# Maize root growth, Oxygen and N availability drive formation of $N_2O$ hotspots in soil

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## Summary

Plant roots modify all major controls of denitrification in soils, particularly the availability of the main substrates ($NO_3^-$ and $C_{org}$), soil moisture, soil $O_2$ content, and root-associated microbial communities, and thus play an important role in $N_2O$ formation. Direct in-situ measurements of $N_2O$ concentrations in the rhizosphere are lacking, yet crucial to understanding how rhizosphere denitrification contributes to overall $N_2O$ emissions from soil. We equipped rhizoboxes with $O_2$-sensitive planar optodes to simultaneously monitor root growth and rhizosphere/soil $O_2$ concentrations. We measured soil surface $N_2O$ fluxes and linked them to root growth, soil moisture, and root/soil $O_2$ concentrations. Based on root growth and $O_2$ concentrations, we identified regions of interest (ROI) and sampled small soil volumes, which were analyzed for C, N, abundance of microbial denitrifiers (*nirK*, *nirS*) and $N_2O$ reducers (*nosZI*, *nosZII*), and soil $N_2O$ concentrations. Plant roots determined depth gradients of nutrients and denitrification gene abundances in the soil of the rhizoboxes with higher resource availability ($NO_3^-$, DOC) and lower soil moisture in the upper soil layers, which also had higher abundances of total bacteria, *nirK* and *nosZII*. We anticipate that these uppermost soil layers largely contributed to $N_2O$ formation. For the first time we were able to show high *in-situ* $N_2O$ concentrations with distinct depth profiles around roots, and $O_2$ and N availability controlling $N_2O$ production at the process scale.

## Introduction

Plant roots play an important role in regulating denitrification, the main process contributing to formation of the climate-relevant greenhouse gas nitrous oxide ($N_2O$) (Butterbach-Bahl *et al.*, 2013; Ciais *et al.*, 2013). Denitrification is the sequential reduction of nitrate ($NO_3^-$) to nitrite ($NO_2^-$), nitric oxide (NO), nitrous oxide ($N_2O$), and molecular nitrogen ($N_2$) by heterotrophic bacteria, fungi, and archaea (Zumft, 1997). Plant roots modify major controls of denitrification through altering soil moisture, $O_2$ content, the availability of its main substrates ($NO_3^-$ and $C_{org}$), and root-associated microbial communities. While the rhizosphere is indicated to be a hotspot of denitrification, the extent of $N_2O$ formation in the rhizosphere and its contribution to $N_2O$ emissions from soils remain unclear.

Increased $N_2O$ losses have been reported from planted compared to bare soils (Woldendorp, 1963; Vinther, 1984; Senbayram *et al.*, 2020; Yankelzon *et al.*, 2024b) and the promoting effect of plant growth on denitrification has been associated with higher $C_{org}$ availability in the rhizosphere (Smith & Tiedje, 1979; Bakken, 1988; Philippot *et al.*, 2009). Plants translocate 20-30 % of assimilated C belowground and about 7-11 % of assimilated C enters the soil as rhizodeposition (Pausch & Kuzyakov, 2018). Higher C availability in the rhizosphere attracts soil microorganisms including bacteria, fungi, oomycetes, and archaea feeding on rhizodeposits making the rhizosphere a hotspot of microbial activity (Starkey, 1958; Philippot *et al.*, 2013). In addition to microbial respiration, root respiration largely contributes to $O_2$ consumption in soil with rates of around 20 mg $O_2$ $m^{-1}$ root $d^{-1}$ for maize roots (Ben-Noah & Friedman, 2018). Root and microbial respiration decrease $O_2$ availability, creating suitable conditions for denitrification (Bateman & Baggs, 2005; Hu *et al.*, 2015). Accordingly, abundance and composition of bacterial communities differ significantly between planted and unplanted soil, and between bulk soil and the rhizosphere (Chèneby *et al.*, 2004; Henry *et al.*, 2008; Philippot *et al.*, 2013). Studies show that denitrifiers are more abundant in the rhizosphere than the bulk soil of a range of different plant species (Knowles, 1982; Chèneby *et al.*, 2004; Ai *et al.*, 2020; Wang *et al.*, 2024). Accordingly, nitrate reduction and denitrification activities were higher in rhizosphere soil compared to bulk soil (Smith & Tiedje, 1979; Klemedtsson *et al.*, 1987; Bakken, 1988; Mahmood *et al.*, 1997; Hamonts *et al.*, 2013; Guyonnet *et al.*, 2017; Malique *et al.*, 2019; Zhao *et al.*, 2020).

Direct in-situ measurements of $N_2O$ concentrations in the rhizosphere are lacking, yet crucial to understanding how roots control denitrification and $N_2O$ formation. Further, it remains unknown to which extent rhizosphere denitrification contributes to overall $N_2O$ emissions from soil and how microscale $N_2O$ production relates to surface $N_2O$ fluxes. Denitrification takes place in microbial hotspots – small soil volumes with faster process rates compared to the average soil conditions (=coldspots) (Groffman *et al.*, 2009; Kuzyakov & Blagodatskaya,

2015). Characterization of these $N_2O$ hot and cold spots is needed to better understand the 'rhizosphere effect' and its significance for $N_2O$ emissions.

This study aimed (I) to elucidate how plant roots control formation of $N_2O$ hotspots in the rhizosphere, (II) to determine spatial distribution of $N_2O$ production (hotspots) in relation to root growth and development, and (III) to characterize microbial N cycling in $N_2O$ hotspots. We hypothesize that (I) root growth promotes microbial growth and respiration, increasing $O_2$ consumption at roots and in the rhizosphere. Accordingly, $N_2O$ fluxes from soil increase with increasing root growth. (II) Increased microbial respiration from root exudation stimulates formation of anoxic microsites ($N_2O$ hotspots) in close proximity of the roots. These $N_2O$ hotspots are characterized by higher availability of C and N, and higher abundance of N reducing microorganisms compared to cold spots with low $N_2O$ concentrations. To address these hypotheses, we equipped rhizoboxes with $O_2$-sensitive planar optodes to simultaneously monitor root growth and rhizosphere/soil $O_2$ concentrations. We further measured surface $N_2O$ fluxes and linked them to root growth, soil moisture, and root/soil $O_2$ concentrations. Finaly, we defined regions of interest (ROI) related to root growth and $O_2$ concentrations, and analyzed them for C, N, abundance of microbial N reducers, and soil $N_2O$ concentrations.

## Material and Methods

### Optode fabrication

Planar optodes are a non-invasive imaging technique based on reversible changes in luminescence properties of analyte-specific fluorophores enabling visualization and quantification of biochemical processes with an emphasis on capturing spatial and temporal heterogeneity (Blossfeld, 2013). The $O_2$ optodes were prepared as previously described by Merl & Koren (2020). 76.5 mg of the indicator dye PtTFPP (Platinum(II)-meso(2,3,4,5,6-pentafluoro)phenyl-porphyrin, Frontier Scientific, Newark, USA), 76.8 mg of the reference dye MY (Macrolex® fluorescence yellow 10GN, Lanxess, Köln, Germany), and 5 g Polystyrene (Sigma Aldrich, Burlington, USA) were dissolved in 50 g Toluene. This 'sensor cocktail' was knife-coated onto a PET foil (Optimont® 501, Bleher Folientechnik GmbH, Ditzingen-Heimerdingen, Germany) using a film applicator (BYK, Wesel, Germany) and a layer of ~10 µm was obtained after the toluene evaporated.

### Soil

The soil was collected from a long-term experimental site in Rotthalmünster, Germany (N48°21′, E13°11′). It was characterized as Haplic Luvisol with a silty loamy texture (19 % clay, 71 % silt, 10 % sand), a pH ($CaCl_2$) of 6.74, with 0.13 % $CaCO_3$. The soil organic carbon

content was 1.21 %, total soil nitrogen was 0.14 %, and C:N ratio was 8.65. Soil was air-dried, sieved to 10 mm mesh size, and stored at 4 °C until the beginning of the experiment.

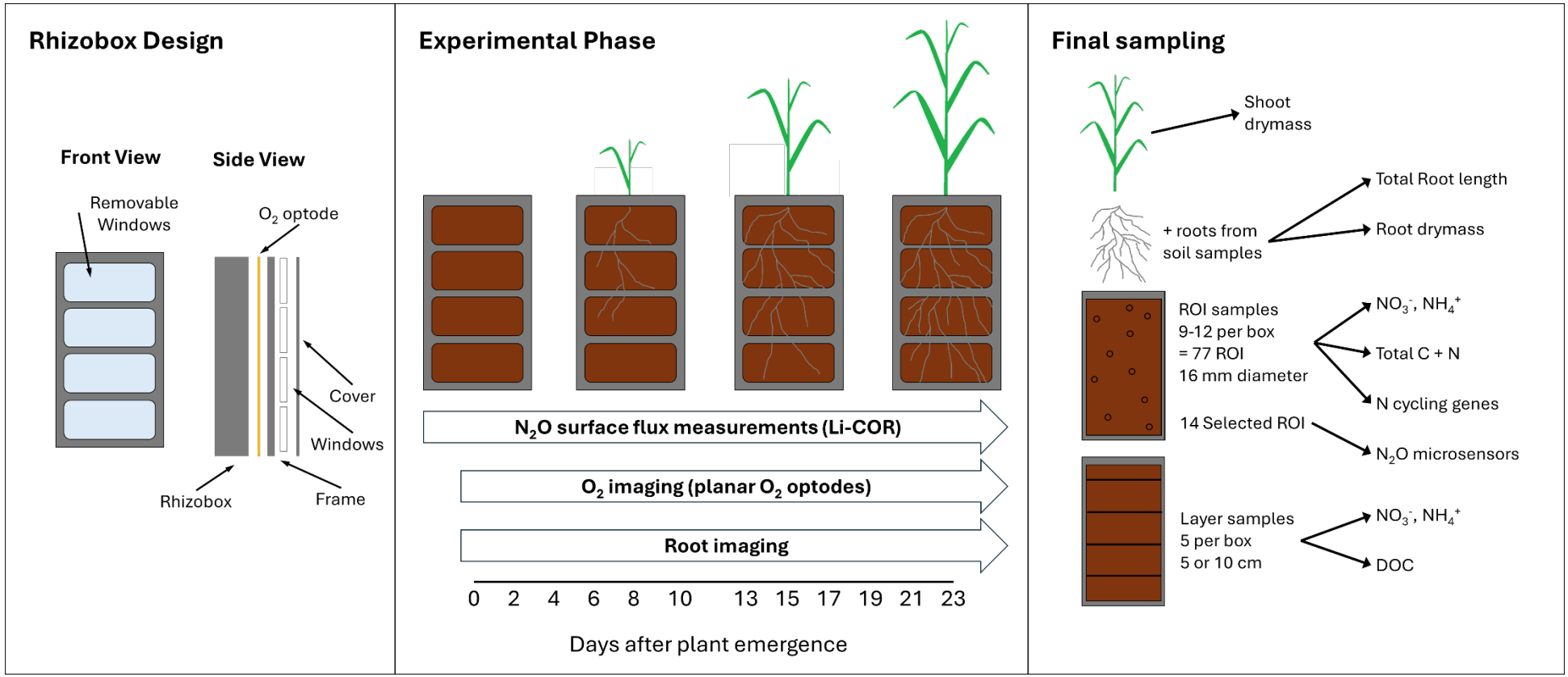



**Figure 1:** Schematic overview of rhizobox design, experimental phase, and final sampling of the experiment. The rhizobox covers consisted of an opaque frame with 4 removable window panels. To monitor $O_2$ concentrations in soil, an $O_2$-sensitive optode was attached to the frame before closing the rhizoboxes.

**Experimental setup**

The experiment was conducted in rhizoboxes with inner dimensions of 20 cm x 2.2 cm x 40 cm (width x depth x height). Each box had three holes (7 mm diameter) in the bottom part allowing for drainage. The inner sides were lined with a polyester wick (Breatex™ 150, Fibertex Nonwovens A/S, Aalborg, Denmark) facilitating rewetting and soil moisture distribution. The “window side” of the rhizoboxes consisted of an opaque frame and transparent, removable acrylic glass windows (Linatex A/S, Herlev, Denmark) (Figure 1). The $O_2$ optode was adhered onto the grid at the window side of the rhizoboxes with insulation tape. Rhizoboxes were closed using a rubber seal and C-clamps.

Dry soil was mixed with finely ground fertilizer (Pioner NPK Makro BLÅ, Azelis A/S, Kgs. Lyngby, Denmark) supplying 200 mg N $kg^{-1}$, 47 mg P $kg^{-1}$, 377 mg K $kg^{-1}$, 64 mg S $kg^{-1}$, and 49 mg Mg $kg^{-1}$. Seven rhizoboxes were filled with 2.2 kg of soil per box to a height of 38 cm, resulting in a bulk density of 1.3 g $cm^{-3}$. Soil was rewetted from the bottom over a period of 112 h and pre-incubated in a climate chamber (Weiss-Umwelttechnik GmbH, Gießen, Germany) for 10 days. One rhizobox was equipped with two FDR sensors (ECH2O 5TE, Decagon Devices, Pullman, USA) at 5-10 cm and 30-35 cm depth measuring volumetric soil water content and soil temperature. Rhizoboxes were weighed and watered every two-to-three days, always after $N_2O$ flux measurements and $O_2$ imaging. Conditions in the climate chamber during preincubation and the experiment were as follows: 16 hours daylight with 300 µmol $m^{-2}$ $s^{-1}$ light intensity, 20 °C day temperature, 15 °C night temperature, 50 % relative humidity day, 60 % relative humidity night.

Maize seeds (*Zey mays* L. cv KWS Stabil) were pregerminated on wet paper for 3 days, and one germinated seed was planted into each box 10 days after rewetting the soil. However, plants emerged in only two rhizoboxes (3 and 4). From the remaining five rhizoboxes, old seeds were removed and small maize seedlings of the same variety were planted. Measurements were conducted on the same days in relation to plant age (i.e., days after emergence) in all rhizoboxes to ensure comparability across replicates.

**$N_2O$ surface flux analysis and calculations**

Surface fluxes of $N_2O$ emitted from soil were measured every two-to-three days using a $N_2O/H_2O$ Trace Gas Analyzer (LI-7820, Li-COR, Lincoln, USA). Rhizoboxes were put into chambers consisting of an opaque bucket and a transparent chamber top. Chamber height was 50 cm, with a total volume of 36.2 L or adjusted to 150 cm (106.9 L) according to plant size. Chamber closing times were 4 and 10 minutes for small and large chambers, respectively. The temperature in the chamber was measured once at closing time and again before opening. The mean temperature was used for flux calculations. $N_2O$ fluxes were

calculated using the R package *goFlux* v0.2.0 (Rheault *et al.*, 2024) and cumulative $N_2O$ emissions were calculated using *agg.fluxes* from the R package *gasfluxes* v0.6-2 (Fuß, 2020).

**$O_2$ and root imaging setup**

The imaging of the $O_2$ optodes was conducted with a single-lens reflex (SLR) camera (EOS 1300D, Canon, Japan) equipped with an objective lens (EFS 18-55 mm Canon, Japan), and an orange 530 nm longpass filter (OG530 SCHOTT, 52 mm × 2 mm) with a plastic filter (#10 medium yellow, LEEfilters.com) attached in front of the long-pass filter to prevent autofluorescence of the longpass filter. For excitation of the optode, a blue LED (470 nm, r-s components, Copenhagen, Denmark) was used. The LED was controlled by a USB-controlled LED driver unit (imaging.fish-n-chips.de). The software look@RGB (imaging.fish-n-chips.de) was used to gather the images and to control the camera and LED. Calibration of the $O_2$ optode was done as described in detail by (Merl & Koren, 2020). $O_2$ imaging was conducted in two-to-three-day intervals, always on the same days as surface $N_2O$ flux measurements.

Image processing and analysis was done using the software ImageJ (imagej.nih.gov/ij/) using a ratiometric approach (Larsen *et al.*, 2011; Merl & Koren, 2020). For the false-color images displaying $O_2$ concentration in % air saturation, the color palette *batlowK* (Scientific Color Maps v8.0.1, (Crameri, 2018) was used. We calculated the anoxic area of the rhizobox window as the proportion of the image area with an $O_2$ concentration < 10 % air saturation (28.4 µmol $O_2$/L) which is in the range of conditions promoting denitrification (Nakajima *et al.*, 1984; Bonin *et al.*, 1989) similar to previous studies (Zhu *et al.*, 2015; Keiluweit *et al.*, 2018).

Prior to $O_2$ imaging, a photo of the rhizoboxes was taken with 'normal light conditions' to monitor root growth on the rhizobox window side. These root images were analyzed with RootPainter (Smith *et al.*, 2022) to segment roots from the soil background. A model trained with randomly selected images was used to segment roots on all the images. Next, root length and root area were estimated from skeletonized images with RhizoVisionExplorer (Seethepalli *et al.*, 2021). For image pre-processing, we used a thresholding level of 200, removed non-root objects that were smaller than 1 mm, an edge smoothing threshold of 2, and a pruning threshold of 5.

**$N_2O$ microsensor measurements**

We used needle-shaped clark-type $N_2O$ microsensors that are based on an indium cathode in organic electrolyte, and an $O_2$-removing compartment measuring $N_2O$ partial pressures (Pa) in both gas and liquid (Andersen *et al.*, 2001; Revsbech, 2021). Sensors were made inhouse, similar to those commercially available from Unisense (Unisense A/S, Aarhus, Denmark). The software SensorTrace Pro (Unisense A/S, Aarhus, Denmark) was used to introduce the sensor into the soil by a computer-controlled micromanipulator and to record the sensor signals. Calibration of the $N_2O$ microsensors was performed in $N_2O$-free water by addition of an $N_2O$-

Standard (27 mM $N_2O$). All microsensors displayed a linear response curve during calibrations.

$N_2O$ microsensor measurements were conducted 23 days after plant emergence. Small holes (2 x 2 mm) were cut into the optode foil and $N_2O$ depth profiles (0-5 mm) were measured in 500 µm steps. In total, 14 regions of interest (ROI) in three different rhizoboxes (1, 4, 5) were chosen for $N_2O$ microsensor measurements based on root development and $O_2$ concentration.

**Harvest and soil sampling**

The two rhizoboxes (Nr. 3 and 4), with initial seeds growing normally, were harvested 39 and 37 days after rewetting, corresponding to 25 and 21 days of plant growth, respectively. Rhizoboxes 1, 2, 5, 6, 7 with replanted seedlings were harvested 53, 49, 52, 50, and 52 days after rewetting, corresponding to 25, 21, 24, 22, and 24 days of plant growth. Due to the large workload of sampling and processing samples, it was not possible to harvest and sample all rhizoboxes on the same day. Shoots were cut above the surface and dried at 60 °C to determine shoot dry mass. Crown roots were added to the root samples.

In each rhizobox, regions of interest (ROI) were defined based on root development and $O_2$ concentration. Soil from ROI was sampled from the window side of the rhizoboxes with small aluminum cylinders (16 mm inner diameter, 10 mm depth) (UGT Umwelt-Geräte-Technik GmbH, Müncheberg, Germany) and the roots were carefully separated from the soil. The soil was then homogenized and divided into three subsamples: ~1 g soil (dry-weight-equivalent) was frozen in Eppendorf tubes ( at – 20 °C for analysis of bacterial functional genes, ~1 g soil (dry-weight-equivalent) was stored in 10 ml centrifuge tubes at 4°C for analysis of mineral N, and the remaining soil (ca. 1-1.2 g) was dried at 105 °C to determine soil water content and total C and N content.

Additional soil samples were taken from rhizoboxes in 5 depths: 0-5 cm, 5-10 cm, 10-20 cm, 20-30 cm, 30-38 cm using a cork borer as auger (inner diameter 2 cm). For each depth layer, eight samples were taken and homogenized into one composite sample, after careful removal of the roots. Each composite sample was then divided into three subsamples: ~50 g soil (dry-weight-equivalent) was stored at 4°C for analysis of mineral N, ~10 g soil (dry-weight-equivalent) was stored at 4°C for analysis of water-extractable $C_{org}$ (WEOC), and ~ 5 g soil (dry-weight-equivalent) were dried at 105° C to determine soil water content.

Roots were washed from the remaining soil and stored in 30 % ethanol at 4°C.

**Root scanning and analysis**

Roots were scanned on a flatbed scanner (Epson, Suwa, Japan) and the software RhizoVisionExplorer (Seethepalli *et al.*, 2021) was used to extract total root length and total root volume from the scanned images. For image pre-processing, we used a thresholding level of 200, removed non-root objects that were smaller than 0.1 px, an edge smoothing

229 threshold of 2, and a pruning threshold of 5. Root diameter classes were classified as <1mm,
230 1-2 mm, and >2 mm. Total root dry weight was determined by drying roots at 105 °C. We refer
231 to root length at the rhizobox window estimated with root imaging as *planar root length* and
232 root length at the final harvest measured with root scanning as *total root length* (Wacker *et al.*,
233 2024).

234 **Soil analysis**

235 Soil mineral N was extracted by shaking the subsample with 1 *M* KCl (ratio 1:5 w:v) on an
236 overhead shaker with 40 rpm for 60 min, filtered (Whatman Grade 42 for depth layer soil
237 samples, 0.8 µm CA syringe filters for ROI samples) and analyzed colorimetrically on a
238 continuous flow analyzer (AA3, Seal Analytical, Norderstedt, Germany). WEOC was extracted
239 by thoroughly shaking soil with ultrapure water (ratio 1:2 w:v) for 60 s, filtered through 0.45 µm
240 PES syringe filters, and analyzed on an elemental analyzer (multi N/C 2100 S, Analytik Jena,
241 Jena, Germany). Total C and N content in ROI samples was analyzed on an elemental
242 analyzer (Vario EL III, Elementar, Langenseibold, Germany).

243 **DNA extraction and quantitative PCR**

244 DNA was extracted from 400 mg of freeze-dried soil for each sample using the NucleoSpin
245 Soil kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions. DNA
246 quality was checked using a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA), before
247 quantification on a Qubit fluorimeter using the Broad Range double stranded DNA kit (Thermo
248 Fisher Scientific). The abundance of total archaea and bacteria (16S rRNA gene), denitrifiers
249 (*nirK* and *nirS*) and $N_2O$ reducers (*nosZ*I and *nosZ*II) were determined using real-time
250 quantitative PCR (qPCR) using specific primers for each gene. The qPCR reactions were
251 performed in two independent runs in a reaction volume of 15 µL containing iQ™ SYBR Green
252 Supermix (Bio-Rad, Hercules, CA, USA), 0.1 % bovine serum albumin (New England Biolabs,
253 Ipswich, MA, USA), primers and 2 ng of template DNA on a CFX Connect Real-Time System
254 (Bio-Rad). Primers, qPCR conditions, and amplification efficiencies are presented in
255 Supplementary Table S1. Standard curves were generated by serial dilutions of linearized
256 plasmids containing a fragment of the specific gene. The amplifications were validated by
257 melting curve analyses and agarose gel electrophoreses. Potential inhibition of PCR reactions
258 was initially checked by amplifying a known amount of the pGEM-T plasmid (Promega,
259 Madison, WI, USA) with the plasmid specific M13F/M13R primer set (Supplementary Table
260 S1) and 2 ng of DNA template or non-template controls for each sample. No inhibition was
261 detected with the amount of DNA used.

### Calculations and statistics

All calculations and statistics were carried out using the statistical software R v4.3.3 (R Core Team, 2024). Simple linear regressions were applied to identify relationships between soil moisture, planar root length, or anoxic soil area. Similarly, we applied simple linear regression models to analyze relationships between soil $N_2O$ concentrations, $O_2$ concentrations, N availability, and abundance of N cycling genes in ROI at the end of the experiment. To identify the factors driving $N_2O$ fluxes, we applied linear mixed effect models (lme) using the lme function from the package nlme v3.1-131 (Pinheiro *et al.*, 2017). We tested models for the experimental period with and without plant growth, respectively, as well as for the whole experimental period. To account for repeated measurements, replicates were set as random effect. Models were compared using maximum likelihood (ML), selected using AIC (Akaike's information criterion), and fitted using restricted maximum likelihood (REML). Pseudo-$R^2$ for lme was calculated using r.squaredGLMM from the package MuMIn v1.42.1 (Barton, 2018).

## Results

### Plant and root growth

Root development followed the same pattern in all rhizoboxes. Planar root length increased slowly during the first week after emergence, then increased almost linearly until harvest (Fig. 2 a). Total root length at harvest was 161.4 ± 48.3, root dry weight was 1.11 ± 0.47 g, and root dry weight was positively correlated with total root length ($R^2$=0.83, $p < 0.01$). Maize shoot dry weight at harvest 4.24 ± 1.16 g, and root:shoot ratio was 0.25 ± 0.03 (Supplementary Table S2). Shoot N content was 3.5 ± 0.4 %.

### Soil Moisture

After rewetting, soil moisture ranged between 84 and 89 % WFPS (Supplementary Fig S1) and was kept at 65-70 % WFPS during plant growth, providing favorable conditions for anaerobic processes such as denitrification (Fig. 2b). Soil moisture sensors showed that soil moisture was very stable in the 30-35 cm layer and fluctuated most in the top 5-10 cm layer (Supplementary Figure S2).

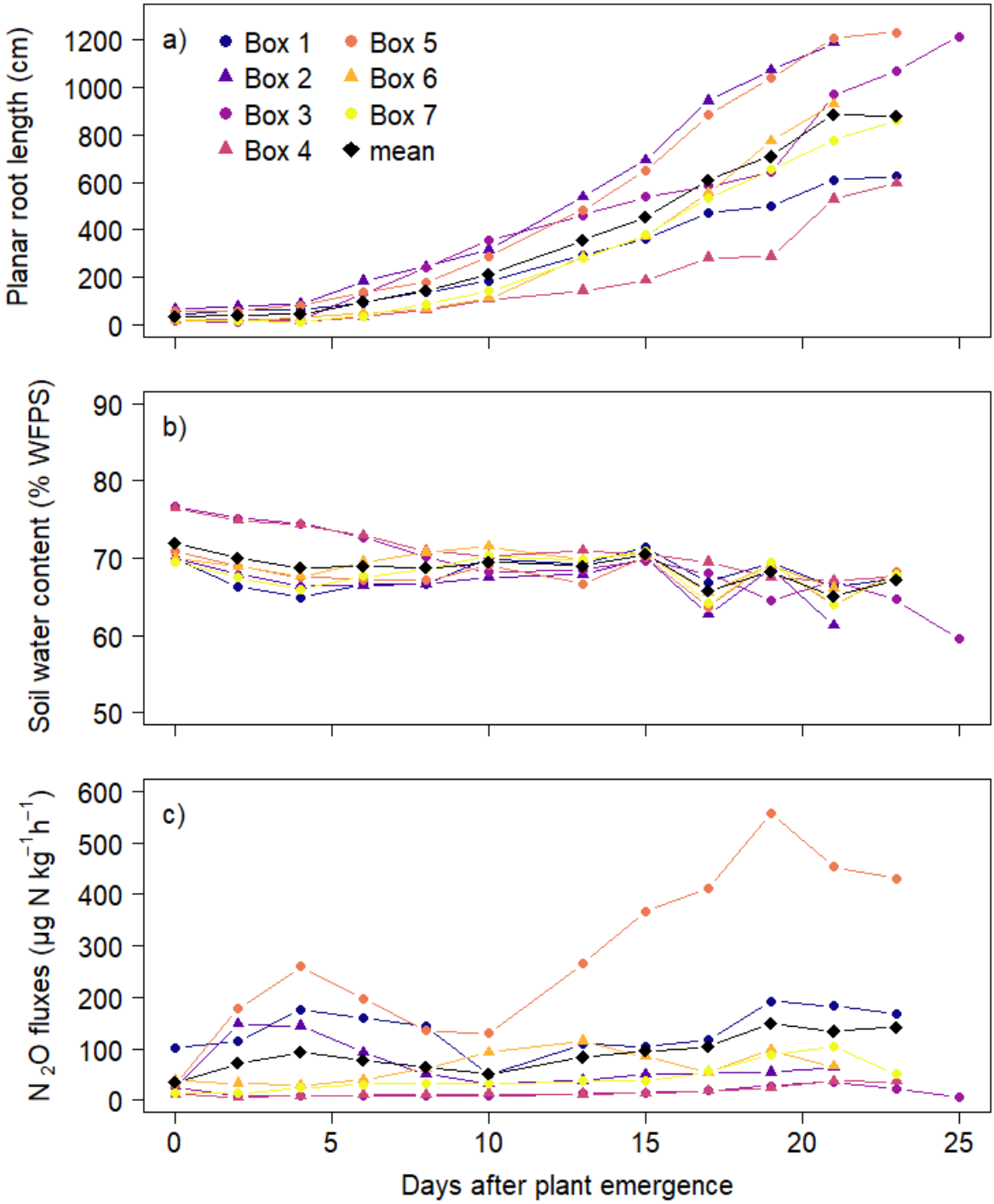



**Figure 2:** (a) Planar root length (cm), (b) soil water content (% water-filled pore space), and (c) $N_2O$ fluxes (µg $N_2O$-N $kg^{-1}$ $h^{-1}$). Colored points and triangles represent each individual rhizobox, black diamonds represent means (n=7).

**$N_2O$ fluxes and cumulative emissions**

The measured $N_2O$ fluxes showed large variations between rhizoboxes (Fig. 2c, Supplementary Fig. S1). During the acclimation phase, $N_2O$ fluxes were comparable in all rhizoboxes and decreased for the first two weeks after rewetting. In the rhizoboxes that had to be replanted, $N_2O$ fluxes remained relatively stable until plant emergence (Supplementary

Fig S1). In rhizoboxes 1, 2, and 5, $N_2O$ fluxes increased directly after plant emergence, in rhizobox 6, $N_2O$ fluxes increased slightly a week after plant emergence, and $N_2O$ fluxes from rhizoboxes 1, 5, and 7 increased towards the end of the experiment (Fig. 2c). $N_2O$ fluxes from box 1 were higher than all other rhizoboxes before plant emergence (Supplementary Fig. S1), and highest from rhizobox 5 after plant emergence (Fig. 2c).

Cumulative $N_2O$ emissions during the planted phase ranged from 8.5 $N_2O$-N $kg^{-1}$ to 157.5 and (Supplementary Fig. S3).

**Soil $O_2$ concentrations and anoxic soil fraction**

$O_2$ imaging started 10 days after rewetting in the rhizoboxes, and most of the optode-soil interface was anoxic at this time (Example for Rhizobox 1 in Figure 3, other data found in Supplementary Figure S4 and S5). Overall, $O_2$ concentrations increased over time in all depths, although watering also led to formation of anoxic conditions in the upper soil layers without root growth (Figure 3). In the deeper soil layers, the rhizobox window area covered with the $O_2$-sensitive planar optode showed almost completely anoxic conditions throughout the experiment. During plant growth, the anoxic fraction decreased in all layers in all rhizoboxes as the soil became increasingly more oxic. $O_2$ concentrations rapidly increased in the topmost soil layer where more than 90 % of the window area was oxic 24 days after plant emergence. $O_2$ concentrations in the depths below followed similar patterns, with a time delay. With increasing root growth, larger areas became oxic, but in the direct vicinity of roots, $O_2$ concentrations were lower compared to surrounding bulk soil.

**Interactions between root growth, soil moisture, soil profile $O_2$ concentrations, and surface $N_2O$ fluxes**

Soil water content and mean anoxic fraction were both negatively correlated with root length ($R^2$=0.27 and 0.85, respectively, $p < 0.0001$), while the anoxic fraction was positively correlated with soil water content ($R^2$=0.42, $p < 0.0001$) (Table 1, Supplementary Figure S6). Linear regressions between surface $N_2O$ fluxes and either soil water content, root length, or anoxic fraction yielded only weak relationships (Table 1, Supplementary Figure S6).

The best model explaining surface $N_2O$ fluxes included an interaction between soil water content and anoxic fraction as fixed factors and the rhizobox as random factor ($p < 0.01$). At a high anoxic fraction, $\ln(N_2O)$ was negatively correlated with soil water content, while $\ln(N_2O)$ was positively correlated with soil water content for low anoxic fractions pointing towards interactive effects of soil water content and anoxia on $N_2O$ formation and reduction. The conditional $R^2$ of the full model was 0.86, while the marginal $R^2$ of the model with only the fixed effect (i.e., without the random factor) was 0.09 highlighting the large differences between rhizoboxes (Table 1, Supplementary Figure S7). Results of regression analyses for the dataset covering the whole experimental period can be found in the Supplementary Table S3.

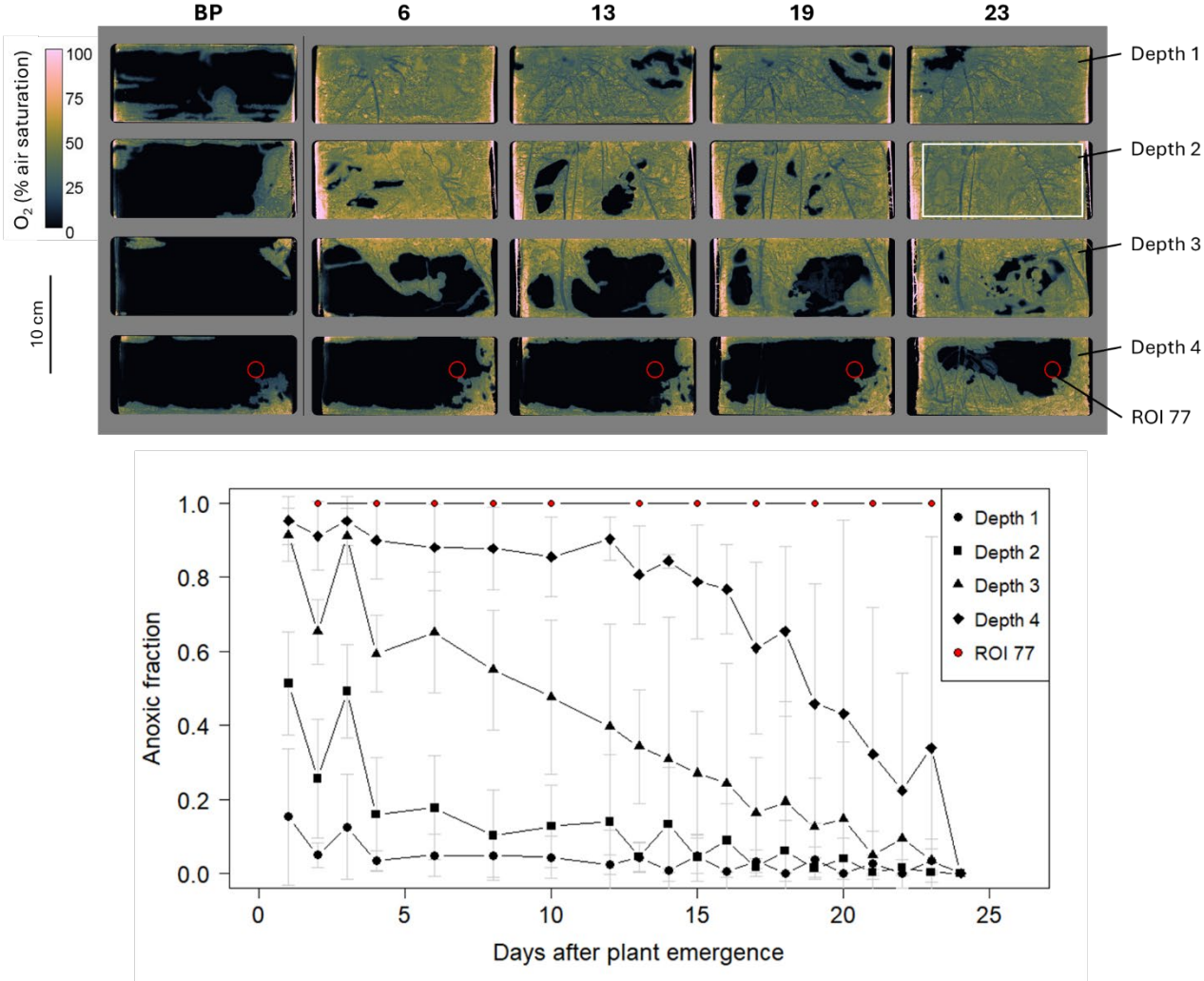



**Figure 3:** Top: False color images of the $O_2$ concentrations in Rhizobox 1 before plant emergence (BP) and on Day 6, 13, 19, and 23 after plant emergence measured with planar $O_2$ optodes. The four 'windows' of the rhizobox correspond to the four depths. The white rectangle is exemplary for the area used to calculate the anoxic fraction of each depth. Lighter areas outside the white rectangle are artifacts at the edges of the optode and were not included in calculations. The red circle represents ROI 77.

Bottom: Fraction of anoxic area (< 10% $O_2$ air saturation = 2.1 % $O_2$) in all rhizoboxes. Different symbols represent the four depths in the rhizoboxes and ROI 77. Mean ± standard deviation for n=7.

**Table 1:** Results of regression analyses (coefficients of determination (Adjusted $R^2$), *p*-values, and sample size *n*) of the relationship between soil water content, root length, anoxic surface fraction, and $N_2O$ surface fluxes (ln transformed) in rhizoboxes.

| Model | *n* | $R^2$ | *p*-value |
|---|---|---|---|
| *Simple linear regressions* | | | |
| Soil water content ~ Root length | 76 | 0.2738 | $7.42 \times 10^{-07}$ |
| Anoxic fraction ~ Soil water content | 76 | 0.4247 | $1.099 \times 10^{-10}$ |
| Anoxic fraction ~ Root length | 76 | 0.8482 | $< 2.2 \times 10^{-16}$ |
| Root dry weight ~ total root length | 76 | 0.8303 | 0.002693 |
| Soil water content ~ total root length | 76 | 0.8899 | 0.0008959 |
| $\ln(N_2O)$ ~ Root length | 76 | 0.06972 | 0.01263 |
| $\ln(N_2O)$ ~ Soil water content | 76 | 0.1058 | 0.002411 |
| $\ln(N_2O)$ ~ Anoxic fraction | 76 | 0.115 | 0.001593 |
| *Linear mixed effect model* | | | |
| $\ln(N_2O)$ ~ Soil water content * Anoxic Fraction<br>Random effect: Rhizobox Nr (replicate) | 76 | 0.857 (cond.)*<br>0.091 (marg.)* | 0.0097 |

*For the linear mixed effect model (lme), the conditional $R^2$ is the variance explained by the entire model, including both fixed and random effects, while the marginal $R^2$ represents only the variance explained by the fixed effects.

**Soil analyses (total CN, mineral N, WEOC)**

Soil analyses showed similar depth patterns for analysis of soil layers and ROI (Figure 4). Soil $NO_3^-$ content decreased with depth, from 320-1299 mg N $kg^{-1}$ in the upper 5 cm to 1.7-502 mg N $kg^{-1}$ in the 5-10 cm layer to 0.04-123 mg N $kg^{-1}$ below 10 cm (Figure 4 a). Soil $NH_4^+$ contents were much lower compared to $NO_3^-$ content. $NH_4^+$ contents were lowest in the 5-20 cm layers and ranged between 0 and 45 mg N $kg^{-1}$ in the others (Figure 4b). Total soil N in ROI ranged between 0.09 and 0.26 % without clear differences in different depths (Supplementary Figure S8). Total soil C in ROI mostly ranged between 1.11 and 1.25 % which was similar to the initial soil $C_{org}$ content (1.21 %). From 77 measured ROI, eight ROI had higher total C contents between 1.5 and 2.3 % which likely included small pieces of roots or $CaCO_3$. DOC content ranged between 24.5 and 188. 7 mg C $kg^{-1}$ with highest values in 0-5 cm layers (Figure 4c).

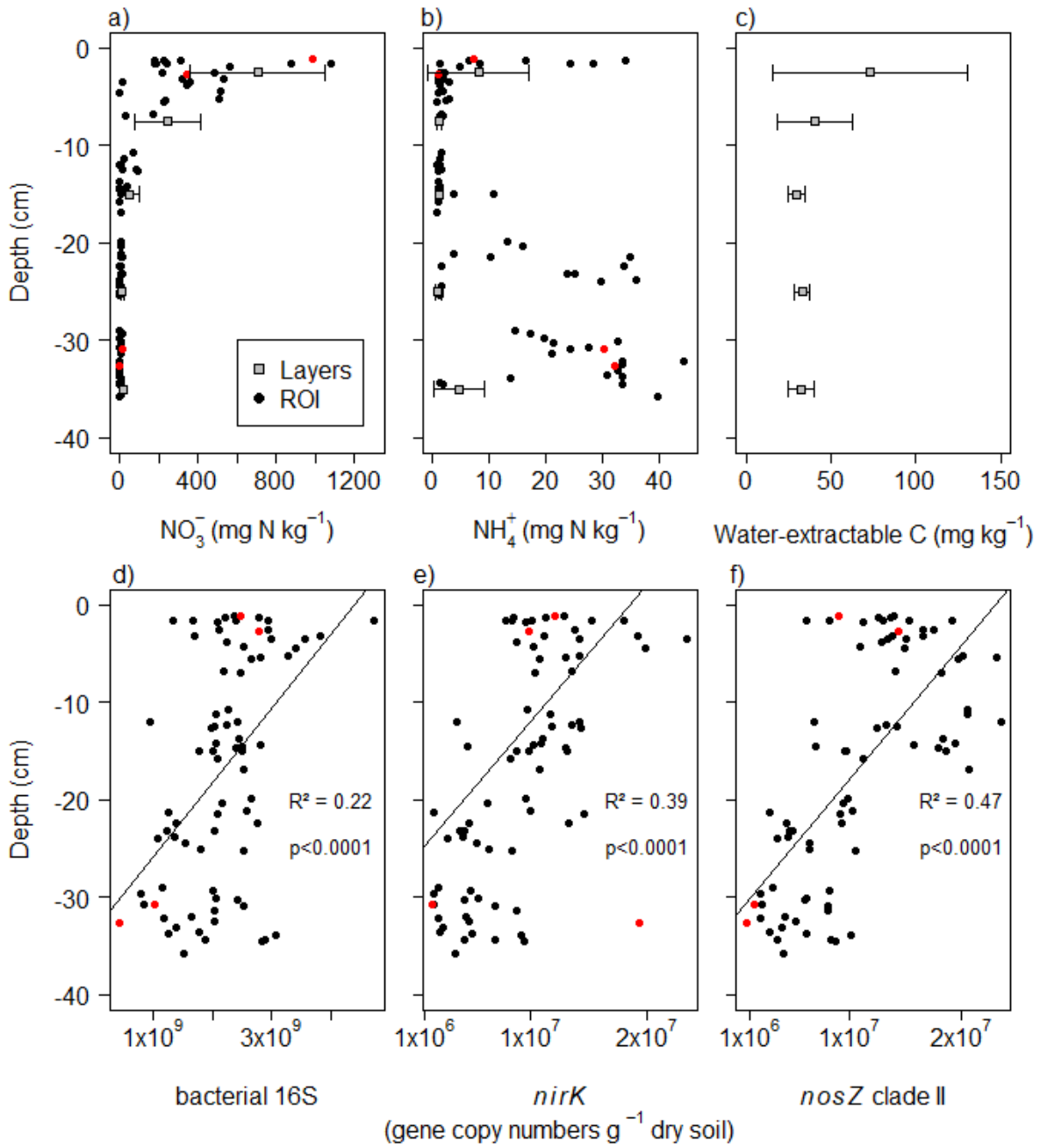



**Figure 4:** Depth distribution of (a) $NO_3^-$, (b) $NH_4^+$, and (c) DOC content in soil layers and ROI, and (d-f) depth distribution of gene abundances in ROI. Each point represents one of 77 ROI, while grey squares represent mean values ± standard deviation for different soil layers (n=7). Red circles represent ROI Nr. 1, 6, 45, and 77. For gene abundances, regression lines, adjusted $R^2$, and *p*-values of simple linear regressions with depth are depicted.

## Functional genes involved in N cycling

Abundance of 16S rRNA genes, a proxy for total archaea and bacteria, decreased with increasing sampling depth ($R^2$=0.22, $p < 0.0001$, Figure 4d, Table 2). Similarly, abundance of *nirK* and *nosZ* clade II genes decreased with increasing sampling depth ($R^2$=0.39 and 0.47, respectively, $p < 0.0001$). For *nirS* and *nosZ* clade I, only very weak correlations with sampling depth were found (Table 2). Except for *nirS*, gene abundances were negatively correlated with

soil $NH_4^+$ concentrations (Supplementary Table S4). However, $NH_4^+$ concentrations were also negatively correlated with soil depth. The ratio of denitrifiers (*nirK* + *nirS*) to $N_2O$ reducers (*nosZ*I + *nosZ*II) increased with increasing $NO_3^-$ content in ROI ($R^2$=0.2, $p < 0.0001$).

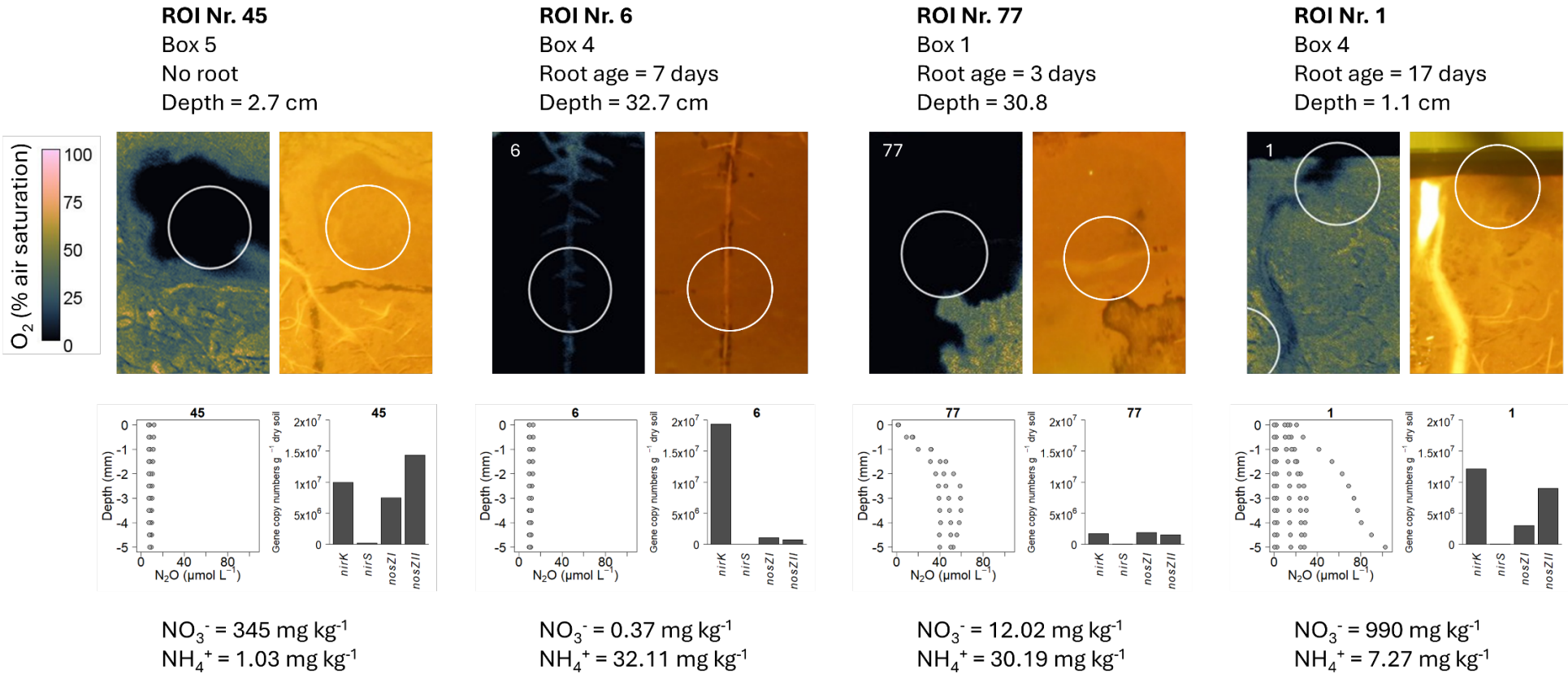
**Table 2:** Results of simple linear regression analyses (coefficients of determination (Adjusted $R^2$), *p*-values, and sample size *n*) of the relationship between $N_2O$, $O_2$, N concentrations, root age, soil depth, and abundance of nitrogen cycling genes in ROI. Significant relationships ($p < 0.05$) are highlighted in bold.

| Model | *n* | $R^2$ | *p*-value |
|---|---|---|---|
| **Min. $N_2O$ ~ $O_2$ max** | **14** | **0.5304** | **0.001893** |
| **Max. $N_2O$ ~ N concentration** | **14** | **0.5227** | **0.002097** |
| **16S ~ Depth** | **77** | **0.2193** | **$1.04 \times 10^{-05}$** |
| ***nirK* ~ Depth** | **77** | **0.3882** | **$8.65 \times 10^{-10}$** |
| *nirS* ~ Depth | 77 | 0.04388 | 0.03745 |
| *nosZI* ~ Depth | 77 | 0.07119 | 0.01085 |
| ***nosZII* ~ Depth** | **77** | **0.4691** | **$3.891 \times 10^{-12}$** |
| **$NH_4^+$ ~ Depth** | **77** | **0.3325** | **$2.436 \times 10^{-08}$** |
| **nirK ~ root age** | **77** | **0.2431** | **$3.127 \times 10^{-06}$** |
| ***nosZII* ~ root age** | **77** | **0.1822** | **$6.433 \times 10^{-05}$** |
| **(*nirK*+*nirS*) / (*nosZI*+*nosZII*) ~ $NO_3^-$** | **76** | **0.2** | **$3.045 \times 10^{-05}$** |

**$N_2O$ microsensor profiles in ROI and their relationship with influencing parameters**

$N_2O$ concentrations measured with $N_2O$ microsensors in ROI at the end of the experiment showed very high variability in $N_2O$ concentrations (Figure 5, Supplementary Figure S9). $N_2O$ concentrations ranged between 0 and 100 µmol $N_2O$ $L^{-1}$ with highest $N_2O$ concentrations measured in ROI 1 close to the shoot and its crown roots in 1.1 cm depth and second highest measured in ROI 77 around a small root at the bottom of the rhizobox at 30.8 cm depth.

For most ROI, changes in $N_2O$ concentration within the same measurement profile were in the range of a few µmol (Supplementary Figure S9). There were exceptions, as ROI 1 where one $N_2O$ profile increased from 20 to 100 µmol $L^{-1}$ and ROI 77 where $N_2O$ concentrations increased from 0 to 40-60 µmol $L^{-1}$ in 2-5 mm depth (Figure 5). Minimum $N_2O$ concentrations in 500 µm depth (i.e, in direct proximity to the optode) were negatively correlated with maximum $O_2$ concentration ($R^2$=0.53, $p < 0.01$, Table 2, Supplementary Figure S10), while maximum $N_2O$ concentrations in 500 µm depth were positively correlated with total N availability in all ROI ($R^2$=0.52, $p < 0.01$, Table 2, Supplementary Figure S10).




$NO_3^-$ = 345 mg kg$^{-1}$
$NH_4^+$ = 1.03 mg kg$^{-1}$

$NO_3^-$ = 0.37 mg kg$^{-1}$
$NH_4^+$ = 32.11 mg kg$^{-1}$

$NO_3^-$ = 12.02 mg kg$^{-1}$
$NH_4^+$ = 30.19 mg kg$^{-1}$

$NO_3^-$ = 990 mg kg$^{-1}$
$NH_4^+$ = 7.27 mg kg$^{-1}$

**Figure 5:** Characterization of four exemplary ROI at the end of the experiment. False-color image showing $O_2$ concentration, photo showing root development, $N_2O$ microsensor depth profiles (µmol $N_2O$), barplot showing gene abundances (gene copy numbers g$^{-1}$ dry soil), and mineral N content (mg N kg$^{-1}$). ROI diameter = 16 mm. Root age refers to the days of root growth in the respective ROI.

## Discussion

### Microsite $N_2O$ formation depended on root growth, $O_2$ and N availability

$N_2O$ microsensor measurements showed very high spatial heterogeneity of $N_2O$ formation in soil. Minimum $N_2O$ concentrations were higher in ROI with lower $O_2$ concentrations confirming that $O_2$ availability was a major control of denitrification at the microsite scale. Oxygen concentration is one of the most important controls of denitrification as it determines when microorganisms switch to anaerobic respiration (Groffman *et al.*, 1988; Schlüter *et al.*, 2024). This, however, does not ascertain that denitrification will occur, as nitrate can be limiting (Smith & Tiedje, 1979; Hojberg *et al.*, 1994; Schlüter *et al.*, 2024), which has been documented in relation to plant growth with rapid N uptake (von Rheinbaben & Trolldenier, 1984; Haider *et al.*, 1985; Rummel *et al.*, 2021). Accordingly, we found higher $N_2O$ concentrations in ROI with higher total N availability indicating that, under $O_2$ limiting conditions, N availability is crucial to determine the magnitude of $N_2O$ emissions. ROI with the highest $N_2O$ concentrations were in the direct vicinity of roots (i.e., ROI Nr. 1, 77). In contrast, ROI 45 was characterized by high $NO_3^-$ content and anoxic conditions, but no roots, and only low $N_2O$ concentrations confirming the importance of roots on formation of $N_2O$ hotspots through C exudation, root and rhizosphere microbial respiration, verifying our second hypothesis. Although functional gene abundances and $N_2O$ concentrations were not significantly correlated in ROI, we found a positive relationship between the (*nirK*+*nirS*)/(*nosZI*+*nosZII*) ratio and $NO_3^-$ availability indicating higher relative genetic potential for $N_2O$ reduction at lower soil $NO_3^-$ content. This relationship was driven by the abundance of *nosZ* clade II nitrous oxide reducers, in line with previous reports (Xu *et al.*, 2020; Jones *et al.*, 2022).

### Roots determined biotic and abiotic gradients in soil

Plant and root growth affected soil moisture, anoxic soil fraction, and distribution of nutrients controlling the abundance of microorganisms. Root growth determined soil moisture through root water uptake as demonstrated by significant negative correlations between soil water content and planar root length throughout the experiment and between soil water content and total root length at final harvest. Further, soil moisture sensors showed that soil moisture was constant at the bottom of the rhizoboxes, while fluctuating more in the upper soil layer with higher root density. Using planar optodes, we were able to show increasing $O_2$ concentrations with increasing plant and root growth. Especially, roots growing into deeper soil layers led to increasing $O_2$ concentrations at the optode-soil interface. We anticipate a combination of several explanations: (1) plant roots can increase the diffusion of $O_2$ through the soil (Jensen & Kirkham, 1963), (2) maize roots form aerenchyma under $O_2$-limited conditions (≤ 3% $O_2$ ≈ 14.3 % $O_2$ air saturation (Gunawardena *et al.*, 2001) facilitating transport of $O_2$ into anoxic soil

435 layers, (3) and roots may have 'pushed' the optode away from the soil surface in some spots
436 creating air-filled cavities between the optode and the soil (Merl *et al.*, 2023). Although roots
437 generally increased aeration and $O_2$ concentrations in soil, roots were always characterized
438 by lower $O_2$ concentrations compared to the surrounding soil confirming our first hypothesis.
439 Most of the added $NO_3^-$ fertilizer was recovered in the upper 10 cm of the rhizoboxes likely
440 due to a combination of initial rewetting the soil in the rhizoboxes from the bottom and
441 evaporation on the soil surface causing a constant upward movement of soil water,
442 transporting $NO_3^-$ to the upper soil layer throughout the experiment. Root growth increased
443 availability of organic C through rhizodeposition leading to higher DOC concentrations in upper
444 soil layers with higher root density. Although root exudation per root surface area decreases
445 with growth/age, total exuded C rates increase with increasing root growth (Santangeli *et al.*,
446 2024). Especially, maize crown/brace roots can exude large quantities of mucilage containing
447 high amounts of sugars (Werner *et al.*, 2022) contributing to high availability of DOC in the
448 uppermost soil layers. Higher availability of DOC and $NO_3^-$ in upper soil layers promoted
449 microbial growth as indicated by decreasing abundances of 16S rRNA, *nirK* and *nosZII* genes
450 with soil depth. Abundances of *nirK* and *nosZII* genes also increased with increasing root age
451 confirming the influence of root growth on microbial denitrifiers. Abundances of *nirS* and *nosZI*
452 were much lower compared to abundances of *nirK* and *nosZII*, respectively, and were not
453 affected by nutrient concentrations or root growth indicating niche differentiation between *nirK*-
454 and *nirS*-type denitrifiers and *nosZ* clade I and II nitrous oxide reducers, respectively (Jones
455 & Hallin, 2010; Achouak *et al.*, 2019; Wang *et al.*, 2024).

456 **Soil moisture and anoxic fraction controlled $N_2O$ fluxes**

457 $N_2O$ fluxes showed distinct patterns with initially high fluxes decreasing until about two weeks
458 after rewetting the soil, followed by a period with lower fluxes. Emergence of plants stimulated
459 $N_2O$ production leading to clear increases in $N_2O$ fluxes within a week following plant
460 emergence. However, correlations between $N_2O$ fluxes and planar root length or cumulative
461 $N_2O$ emissions and total root length at harvest did not yield significant relationships as we
462 would have expected. Nonetheless, our study confirmed intercorrelation of root growth and
463 $N_2O$ formation as $N_2O$ fluxes were controlled by an interaction of the anoxic fraction and soil
464 moisture, confirming interacting effects of root water uptake and anaerobicity on $N_2O$ formation
465 and reduction. For low anoxic fractions, increasing soil moisture led to increasing $N_2O$ fluxes,
466 while for high anoxic fractions higher soil moisture led to lower $N_2O$ fluxes due to the reduction
467 of $N_2O$ to $N_2$ (Davidson, 1991; Rohe *et al.*, 2021). In addition, differences between replicates
468 explained most of the variance in $N_2O$ fluxes. Soil heterogeneity is known to strongly control
469 the formation of denitrifying $N_2O$ hotspots causing very high variation within replicates of the
470 same treatment (Folorunso & Rolston, 1984; Christensen *et al.*, 1990; Schlüter *et al.*, 2024).

Plant and root growth further increased heterogeneity in soil but also differences between replicates.

**The uppermost soil layers contributed largely to $N_2O$ fluxes**

We anticipated that some zones of the rhizoboxes were more likely to contribute to $N_2O$ formation – the uppermost and the lowest soil layers. The upper 5 cm soil layer was characterized by very high $NO_3^-$ concentrations (up to >1000 mg $NO_3^-$-N $kg^{-1}$), highest concentrations of DOC, and highest abundances of denitrifying and $N_2O$ reducing genes (*nirK* and *nosZII*). Although $O_2$ concentrations in the uppermost soil layer increased fast, small anoxic areas were identified with the $O_2$ optodes at the later/last measurement days. Furthermore, rewatering from the top kept soil continuously wet which (together with high $NO_3^-$ and DOC) provided optimal conditions for denitrifiers as confirmed by microsensor measurements (ROI 1). Short distance to the soil surface facilitated $N_2O$ diffusion to the surface and its loss to the atmosphere. Accordingly, cumulative $N_2O$ emissions were positively correlated with soil $NO_3^-$ content in the uppermost soil layer. In contrast, the bottom layer of the rhizoboxes had larger areas that remained anoxic throughout the experiment. $NO_3^-$ concentrations in the bottom layer were low (15 mg $NO_3^-$-N $kg^{-1}$, 200 µM $NO_3^-$), but sufficient to sustain denitrifying microorganisms (Palmer & Horn, 2015) as confirmed by $N_2O$ microsensor profiles indicating substantial $N_2O$ formation around a small root in ROI 77. Continuous high soil moisture and long diffusion pathways from the lower layers of the rhizobox to the soil surface likely promoted $N_2O$ reduction to $N_2$, which has been reported for this particular soil (Malique *et al.*, 2019; Rummel *et al.*, 2021; Yankelzon *et al.*, 2024a).

## Conclusions

We provide the first *in-situ* measurements of $N_2O$ concentration profiles showing distinct patterns with highest $N_2O$ concentrations around roots compared to bulk soil. Our study confirmed that $O_2$ and N availability control $N_2O$ production at the process scale while emphasizing the importance of roots in shaping $N_2O$ hotspots. Plant roots determined depth gradients in the soil of the rhizoboxes with higher DOC and lower soil moisture in the upper soil layers. Abundances of total bacteria, denitrifying *nirK* and $N_2O$ reducing *nosZII* responded to resource availability with higher abundances in upper soil layers with high nutrient contents and more root growth. We argue that these uppermost soil layers largely contributed to $N_2O$ formation and $N_2O$ fluxes measured at the soil surface. Combining $O_2$ optode and root imaging proved to be a successful approach to monitor major controls of rhizosphere N cycling and $N_2O$ formation at high spatial and temporal resolution. Carefully choosing ROIs allowed reducing the number of samples, while covering a broad range of soil conditions revealing

correlations between $N_2O$ concentrations, substrate availability, and microbial gene abundances.

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## Competing interests

The authors declare no competing interests.

## Author contributions

Conceptualization: PSR, CWM, KK; Investigation: PSR, MRR, AS; Data analysis (soil, root, $N_2O$): PSR; Data analysis (optodes): PSR, TM; Data analysis (qPCR): AS; Writing – original draft preparation: PSR; Writing – review and editing: MRR, AS, TM, SH, CWM, KK. All authors have read and agreed to the published version of the manuscript.

## Data availability

The data that support the findings of this study will be made openly available at http://doi.org/10.5281/zenodo.15537545.

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