2 consumed by roots themselves and by rhizomicrobial respiration. Microbial respiration can constitute more than 50 % of rhizosphere respiration (Kuzyakov and Larionova 2005) and is strongly enhanced when high amounts of organic compounds are available, e.g. via rhizodeposition and root exudation. Yin et al. (2020) showed via non-invasive imaging of white lupine root systems grown in soil that root allocated C was released in hotspots where cluster roots were present. Also, cluster roots release higher amounts of citrate into the

rhizosphere than non-cluster roots (Dessureault-Rompré et al. 2007). This could lead to increased microbial respiration activity specifically in the areas directly surrounding these root structures and explain the strong increase of pCO_2 we observed around cluster roots.

Magnitude and extent of CO_2 accumulation and O_2 depletion zones are highly sensitive to soil water content

Soil water content strongly altered pCO_2 and pO_2 at the root surface as well as the extent of CO_2 and O_2 gradients around the roots. Around cluster roots, CO_2 and O_2 gradients extended up to 9.5 mm and more than 10 mm, respectively, in moist soil (0.30 cm^3 cm^{-3} volumetric soil water content), but decreased to 0.3 mm and 1 mm in dry soil (0.10 cm^3 cm^{-3}). This confirms results by Rudolph-Mohr et al. (2017) where O_2 gradients measured via optode imaging extended up to 8 mm from the surface of maize roots in wet soil but only approx. 2 mm in dry soil. For young maize roots, Holz et al. (2020) reported a CO_2 concentration gradient in saturated soil of ~ 3 mm, close to values we measured for non-cluster lateral roots of lupine in wet soil. Two processes might explain the sensitivity of absolute rhizosphere CO_2 and O_2 concentration to soil water content: First, a high ratio of water-filled pore space results in accumulation of CO_2 and formation of oxygen depletion zones due to rhizosphere respiration as diffusive gas transport is restricted in contrast to dry, well-aerated soil. Second, volumetric soil water content positively correlates with respiration rates in soils (Morillas et al. 2017) as low moisture content may inhibit microbial population growth and respiration activity (Unger et al. 2010). Pronounced intensification of respiration in vegetated soil after rewetting of dry soil has been reported as strong and fast increase of soil moisture can boost decomposition of dead microbial biomass (Unger et al. 2010; Morillas et al. 2017). The severity of soil water content change (or soil water potential change) also impacts respiration rates: Zhu and Cheng (2013) found that rhizosphere respiration declined by up to 23 % in a sunflower field subject to prolonged severe drying-rewetting cycles but was not significantly altered in a soybean field under moderate drying-rewetting conditions.

In summary, the actual soil water content, as well as its variations prior and during respiration measurements, needs to be reported along with CO_2 and O_2 concentration in order to enable informed comparisons of absolute values and respiration activity.

Diurnal variation of rhizosphere respiration

We observed that CO_2 concentration in the rhizosphere locally increased from morning to afternoon along with the diurnal course of illumination and temperature. Allocation of assimilates from photosynthesis to roots for respiration often follows the diurnal course of photosynthesis (Ben-Noah and Friedman 2018) and both autotrophic respiration by roots and rhizomicrobial respiration are linked to photosynthetic activity of plants (Kuzyakov and Gavrichkova, 2010). Exudation of C-compounds supplying the substrate for microbial respiration also varies throughout the day. Dessureault-Rompré et al. (2007) report a diurnal pattern and multiple instances of increased exudation in the afternoon by cluster roots of white lupine plants. Watt and Evans (1999) measured the highest exudation rates of white lupine roots during the

photoperiod. These results could explain why we observed an increase in pCO_2 in distinct regions during the photoperiod. However, some roots showed CO_2 concentration peaking at noon and declining in the afternoon, or even no diurnal variation of respiration. The underlying spatial heterogeneity of respiration within the individuals' root systems indicates that also root age, development stage and growth rate could have affected the respiration activity. Several studies suggest that respiration rates of cluster roots decrease with progressing senescence: Massonneau et al. (2001) measured a decline in O_2 consumption rate in mature and senescent cluster roots (where no further growth occurs) by up to 80 %, Kania et al. (2003) report a decrease of 60 % compared to respiration rates measured in juvenile, still developing cluster roots. Similar to respiration rates, exudation rates of cluster roots are linked to their development stage: mature cluster roots excrete more citrate than young cluster roots (Wasaki et al. 2005; Massonneau et al. 2001). Yin et al. (2020) show that even though hotspots of C-exudation occurred where cluster roots were present, not all cluster roots formed hotspots, likely because they differed in their stage of development. In our study, we could not clearly separate the cluster roots by their development stage, as we did not remove the optodes before the last day of the experiment. However, the first cluster roots close to the CO_2 optodes emerged on day 10 after planting and more cluster roots developed over time, so that by day 14 (where we quantified diurnal variations) cluster roots of different development stage were certainly present. As we only compared CO_2 concentrations within the photoperiod, further investigations of a full diurnal cycle and a combination of pH optode imaging (e.g. Rudolph-Mohr et al. 2013, 2014) now also with CO_2 optode imaging could reveal further insights.

Hotspots of respiration and root tissue N content

We observed the formation of distinct hotspots of increased respiration after rewetting the soil from 0.10 $cm^3\ cm^{-3}$ to 0.30 $cm^3\ cm^{-3}$ on DAP 19 and compared C and N content as well as C:N ratio from roots sampled in hotspots to regions of low respiratory activity. The N content of roots attributed to respiration hotspots was significantly lower than that of roots sampled from regions of lower respiration activity after rewetting (26 mg N g^{-1} dry weight vs. 37 mg N g^{-1} dry weight). This is contrary to the findings of Funayama-Noguchi et al. (2020) who reported a positive correlation of root respiration rate and tissue N content of white lupine roots. Several studies investigating fine roots of trees also found that root respiration rates increase with root N content (Hishi 2007; Jia et al. 2013; Pregitzer et al. 1998). In contrast to these studies, we did not determine specific root respiration rates based on root weight, but measured changes of CO_2 and O_2 concentration induced by root and rhizomicrobial respiration. As we observed strong variations and heterogeneity of respiration not only after rewetting, but also throughout the previous growing period, it is likely that the regions we classified as "low" vs. "high" respiratory activity where roots were sampled for C/N analysis were representative of the time of sampling rather than the conditions over the duration of the experiment. For example, we observed that one cluster root formed a CO_2 hotspot on DAP 16 but showed lower respiration activity after rewetting compared to other regions that previously had not been classified as hotspots. Furthermore, we did not differentiate between younger and older root tissue or cluster vs. non-cluster roots for sampling. However, Funayama-Noguchi et al. (2020) did not find significant differences of cluster root tissue C and N content compared to non-

cluster roots of white lupine under P-deficiency. Thus, it is not fully clear if our sampling concept was not able to represent the general behavior or if exudation with the inherent transfer of C into the rhizosphere induces a higher respiration activity outside roots leading to an overall higher respiration activity.

Methodological considerations

We have demonstrated that non-invasive imaging with planar optodes is suitable for the quantification of rhizosphere respiration at the root system scale over several weeks and that this imaging technique can be applied to measure pCO_2 and pO_2 at soil moisture levels between 0.10 and 0.30 $cm^3\ cm^{-3}$. In contrast to the O_2 optodes we used, which can be applied in both the gaseous and aqueous phase, it is important to notice that the CO_2 optodes (PreSens SF-CD1R) only function as long as the sensor matrix remains hydrated. As the CO_2 optodes can be irreversibly damaged by desiccation, we conducted several preliminary experiments where we repeatedly dried and rewetted test samples to different volumetric soil water contents. We found that in our set-up the CO_2-optodes still function when the soil is at 10 % volumetric water content (average, determined gravimetrically), but were irreversibly damaged for a volumetric soil water content falling below 5 %. As discussed in Holz et al. 2020, comparison of pCO_2 measurements between plants or treatments can be biased by variations of soil water content, which was avoided in our experiment by daily adjustment of the water content to 0.30 $cm^3\ cm^{-3}$ 30 minutes prior to imaging. During the drying-rewetting experiment we specifically addressed the variation in pCO_2 along with soil moisture changes, but the lowest water content during the course of the experiment was 10 % to protect optodes from damage. Since we found a strong sensitivity of CO_2 concentrations to soil water content, the precise measurement of local soil moisture distribution within the rhizotron could be addressed additionally by complementary neutron radiography. Combination of O_2 optodes and neutron radiography showed that oxygen consumption gradients towards maize roots were linked to gradients of water content (Rudolph-Mohr et al. 2017). Moradi et al. (2012) show that local water content at the root-soil interface is altered during drying-rewetting cycles affecting gas transport close to the root surfaces, similar to the presence of a mucilage layer slowing down diffusive gas transport (Ben-Noah and Friedman 2018). Furthermore, optical imaging alone cannot detect root structures growing in greater distance to the optodes. We could not quantify the impact of rhizosphere respiration of roots located not directly close to the optode on measured soil pCO_2 in this study, but observed a significant increase in “bulk soil” concentration over the course of the day and after rewetting. However, the regions we classified as bulk soil could also be influenced by roots close, but not directly growing at the optode surface. Recently, Rudolph-Mohr et al. (2021) combined O_2 optodes, neutron radiography and 3D neutron laminography and demonstrated that oxygen consumption by maize roots growing in up to 7.5 mm distance to the rhizotron window (where the O_2 optode was attached) could be detected.

Conclusions

In this study, we have illustrated the spatiotemporal heterogeneity of CO_2 and O_2 concentrations in the rhizosphere of white lupine plants and demonstrated that planar optodes can be applied to monitor

rhizosphere respiration over the course of several weeks at different soil moisture contents. We conclude from our results that respiration activity strongly differs within the root system, where particularly cluster roots represent respiration hotspots. However, measurements of rhizosphere respiration, specifically CO_2 concentrations, are highly sensitive to water content and fast changes of soil moisture. We therefore suggest to also indicate and track the course of soil water content prior and during measurement of rhizosphere respiration. A simultaneous monitoring of pCO_2, pO_2, local soil moisture distribution and root system architecture is desirable for a quantitative analysis of rhizosphere respiration and could be achieved by complementary 2D optode and 2D or 3D neutron imaging in the future.

Declarations

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Competing Interests

The authors have no competing interests to declare that are relevant to the content of this article.

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Figures

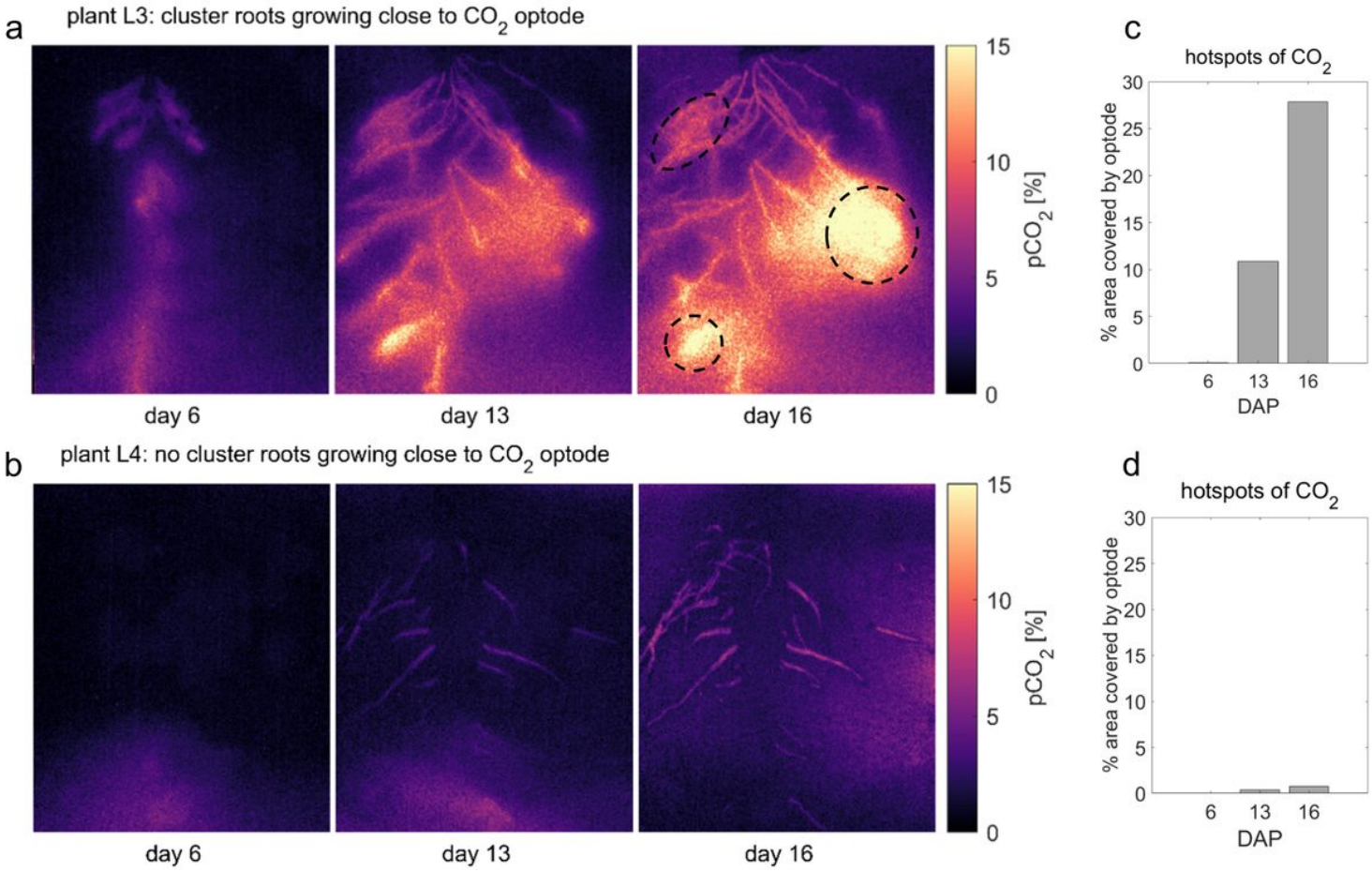

Figure 1

Time series of pCO_2 [%] measured on day 6, 13 and 16 after planting at 0.30 cm^3 cm^{-3} volumetric soil water content. **a)** pCO_2 is shown for plant L3, where cluster roots grew close to the CO_2 optode (exemplary regions with cluster root abundance indicated by black ellipses, see also Fig. S1). **b)** shows pCO_2 of plant L4, where close to the CO_2 optode there were only lateral roots without clusters. **c)** and **d)** indicate the hotspot area in % of the area covered by the optode. Background value and hotspots (mean

of background + 3SD) were statistically separated in the gray value images as described in Bilyera et al. (2020).

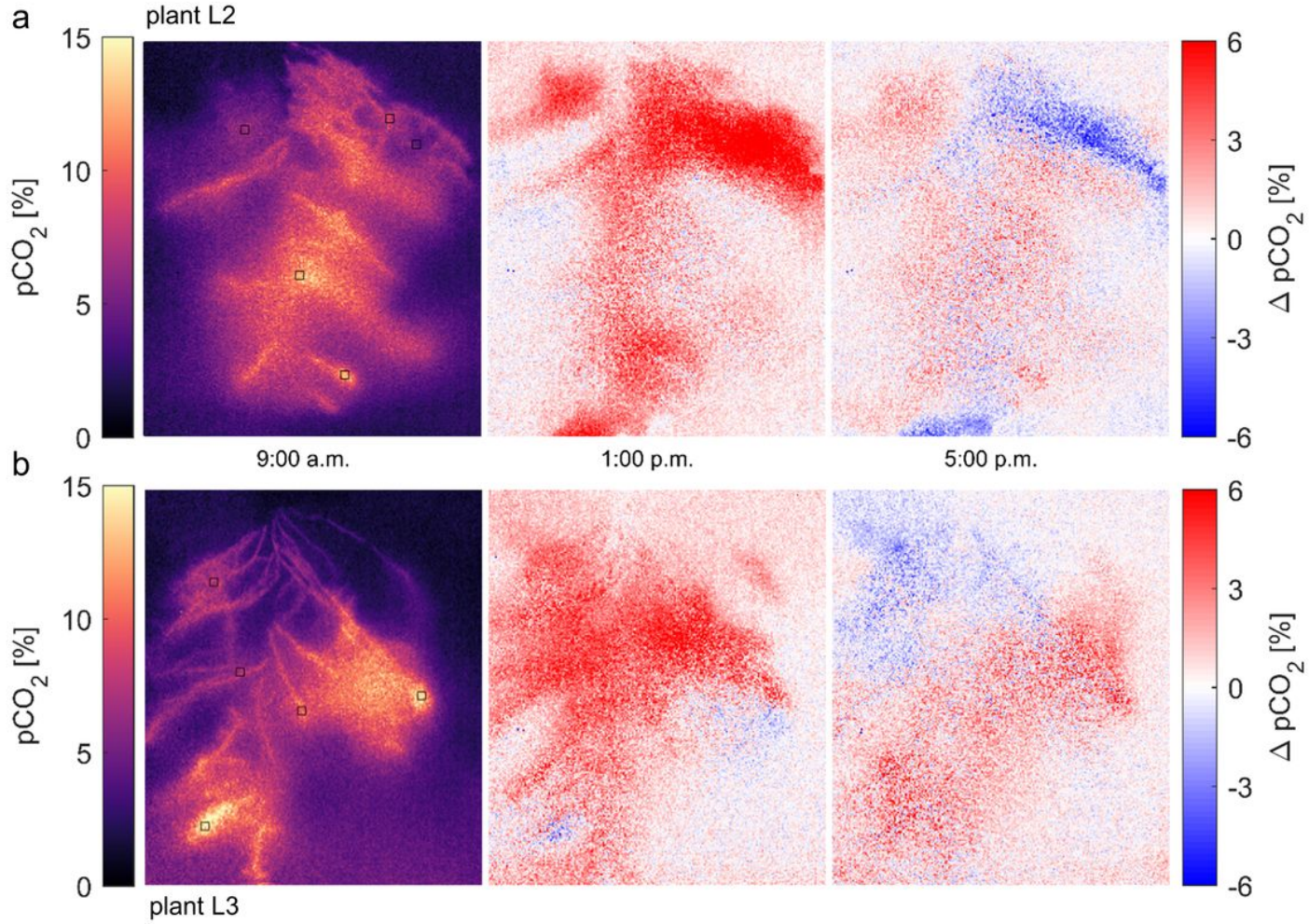

Figure 2

Changes of pCO_2 from morning to afternoon on day 14 after planting for plant L2 (**a**) and L3 (**b**). The plants were irrigated to 0.30 cm^3 cm^{-3} at 8:30 a.m., and if water content changed by more than 0.02 cm^3 cm^{-3}, soil moisture was re-adjusted at noon and in the afternoon. Left panels: pCO_2 measured 30 minutes after watering at 9 a.m.; center panels: difference (Δ pCO_2) between 1 p.m. and 9 a.m.; right panels: difference (Δ pCO_2) between 5 p.m. and 1 p.m. Regions for quantitative analysis of root zone pCO_2 (see **Fig. 3**) are indicated as black squares in the first panels (regions for bulk soil comparison not shown).

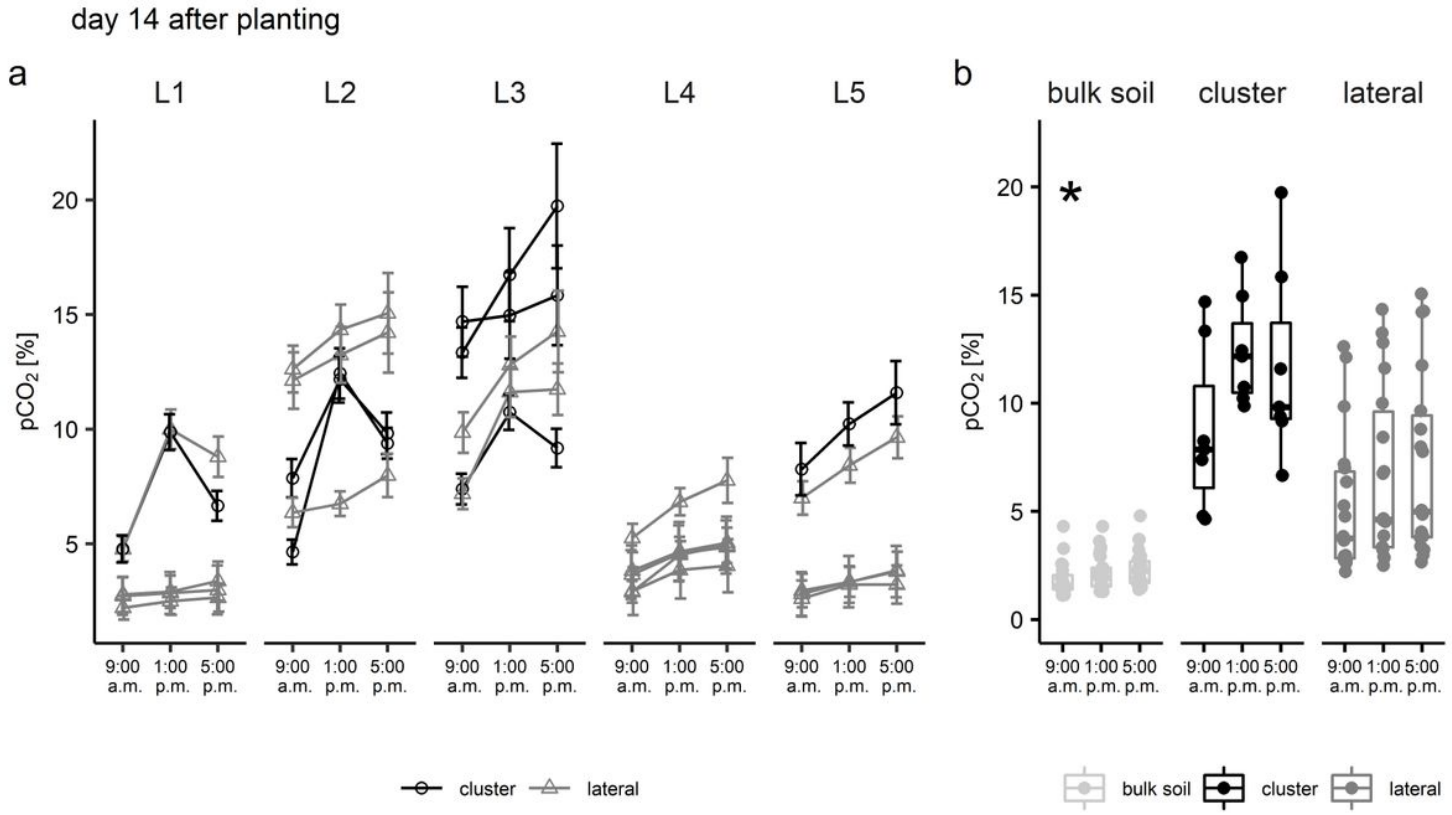

Figure 3

a) Mean CO_2 concentration (error bars indicate ± standard deviation) in each five 4 mm² regions selected within the root zones for plants L1 to L5 on day 14 after planting (cluster roots: n = 7, lateral roots: n = 18). Measurements were repeated in the morning at 9 a.m., at noon (1 p.m.) and in the afternoon (5 p.m.) to capture the diurnal variation in rhizosphere respiration. Water content was adjusted to 0.30 $cm^3\ cm^{-3}$ in the morning and, if water content changed by more than 0.02 $cm^3\ cm^{-3}$, again at noon and / or in the afternoon. Plant L4 did not grow cluster roots close to the CO_2 optode. **b)** Diurnal variation of pCO_2 in selected 4 mm² regions in bulk soil (n = 25), around cluster roots (n = 7) and lateral roots (n = 18) on day 14 after planting. Significant differences following Wilcoxon test ($\alpha < 0.05$) are highlighted with * ($p \leq 0.05$).

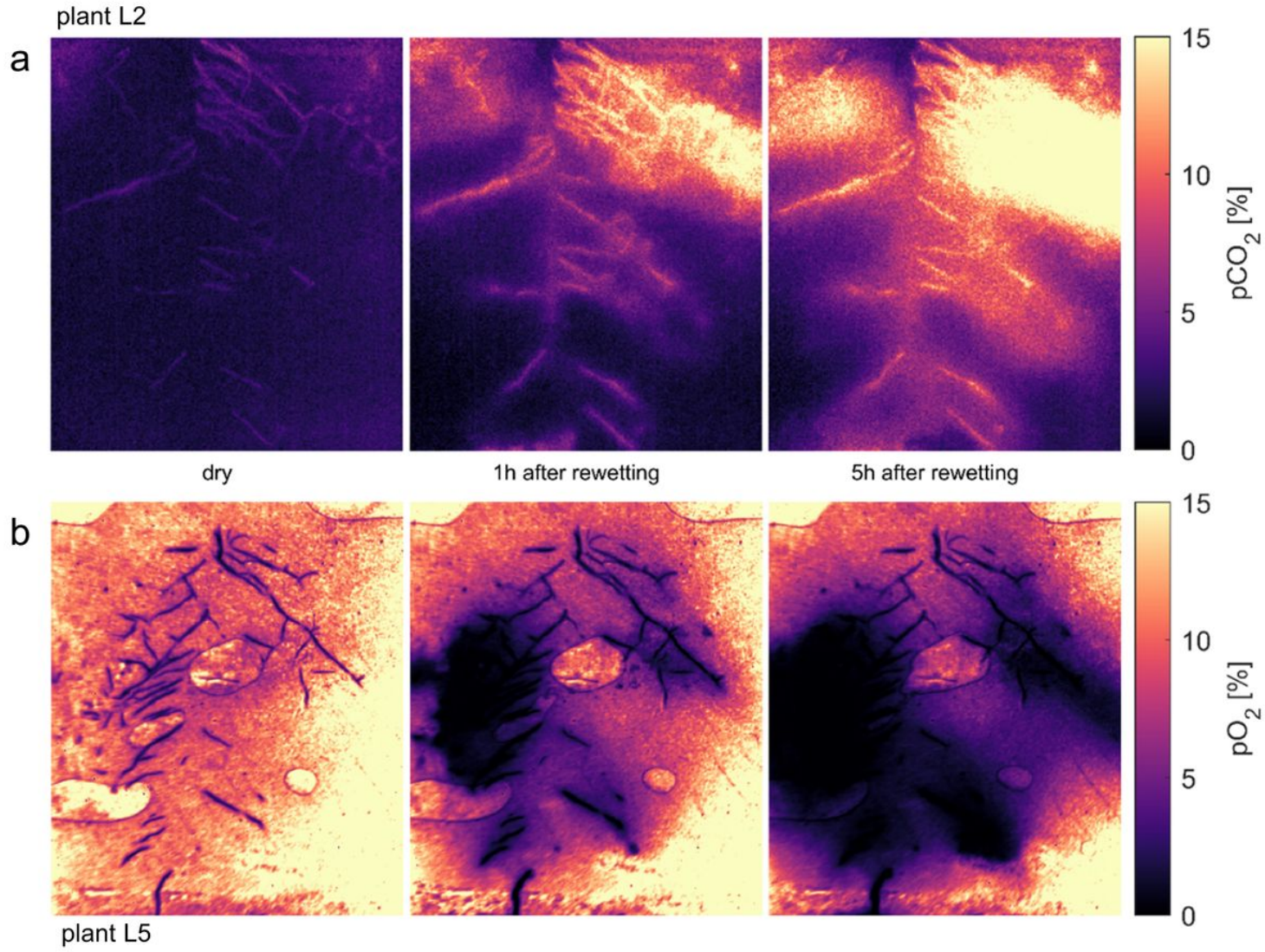

Figure 4

a) Time series of CO_2 concentration (plant L2) and **b)** time series of O_2 concentration (plant L5) throughout the drying-rewetting experiment at day 19 after planting. Gluing the O_2 optodes to the rhizotron window resulted in some “bubble-like” structures in the oxygen images; those regions were not selected for quantitative analysis. The first panel shows pCO_2 resp. pO_2 prior to rewetting (dry soil, soil water content 0.10 $cm^3\ cm^{-3}$). The rhizotrons were rewetted to 0.30 $cm^3\ cm^{-3}$ via capillary rise from the bottom.

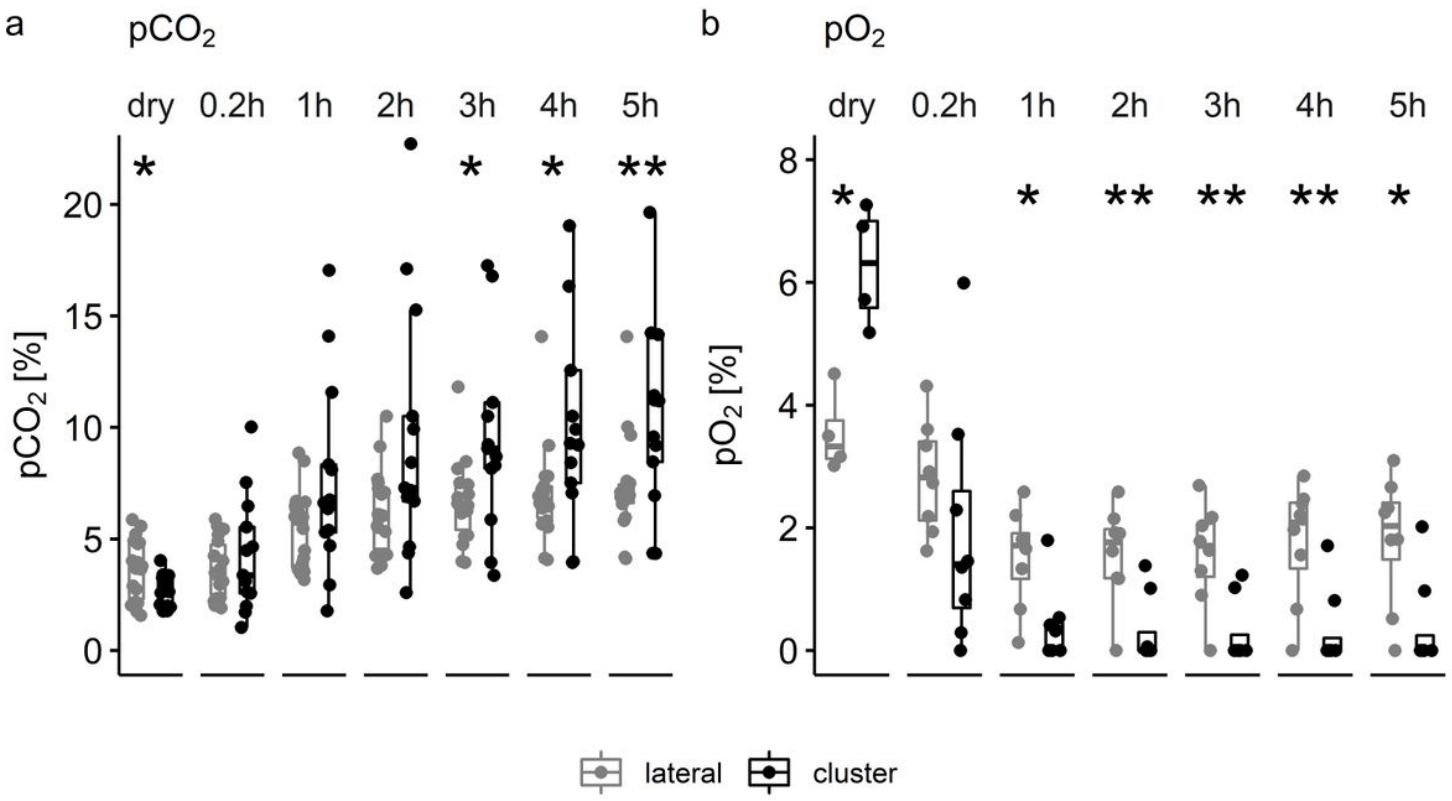

Figure 5

Change of pCO_2 and pO_2 from 0.2 h to 5 h after rewetting from dry conditions. **a)** CO_2 concentration at the surface of different cluster roots (n = 13) and lateral roots (n = 18); (cluster roots from four plants, laterals chosen from five plants); **b)** O_2 concentration at the surface of different cluster roots (n = 8) and lateral roots (n = 8) (2 plants). Significant differences between cluster and lateral roots following pairwise Wilcoxon test ($\alpha < 0.05$) are highlighted with * ($p \leq 0.05$) and ** ($p \leq 0.01$).

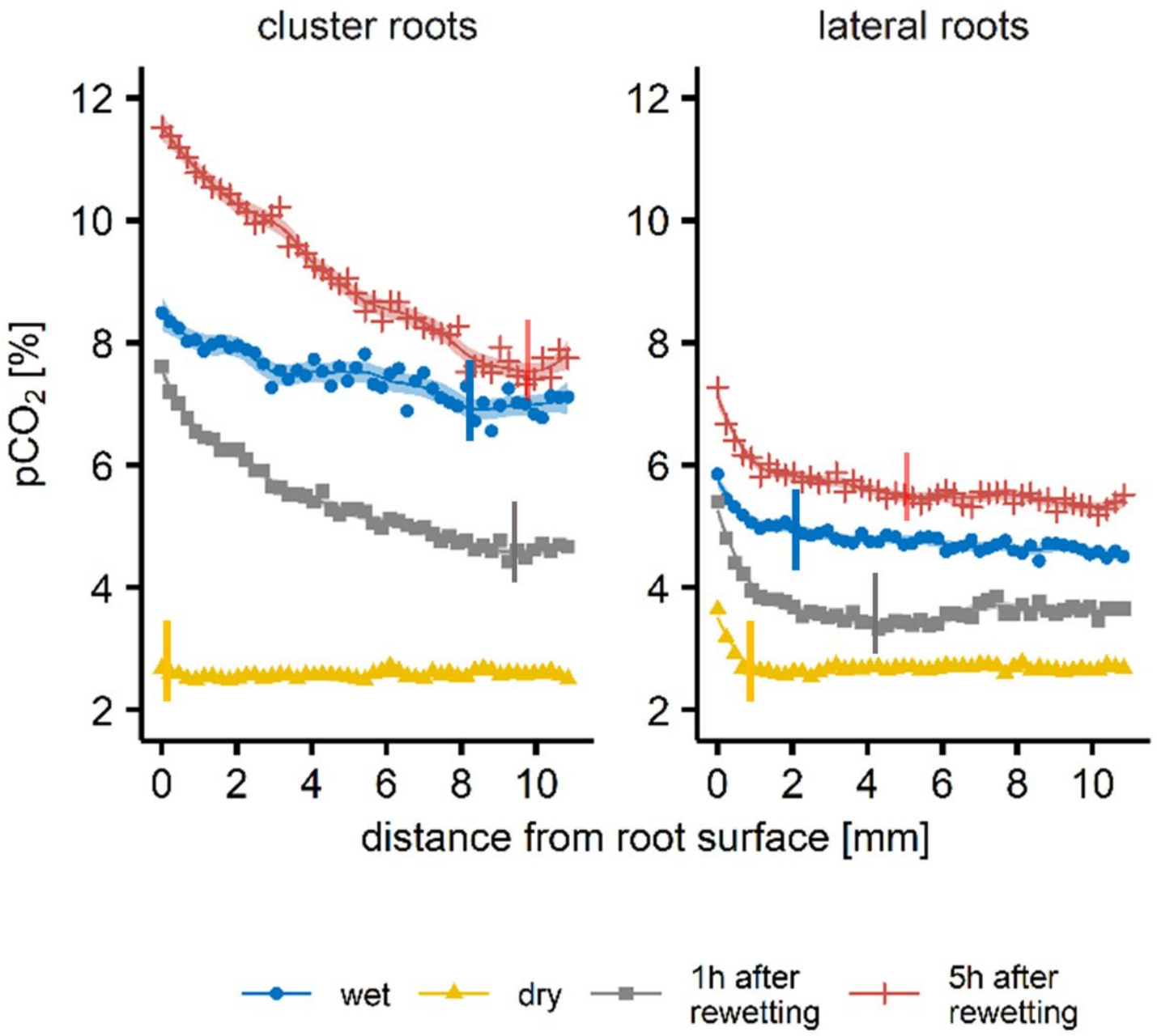

Figure 6

Extent of CO_2 accumulation zones (borders indicated by colored vertical bars) around cluster and lateral roots compared in wet soil (0.30 cm^3 cm^{-3}, day 16 after planting), dry soil (0.10 cm^3 cm^{-3}, day 19 after planting) and in rewetted soil (1h and 5 h after rewetting to from 0.10 cm^3 cm^{-3} to 0.30 cm^3 cm^{-3}, day 19 after planting). Data points indicate mean pCO_2 [%] of cluster roots (*n* = 13, left panel) and lateral roots (*n* = 18, right panel). Cluster roots were chosen from four plants, laterals from all five plants. Lines and shaded area indicate the local regression curve ("loess" function in RStudio, package: "stats") with 95% confidence interval.

Supplementary Files

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- SupplBereswilletalNoninvasiveimaginglupine.pdf